Effects of Antibiotics on Aquatic Microbes

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Abstract

Antibiotics are designed to inhibit the growth of or kill bacteria. Of the many classes of antibiotics that have been synthesized two particular classes of antibiotic have been frequently detected in the natural environment, namely the tetracyclines and sulfonamides. Following use, these compounds can enter the environment *via* the application of animal manures or domestic seawage sludge to land as a fertiliser or from wastewater treatment plant effluents. Once in the environment the compounds can persist and, depending on their properties, are distributed around the different environmental media (i.e. surface waters, groundwaters, soils and sediments). Several studies have suggested that tetracyclines and sulfonamides may have significant impacts on microbial function in the natural environment. However most of these studies have used existing standardised test procedures and/or unrealistic exposure conditions. The aim of the current study therefore was to develop a more environmentally realistic test system for assessing the effects of antibiotics on aquatic microbial communities and to apply this to assess the potential impacts of a sulphonamide and a tetracycline antibiotic on aquatic microbial communities. The developed system, which was an aquatic microcosm composed of fresh river water, return line treated sewage and OECD synthetic sewage, was initially used to assess the effects of 3, 5-dichlorophenol, an OECD reference toxicant. Effects of DCP were seen within the concentration range that is considered valid by other standard microbial toxicity tests, such as OECD method 209 (3.2-32 mg/L). Since the developed system was able to quantify effects on aquatic microbial communities, it can be seen as being more environmentally relevant than existing standard tests, which rely on testing single microbial species or sludge communities. The test system was then used to test the effects of chlortetracycline (CTC) and sulfamethoxazole (SMX) on multisubstrate utilization. Following addition of the study antibiotics, effects were observed for both antibiotics on total substrate utilization and on the utilization of specific, ecologically relevant end-points (such as nutrient cycling and polymer degradation) also. Effects were also observed on the structure of microbial communities and on the functional diversity of substrate utilization (especially in the case of SMX exposure) and these effects generally persisted for up to 3 d after addition of the study compounds at a lower concentration of 0.1 mg/L for both compounds. The results indicate that both antibiotics were inhibiting various aspects of substrate utilization, most of which were related to ecologically relevant processes that occur in the aquatic environment. The results also suggest that SMX and CTC exposure result in changes in the community structure of ecologically relevant groups of microbes (such as bacteria involved in the nitrogen and carbon cycle bacteria). Microbial communities that were exposed to CTC and SMX showed a varying degree of recovery, although more long term effects were seen in SMX exposures; functional effects were observed for the duration of the exposure. It was hypothesized that the observed recovery may have been caused by the selection of antibiotic resistance in the exposed communities. Culture based resistance studies showed that there was a significant temporal rise in CTC resistance at all CTC concentrations, but

not SMX resistance, across the dose range. In addition, a greater number of distinct microbial morphotypes could be isolated from combined microcosms on day 7 compared with day 1. An increase in CTC and SMX resistance in control exposures was also observed. In addition, distinct resistant morphotypes developed in both dosed and undosed microcosms on day 7 of the study. Furthermore, the results of a series of different culture based experiments suggested that multidrug resistance was present in morphotypes that were isolated from dosed and control microcosms. These data therefore suggest that multidrug resistance was present at the start of the exposure and may also have been co-selected by exposure to CTC and SMX. In general, CTC and SMX show signs that they may pose a risk to the environment and possibly the wider health of humans and animals.

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Declaration

Submitted by Iain Andrew Davies, to the University of York as a thesis for the degree of Doctor of Philosophy in Environmental Sciences, October 2010.

I certify that all material present in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for award of a degree by this or any other university.

Iain Andrew Davies, October 2010

Chapter 1: General Introduction

1. Introduction

The problem of aquatic pollution is not a new issue. In 1961 legislation was passed to eliminate the use of branched surfactants due to cases of "foaming rivers," which resulted from emissions of compounds such as alkyl benzene sulfates (Scheilbel, 2004). An emerging issue in the present day is the pollution of aquatic systems with active pharmaceutical ingredients (APIs) and the effects they exert on ecosystems.

One class of pharmaceutical compound that has been detected in the aquatic environment (and other environmental compartments) is the antibiotics. Gustafson and Bowen (1997) describe antibiotics as a wide range of natural and semi-synthetic compounds that are used in the treatment and prevention of bacterial diseases. Alexander Fleming observed the first documented antibiotic activity in a laboratory setting in 1928 when he accidentally noticed that the *Penicillium* fungus inhibited the growth of *Staphylococci sp.* on agar plates (Fleming, 1929). During the next decade the sulfonamides became the first group of antibiotics to be administered therapeutically. A number of new antibiotic compounds have since been discovered (summarised in table 1.1). The most recent, tigecycline, was fast-tracked onto the market in 2005.

Antibiotics can be separated into different classes. Compounds from each antibiotic class have a distinct cellular target (or targets) and therefore mechanisms of action (Table 1.2). Bacitracin is a mixture of related cyclic polypeptides and as such does not belong to a specific group. Antibiotic mechanisms of action can be sub-divided into four general mechanisms; protein synthesis inhibition, DNA synthesis inhibition, inhibition of RNA synthesis and inhibition of cell wall synthesis (Retsema and Fu, 2001).

Table 1.1: Chronology of antibiotic discovery.

Discovery Event	Discovery Date	Country
Penicillins (discovered)	1928	UK
Sulfonamides (discovered)	1932	Germany
Gramicidin (discovered)	1939	USA
Penicillin (introduced)	1942	UK and USA
Streptomycin (discovered)	1943	USA
Bactiracin (discovered)	1943	USA
Cephalosporins (discovered)	1945	Italy
Chloramphenicol (discovered)	1947	USA
Clortetracycline (discovered)	1947	USA
Neomycin (discovered)	1949	USA
Oxytetracycline (discovered)	1950	USA
Erythromycin (discovered)	1952	USA
Vancomycin (discovered)	1956	USA
Kanamycin (discovered)	1957	Japan
Methicillin (introduced)	1960	UK and USA
Ampicillin (introduced)	1961	England
Spectinomycin (reported)	1961	USA
Gentamicin (discovered)	1963	USA
Cephalosporin (introduced)	1964	UK
Doxycycline (introduced)	1966	USA
Clindamycin (reported)	1967	USA
Tobramycin (discovered)	1971	USA
Cephamycins (discovered)	1972	USA
Minocycline (introduced)	1972	USA
Tigelcycline (introduced)	2005	USA

Table 1.2: Mechanism of action of major antibiotic groups.

Antibiotic Group	Example	Mechanism of Action	Reference(s)
Aminoglycosides	Streptomycin	Competitive binding to 30S ribosomal subunit; causes codon misreading & failed translocation. Protein synthesis is halted.	Davies *et al*., 1965; Davies & Davis, 1968
Bacitracin	Bacitracin	Inhibits dephosphorylation of C55-isoprenyl pyrophosphate preventing peptidoglycan transport across cytoplasmic membrane; results in inhibition of cell wall synthesis	Anderson *et al*., 1966; Stewart & Stromiger, 1967
Beta-Lactams	Dicloxacillin	Act as analogues of the terminal D-alanyl-D-alanine terminal of the peptidoglycan precursor NAM/NAG-peptide subunits. Cell wall biosynthesis is prevented. Also causes depolarisation of cell wall; acts as cellular signal to commence autlysis.	Ghysen, 1991; Penyige *et al*., 2001
Cephems	Ceftobiprole	Inhibit enzymes responsible for cross-linking of oligopeptides that form long chain peptidoglycan polymers in Gram positive cell wall.	Smith, 1985
Chloramphenicol	Chloramphenicol	Prevents peptidyl transfer activity by competitive inhibition of 23s ribosomal subunit; protein synthesis halted.	Hansen *et al*., 2003
Glycopeptides	Vancomycin	Bind to D-alanyl–D-alanine C-terminus of nascent peptidoglycan, stopping it from becoming cross-linked by reactions catalysed by transglycosylases and transpeptidases. Cell wall is weakened and cell lysis occurs.	Nieto & Perkins, 1971; Arthur & Courvalin, 1993; Walsh, 1993; Ge *et al*., 1999
Macrolides	Erythromycin	Bind reversibly to the 50s ribosomal sub-unit preventing elongation of polypeptide chain and premature detachment from ribosomal binding site. Production of premature peptides causes cell death.	Cocito *et al*., 1997
Quinolones	Trovafloxacin	Inhibit type II Topoisomerase (DNA Gyrase & Topoisomerase IV) activity. Resultant build-up of double stranded breaks in chromosomes triggers SOS cellular response and subsequent cell death.	Hooper, 1998
Rifampins	Rifampin	*E. Coli* bacteriocidal activity thought to be additionally caused by apoptosis via activiation of suicide gene *mazEF*.	Hartmann *et al*., 1985

Table 1.2 (cont).

Antibiotic Group	Example	Mechanism of Action	Reference(s)
Sulfonamides	Sulfamethoxazole	Competitively inhibits the enzyme dihydropteroate synthetase (DHPS) by acting as a structrural analogue of Para-aminobenzoic acid (PABA). Folic acid therefore cannot be incorporated into essential nucleic acids and proteins and cell growth is inhibited.	Henry, 1943
Tetracyclines	CTC	Transiently bind to the ribosomal 30S sub-unit. Polypeptide elongation is therefore inhibited and protein synthesis cannot proceed normally.	Gale *et al*., 1981

1.2. Antibiotic Usage

The usage of antibiotic compounds can be split into two distinct categories, human and veterinary. Antibiotics are used to treat a wide array of infections caused by pathogenic bacteria such as *Escherichia coli* 0157 and *Pneumonocystis pneumonia*. Such drugs can be administered orally, topically or *via* an injection. Some countries also use antibiotics (such as tetracyclines) as dietary growth-promoting agents. Volumes of antibiotics that enter the environment can be estimated from sales data and information on metabolism in the treated human or animal. The British Veterinary Index provides veterinary sales data in the UK. As mentioned by Boxall *et al.* in 2002 however, this data does not take into account sales by companies to veterinary surgeons, pet shop sales or sales made by the AHDA (Animal Health Distributors Association). Human sales figures in the EU are estimated from prescription data. This data is collated and published by IMS-MIDAS. However, these figures do not take into account factors such as "over the counter" sales and illegal (and therefore unregistered) drug production (Boxall *et al*., 2001; Daughton and Ternes, 1999).

Despite anomalies in data, antibiotic usage data is one of the best recorded in the EU compared with other pharmaceutical classes (Stege *et al*., 2003). There have been only limited attempts however to collate available data on total usage values. For example, Kools *et al.* (2008) were able to collate data from a wide array of sources to estimate antibiotic usage within Europe in 2004. It was found that total veterinary antibiotic use (in 2004) was 4634 tonnes. This represented a 33% increase compared with 1997 figures and an 18% rise on 1999 usage data. These changes highlight the fact that antibiotic usage from year to year can be extremely variable due to changing usage profiles. For example, the use of antibiotics as growth promoters has dropped by 50% whereas their therapeutic use in Denmark and the Netherlands increased between 1997 and 2004 (KTBL, 2005; MANRAN, 2004). The most widely used veterinary antibiotics in the EU were the tetracyclines, followed by beta-lactam / cephalsporin mixtures.

With regards to human antibiotic use, EU figures indicate that humans are medicated by 10 more days per year compared with animals. The total volume of antibiotics sold for human consumption was 7659 tonne in 1997. EU figures indicate that human antibiotic consumption was approximately 6.3 fold higher than veterinary usage; human usage was 342 mg antibiotic/kg body mass/year compared with 54 mg antibiotic/ kg body mass/year for animal usage (Ungemach *et al*., 2006; Kools *et al*., 2008).

1.3. Exposure Routes of Antibiotics into the Environment

Antibiotics may enter the environment in a number of ways (Figure 1). Sewage treatment plants (STPs) represent the most significant point source of antibiotics in the aquatic environment. Human APIs are transported into STPs as domestic waste (in faeces, urine or from medicines that

have been discarded into toilets or sinks). If a drug entering the STP is hydrophilic it may pass through the sewage treatment process without being fully degraded. Drugs or partially metabolised drugs will then enter the aquatic environment when the sewage is discharged into surface waters (Fent *et al*., 2006). Lipophilic or very polar antibiotics tend to adsorb to particulate matter in STPs (ICON, 2001). This particulate matter is often spread onto fields as a source of nutrients with the only restrictions on this being the nutrient and heavy metal contents (86/278/EEC., 2000; Petrovic and Barcelo 2004).

Antibiotics can also be directly emitted to fields and adjacent water bodies by medicated animals. Excreta from these animals may contain the parent antibiotic, metabolites of the antibiotic or a mixture of both (Kemper, 2008). Antibiotics that are applied topically (such as tetracycline and lincomycin) may also enter the terrestrial and aquatic environment *via* run-off from the animal skin during rainfall events (Armstrong and Phillips, 2008). Antibiotics entering the environment in this manner are generally in the form of parent compounds as they have not been in contact with phase I or phase II enzymes that metabolise the antibiotic in the body (Magnusson *et al*., 2001). Phase 1 enzymes generally catalyse oxidative reactions or reactions which make a xenobiotic more polar, whereas phase II enzymes generally catalyse reactions which conjugate xenobiotic compounds with proteins (Akagah *et al*., 2008).

Antibiotics may also be released on purpose to the aquatic environment when they are used as aquaculture treatments. For example, oxytetracycline and oxolinic acid are routinely administered to aquaculture sites as a preventative measure against microbial pathogens (Bjorkland *et al*., 1991; Primavera *et al*., 1993; Hirsch *et al.*, 1999). Halling Sørensen *et al* (1998) calculated that 70-80% of drugs administered in aquaculture remain in the environment. Antibiotics that enter aquatic systems can also associate with freshwater and marine sediments where they have been shown to accumulate over time (Richardson and Bowron, 1985; Halling Sørensen *et al*., 1998).

Effluent from drug production plants can also act as a point source of antibiotics into the aquatic environment. Despite claims that tight manufacturing processes would only result in minor levels of APIs being released into the aquatic environment, extremely high levels of antibiotics have been detected in effluent from drug manufacturing plants in India. Ciprofloxacin for example has been detected at levels of 31 mg/L in surface waters receiving effluent from drug manufacturing (Williams, 2005; Larsson *et al.*, 2007). More recently, higher levels of pharmaceuticals have also been found in aquatic systems receiving industrial effluent in the USA (Phillips *et al*., 2010).

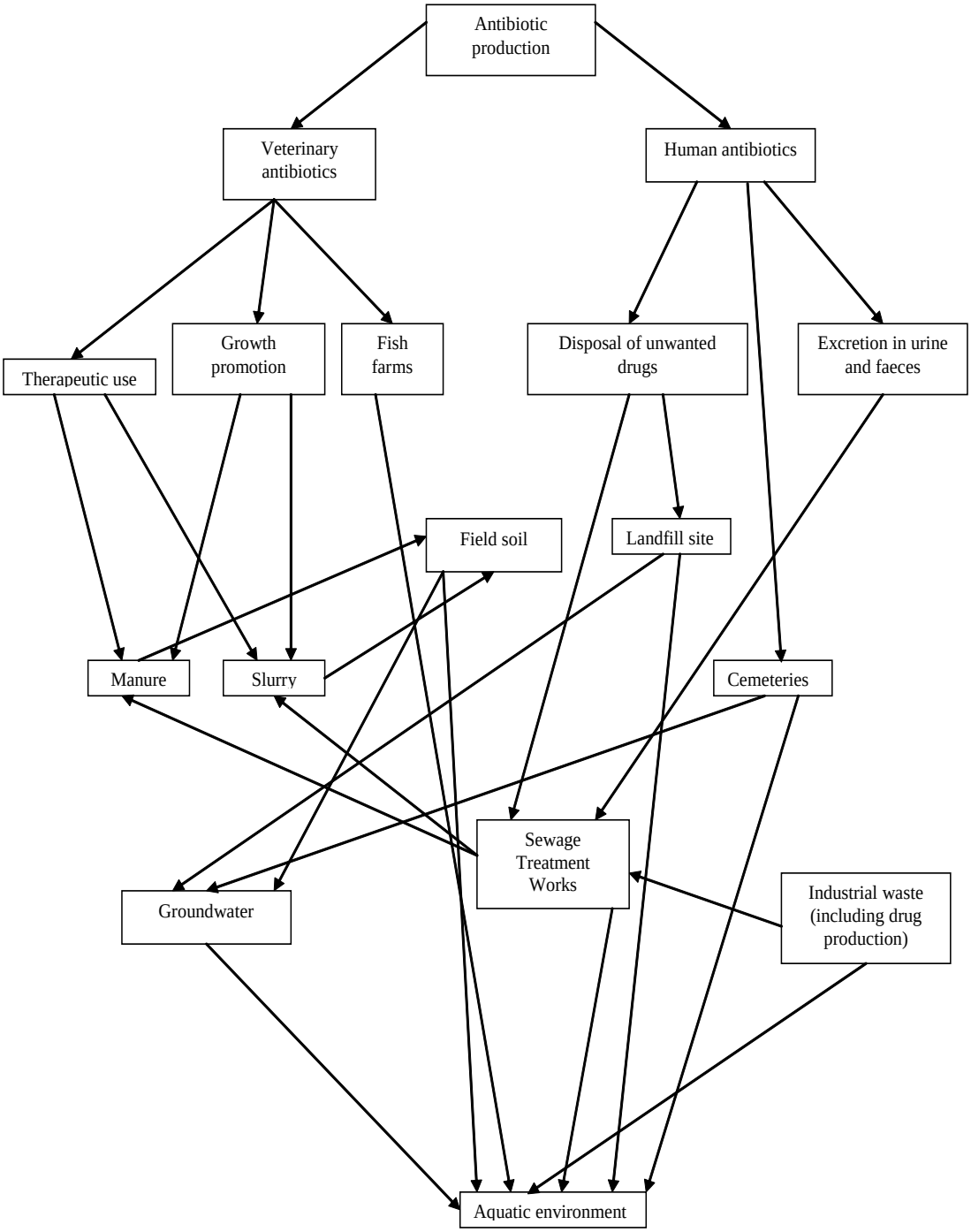


Figure 1.1: Exposure routes for antibiotics into the environment.

1.4. Reported Occurrence of Antibiotics in the Environment

Much research has been conducted to quantify the extent to which antibiotics are present in the environment since Watts *et al.* (1983) first detected antibiotic compounds in the aquatic environment. This has been facilitated by the development of increasingly more sensitive analytical techniques in the last 40 years (Erikson, 2002). It has been estimated that over one million tonnes of antibiotics have been released into the biosphere (25 $x10^6$ kg/year) between the start of antibiotic therapy and 1984 (Verma *et al*., 2007). Consequently, APIs have been detected in several environmental matrices at concentrations generally between 10^{-5}–10^{-9} g l^{-1} (Table 1.3).

Antibiotics have been detected in several matrices, including soil (eg: Jacobsen *et al*., 2004), surface water (eg: Kolpin *et al*., 2002), sediment (eg: Lalumera *et al*., 2004) groundwater (eg: Karthikeyan and Bleam, 2003) and biota (eg: Chafer-Pericas *et al.*, 2010). The majority of these monitoring studies have focused on detecting the occurrence of antibiotics in surface waters and groundwater, with only a small number of studies looking at soil, sediment or the marine environment. Across all environmental matrices the sulfonamides, tetracyclines, macrolides and fluoroquinolones represent major antibiotic groups that have all been frequently detected. The most commonly detected individual antibiotic compounds are sulfamethoxazole and trimethoprim.

Studies that have investigated antibiotic occurrence upstream and downstream of an STP (sewage treatment plant) have often found higher concentrations downstream (Bendz *et al*., 2005; Ashton *et al.*, 2004; Hilton and Thomas, 2003). Higher antibiotic concentrations have also been reported in groundwater and sediment below landfill sites (Holm *et al*., 1995). Water bodies near agricultural land and soils amended with manure have also been shown to contain higher concentrations of antibiotics than in more pristine environments (Yang and Carlson, 2004; Jacobsen *et al*., 2004; Hamsher *et al.*, 2002).

Table 1.3: Reported occurrence of major antibiotic class representatives in various environmental compartments. Data in italics corresponds to limits of detection.

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
tetracyclines					
chlortetracycline	Soil amended with liquid manure	Denmark	0.6×10^{-3} mg/kg *$1.1x\ 10^{-3}$ mg/kg*	nd - 15.5×10^{-3} mg/kg	Jacobsen *et al.*, 2004
	Soil amended with liquid manure	Germany	2×10^{-3} mg/kg *$5x\ 10^{-3}$ mg/kg*	$4.6 - 7.3 \times 10^{-3}$ mg/kg	Hamsher *et al.*, 2002
	Stream water	USA	0.05 mg/kg	0.49 - 0.69 mg/kg	Yang and Carlson, 2004b
	Stream water	USA	*0.10* µg/L	0.16 µg/L	Kolpin *et al.*, 2002
oxytetracycline	Surface water	Italy	*<0.0003 µg/L*	0.01435 µg/L	Calamari *et al*., 2003
	Surface water	Italy	*<0.0003 µg/L*	nd – 0.01920 µg/L	Calamari *et al.*, 2003
	Stream water	USA	*0.10 µg/L*	0.34 µg/L	Kolpin *et al.*, 2002
	Lake water (near agricultural land)	USA	*0.05 µg/L*	0.13 µg/L	Yang and Carlson, 2004b
	Lake water	USA	0.05	0.09 µg/L	Yang and Carlson, 2004b
	Marine sediment	Italy	0.061 µg /kg	246.3 µg /kg	Lalumera *et al.*, 2004
tetracycline	Soil amended with liquid manure	Germany	1×10^{-3} mg/kg *($5x\ 10^{-3}$) mg/kg*	$(86.2 - 198.7) \times 10^{-3}$ mg/kg	Hamsher *et al*., 2002
	Surface water	USA	0.05 µg/L	0.14 µg/L	Yang and Carlson, 2004b
	Surface water	USA	0.05 µg/L	0.06 and 0.12 µg/L	Yang and Carlson, 2004b
	Stream water	USA	*0.10 µg/L*	0.11 µg/L	Kolpin *et al.*, 2002
	Groundwater	USA	*0.05 µg/L*	0.5 µg/L	Karthikeyan and. Bleam, 2003

Table 1.3 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
Doxycycline	Lake water	USA	0.05 µg/L	0.05 µg/L	Yang and Carlson, 2004b
Doxycycline	Lake water	USA	0.05 µg/L	0.05 µg/L	Yang and Carlson, 2004b
Doxycycline (cont)	Surface water	USA	0.05 µg/L	0.08 µg/L	Yang and Carlson, 2004b b
	Surface water	USA	0.05 µg/L	nd and 0.05	Yang and Carlson, 2004b
democlocycline	Surface water	USA	0.05 µg/L	0.12 µg/L and 0.32 µg/L	Yang and Carlson, 2004b
	Surface water	USA	0.05 µg/L	0.44 µg/L	Yang and Carlson, 2004b
sulfonamides					
sulfamerazine	Surface water	USA	0.05 µg/L	nd and 0.05 µg/L	Yang and Carlson, 2004b
	Lake water	USA	0.05 µg/L	0.19 µg/L	Yang and Carlson, 2004b
sulfamethazine	Stream water	USA	0.02 µg/L - 0.12 µg/L	*0.05 µg/L*	Kolpin *et al*., 2002
	Stream water	USA	0.22 µg/L	*0.05 µg/L*	Kolpin *et al.*, 2002
	Lake water	USA	0.22 µg/L	0.05 µg/L	Yang and Carlson, 2004b
	Surface water	USA	0.0010 µg/L	< 0.0010 µg/L	Skadsen *et al.*, 2004
	Groundwater (landfill site)	Denmark	*20 µg/L*	< 20 µg/L – 900 µg/L	Holm *et al.*, 1995
	Groundwater	Germany	*0.02 µg/L*	0.16 µg/L	Hirsch *et al.*, 1999
sulfamethoxazole	Surface water	Germany	*0.02 µg/L*	0.03 - 0.48 µg/L	Hirsch *et al.*, 1999
	Surface water (tributaries)	Germany	*0.030 µg/L*	< 0.030 µg/L & 0.040 µg/L	Wiegel *et al*., 2004
	Surface water (in April 1998)	Germany, Czech Rep.	*0.030 µg/L*	0.030 - 0.070 µg/L	Wiegel *et al.*, 2004
	Surface water (upstream STP) discharge)	Sweden	NS	< 0.001	Bendz *et al*., 2005
	Surface water (downstream STP discharge)	Sweden	NS	0 - 0.01 µg/L	Bendz *et al*., 2005

Table 1.3 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
Sulfamethoxazole (cont)	Surface water (upstream STP)	UK	<0.050 µg/L	0.050 µg/L	Ashton *et al.*, 2004
	Surface water (downstream STP)	UK	<0.050 µg/L	0.050 µg/L	Ashton *et al.*, 2004
	Surface water	UK	<0.050 µg/L	0.050 µg/L	Hilton and Thomas, 2003
	Stream water	USA	*0.05 µg/L*	0.15 - 1.9 µg/L	Kolpin *et al*., 2002
	Stream water	USA	*0.023 µg/L*	0.066 µg/L - 0.52 µg/L	Kolpin *et al*., 2002
	Surface water	USA	0.0010 µg/L	0.010 ± 0.007 µg/L	Skadsen *et al.*, 2004
	Lake water	USA	0.05 µg/L	0.06 µg/L	Yang and Carlson, 2004b
	Surface water	USA	0.007 µg/L	0.023 µg/L	Heberer *et al.*, 2001
	Surface water	USA	0.05 µg/L	0.05 µg/L & 0.12 µg/L	Yang and Carlson, 2004b
	Groundwater	Germany	0.0018 µg/L *0.0062 µg/L*	0.410 µg/L	Sacher *et al.*, 2001
	Groundwater	USA	0.023 µg/L	0.002 µg/L	Heberer *et al.*, 2001
	Groundwater	Germany	0.025 µg/L	< LOQ – 0.11 µg/L ± 0.07 µg/L	Ternes *et al*., 2007
	Groundwater	USA	*0.05 µg/L*	0.01 µg/L & 0.08 µg/L	Karthikeyan and Bleam, 2003
	Groundwater	Germany	0.02 µg/L	0.47 µg/L	Hirsch *et al.*, 1999
sulfathiazole	Surface water	USA	0.0010 µg/L	< 0.0010 µg/L	Skadsen *et al.*, 2004
sulfamethizole	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 330 µg/L	Holm *et al*., 1995
Sulfadiazine	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 1160 µg/L	Holm *et al.*, 1995
sulfanilamide	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 300 µg/L	Holm *et al*., 1995
sulfaguanidine	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 1600 µg/L	Holm *et al*., 1995
sulfanilic acid	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 10440 µg/L	Holm *et al.*, 1995

Table 1.3 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
acetyl-sulfamethoxazole	Surface water (upstream STP)	UK	0.050 µg/L	< 0.050 µg/L	Ashton *et al.*, 2004
	Surface water (downstream STP)	UK	0.050 µg/L	< 0.050 µg/L – 0.239 µg/L	Ashton *et al*., 2004
	Surface water	UK	0.050 µg/L	< 0.050 µg/L - 0.240 µg/L	Hilton and Thomas, 2003
fluoroquinolones					
ciprofloxacin	Soil amended with sludge	Switzerland	0.05 mg /kg	0.27 + 0.04 - 0.40 + 0.03 mg /kg	Golet *et al.*, 2002
ciprofloxacin (cont)			*0.18 mg /kg*		
	Stream water	USA	*0.02 µg/L*	0.02 µg/L - 0.03 µg/L	Kolpin *et al.*, 2002
	Surface water	Italy	*<0.0003 µg/L*	nd – 0.0262 µg/L	Zuccato *et al.*, 2005a
	Surface water	Italy	*<0.0003 µg/L*	0.01436 µg/L	Calamari *et al*., 2003
Norfloxacin	Soil amended with sludge	Switzerland	0.05 mg /kg *0.18 mg /kg*	0.27 + 0.01 mg /kg - 0.40 + 0.03 mg /kg	Golet *et al*., 2002
Flumequine	Sediment (trout & Fsea-bass farms)	Italy	0.012 mg /kg	578.8 mg /kg	Lalumera *et al*., 2004
Ofloxacin	Surface water	Italy	*<0.0003 µg/L*	0.3061 µg/L	Zuccato *et al.*, 2005a
	Surface water	Italy	*<0.0003 µg/L*	0.0331 – 0.0370 µg/L	Zuccato *et al.*, 2005a
ciprofloxacin + norfloxacin	Surface water	Switzerland	0.0025 µg/L *0.009 µg/L*	nd - 0.015 µg/L ± 0.003 µg/L	Golet *et al.*, 2002a
Macrolides					

Table 1.3 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
clarithromycin	Surface water	Germany	*0.02 µg/L*	nd - 0.26 µg/L	Hirsch *et al.,* 1999
	Surface water	Italy	*<0.0003 µg/L*	0.0016 µg/L – 0.0203 µg/L	Zuccato *et al.,* 2005a
	Surface water	Italy	*<0.0003 µg/L*	0.00831 µg/L	Calamari *et al.,* 2003
	Surface water	Germany, Czech Rep.	*0.030 µg/L*	< 0.030 µg/L - 0.030 µg/L	Wiegel *et al.,* 2004
	Surface water (tributary)	Germany	*0.030 µg/L*	0.030 µg/L & 0.040 µg/L	Wiegel *et al*., 2004
dehydrated erythromycin	Stream water	USA	*0.05 µg/L*	0.1 - 1.7 µg/L	Kolpin *et al*., 2002
	Surface water	Germany, Czech Rep.	*0.030 µg/L*	0.030 - 0.040 µg/L	Wiegel et al., 2004
dehydrated erythromycin (cont)	Surface water (tributary)	Germany	*0.030 µg/L*	0.040 µg/L & 0.070 µg/L	Wiegel *et al.,* 2004
	Surface water	Germany	0.02 µg/L	0.15 µg/L - 1.70 µg/L	Hirsch *et al.,* 1999
	Surface water	USA	0.07 µg/L	0.17 ± 0.03 µg/L	Yang and Carlson, 2004a
	Surface water	Italy	*<0.0003 µg/L*	0.00450 µg/L	Calamari *et al.,* 2003
	Surface water (upstream & downstream STP)	UK	0.010 µg/L	0.057 µg/L & 1.000 µg/L	Hilton and Thomas, 2003
	Surface water	Italy	*<0.0003 µg/L*	0.0032 µg/L – 0.0159 µg/L	Zuccato *et al.,* 2005a
	Surface water (upstream STP)	UK	0.010 µg/L	0.010 µg/L - 0.057 µg/L	Ashton *et al.,* 2004
	Surface water (downstream STP)	UK	0.010 µg/L	0.010 µg/L - 1.022 µg/L	Ashton *et al.,* 2004
	Groundwater	Germany	0.0042 µg/L *0.014 µg/L*	0.049 µg/L	Sacher *et al.,* 2001
Spiramycin	Surface water	Italy	*<0.0003 µg/L*	0.0098 µg/L – 0.0438 µg/L	Zuccato *et al.,* 2005a
	Surface water	Italy	*<0.0003 µg/L*	0.07420 µg/L	Calamari *et al*., 2003

Table 1.3 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
Lincomycin	Surface water	USA	0.0010 µg/L	< 0.0010 µg/L	Skadsen *et al*., 2004
	Surface water	Italy	*<0.0003 µg/L*	0.0326 µg/L – 0.2489 µg/L	Zuccato *et al.*, 2005
	Stream water	USA	*0.05 µg/L*	0.06 µg/L – 0.73 µg/L	Kolpin *et al*., 2002
	Surface water	Italy	*0.0003 µg/L*	0.02440 µg/L	Calamari *et al.*, 2003
roxithromycin	Stream water	USA	*0.03 µg/L*	0.05 µg/L - 0.18 µg/L	Kolpin *et al*., 2002
	Surface water	Germany	*0.02 µg/L*	n.d. - 0.56 µg/L	Hirsch *et al.*, 1999
	Surface water (site 1)	USA	0.03 µg/L	0.04 µg/L ± 0.001 µg/L	Yang and Carlson, 2004a
	Surface water (site 2)	USA	0.03 µg/L	0.06 µg/L ± 0.002 µg/L	Yang and Carlson, 2004a
	Surface water (tributary)	Germany	*0.030 µg/L*	0.030 µg/L & 0.30 µg/L	Wiegel *et al.*, 2004
	Surface water	Germany, Czech Rep.	*0.030 µg/L*	0.030 µg/L - 0.040 µg/L	Wiegel *et al.*, 2004
chloramphenicol					
chloramphenicol	Surface water	Germany	*0.02 µg/L*	n.d. – 0.06	Hirsch *et al.*, 1999
trimethoprim					
Trimethoprim	Surface water	Canada	0.001 µg/L – 0.010 µg/L	0.134 µg/L ± 0.008 µg/L	Metcalfe *et al*., 2003
	Marine (harbour)	Canada	0.001 µg/L – 0.010 µg/L	0.043 µg/L ± 0.004 µg/L	Metcalfe *et al*., 2003
	Estuary (Tees River Estuary	UK	0.004 µg/L	< 0.004 µg/L - 0.017 µg/L	Thomas and Hilton, 2004
	Estuary (Tyne River Estuary)	UK	0.004 µg/L	< 0.004 µg/L - 0.046 µg/L	Thomas and Hilton, 2004
	Estuary (Mersey River Estuary)	UK	0.004 µg/L	< 0.004 µg/L - 0.569 µg/L	Thomas and Hilton, 2004

Table 1.3 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference(s)
trimethoprim (cont)	Surface water (tributary)	Germany	*0.030 µg/L*	< 0.030 µg/L & 0.040 µg/L	Wiegel *et al.*, 2004
	Surface water	Germany, Czech Rep	*0.030 µg/L*	< 0.030 µg/L - 0.030 µg/L	Wiegel *et al.*, 2004
	Surface water (upstream STP)	UK	*0.010 µg/L*	< 0.010 µg/L - 0.036 µg/L	Ashton *et al.*, 2004
	Surface water (downstream STP)	UK	*0.010 µg/L*	< 0.010 µg/L - 0.042 µg/L	Ashton *et al.*, 2004
	Surface water (upstream STP)	Sweden	NS	< 0.001µg/L	Bendz *et al.*, 2005
	Surface water (downstream STP)	Sweden	NS	0.01 µg/L - 0.02 µg/L	Bendz *et al.*, 2005
	Stream water	USA	*0.03 µg/L*	0.15 µg/L - 0.71 µg/L	Kolpin *et al.*, 2002
	Stream water	USA	*0.014 µg/L*	0.13 µg/L - 0.30 µg/L	Kolpin *et al.*, 2002
	Surface water	USA	0.0010 µg/L	<0.0010 µg/L	Skadsen *et al.*, 2004
	Surface water (Belfast Lough Estuary)	UK	0.004 µg/L	0.024 µg/L - 0.032 µg/L	Thomas and Hilton, 2004
	Surface water (Thames River Estuary)	UK	0.004 µg/L	< 0.004 µg/L	Thomas and Hilton, 2004

LOD = limit of detection; LOQ = limit of quantification; nd = not detected

1.5. Environmental Fate of Antibiotics

1.5.1. Adsorption and Absorption

The environmental compartment to which an antibiotic compound is transported, or the extent of transport, depends on the adsorption and absorption of an antibiotic (Boxall, 2004). Adsorption is the propensity for a molecule to sorb to the surface of soil, sediment structures, sludge, microorganisms and other particulate matter. In contrast, absorption involves the penetration of the antibiotic into the particulate matter (Ter Laak, 2005). This degree of absorption or adsorption of an antibiotic will be dependent on the charge, hydrophobicity and polarity of the antibiotic molecule as well as the characteristics of the natural environment, including the pH, organic carbon content of particulate matter (e.g. suspended solids, soil particles, sediment particles) and the charge on the surface of the particulate matter (Ter Laak, 2005). The extent to which an antibiotic will be bound to particulate matter can be predicted from the physio-chemical characteristics of the antibiotic, particularly the octanol water distribution coefficient (D_{ow}). (D_{ow}) is defined as a concentration ratio of a compound between two distinct phases, *n*-octanol and water at a given pH. D_{ow} is then modified to account for ionisable species at a given pH to give the octanol water coefficient (Log K_{ow}). If the (D_{ow}) of a compound is < 1 then a compound is not likely to sorb significantly. Values greater than 1 indicate sorption is likely (Kummerer, 2004).

1.5.2. Environmental Transport

Once antibiotics have entered the terrestrial environment they can be further transported to water bodies by three routes. The first of these, surface run-off, occurs when there is heavy rainfall or when additional water has been applied. Antibiotic residues (from soil amendment with manure, dung or slurry) on the soil surface can be carried into an adjacent water bodies after a rainfall event (Boxall *et al*., 2001). Antibiotics can also enter groundwater via leaching. During this process compounds are carried downward through soil horizons and are eventually carried into groundwater beneath soil or adjacent water bodies via drainage systems. If fissures exist within soil (due to dry weather or between rock formations for example) then preferential macropore flow may occur; antibiotics transported in this manner enter the aquatic environment more rapidly compared with leaching (Heberer, 2002).

Leaching of antibiotic compounds into the environment has also been reported from landfill sites. Holm *et al.* (1995) for example detected sulfamethazine in groundwater under a landfill site in Denmark. Daughton (2003) has also reported the potential for APIs to leach into the environment from medicated corpses.

1.5.3. Degradation

Antibiotics (and potential metabolites and transformation products of antibiotics) and other xenobiotic compounds can be subjected to a range of degradative processes in the aquatic and terrestrial environment, resulting in the dissipation of the parent compound. Degradative processes may result in the formation of one or more metabolites, one or more transformation products (such as R or S enantiomers) or the complete mineralisation of the compound into natural products. If a compound is recalcitrant to degradation then it is said to be persistent and will remain in the environment chemically unchanged (ECOTOC, 2005). Degradation can occur *via* either biotic or abiotic processes.

1.5.3.1. Abiotic Degradation

Abiotic degradation of antibiotics and other xenobiotic compounds occurs mainly *via* hydrolysis and photolysis. Hydrolysis reactions occur when a molecule reacts with the H_3O^+ and OH^- in water. Hydrolysis reactions result in two products, one that has gained electrons from OH^- ions and one that has donated electrons to H_3O^+ ions. Hydrolysis reactions are generally pH dependent since they depend on the H_3O^+/OH^- ratio of an environment. Temperature can also affect hydrolysis rates as can the combined effects of temperature and pH. For example Loftin *et al.* (2008) observed increased rates of oxytetracycline, tetracycline and CTC hydrolysis as a result of increasing temperature and pH. Several antibiotics have been shown undergo hydrolysis in the environment. These include oxytetracycline (Loftin *et al*., 2008; Pouliquen *et al*., 2007), fluorfenicol (Pouliquen *et al.*, 2007), oxolinic Acid (Pouliquen *et al.*, 2007), flurnequine (Pouliquen *et al.*, 2007), amoxicillin (Andreazzi *et al*., 2004), penicillin G (Li *et al.*, 2008) and sulfadiazine (Yang *et al.*, 2009).

Photolysis of antibiotics and other xenobiotic compounds involves the dissociation of a molecule as the result of contact with photons. Since a photon's energy is inversely proportional to its wavelength, photolysis can also involve electromagnetic waves with the energy of visible light or higher, such as ultraviolet light, x-rays and gamma rays. Two types of photolysis can occur in the environment, direct photolysis or indirect photolysis. Both reactions can occur simultaneously. Direct photolysis occurs when a molecule absorbs electromagnetic waves, causing decomposition of the excited state molecule. Indirect photolysis results from the chemical or electronic transfer from light-absorbing humic substances in natural water bodies. Iron compounds present in the environment can also result in indirect photolysis of antibiotics via the Photo-Fenton Process. Bautitz and Nogueira (2007) for example demonstrated that tetracycline photodegradation was favoured by the presence of an iron source under light conditions.

Photodegradation of several antibiotic classes has been shown to occur in the environment, including sulfonamides (Sukul *et al*., 2008; Thiele-Bruhn and Peters, 2007; Boreen *et al*., 2004),

tetracyclines (Thiele-Bruhn and Peters, 2007; Bautitz and Nogueira, 2007; Sanderson *et al*., 2005), amoxicillin (Andreozzi *et al.*, 2004), fluoroquinolones (Belden *et al*., 2007; Cardoza *et al*., 2005; Knapp *et al.,* 2005; Ferdig *et al*., 2005) and macrolides (Andreozzi *et al.,* 2006; Di Paola *et al*., 2006).

1.5.3.2. Biodegradation

Biotic degradation (biodegradation) has been described as the "molecular degradation of a substance, resulting from the complex action of living organisms" (Leisinger *et al*., 1981). As a result, environmental microbes are able to utilise xenobiotic molecules as substrates within their metabolism (Limbert and Betts, 1996). Microbial communities in the environment play distinct roles in the decomposition of organic xenobiotics, acting as degradative consortia (Pelz, 1999). Some members of the consortia may not be able to degrade a particular molecule but may be able to biochemically transform it. Another member of the consortia may be able to degrade a transformation product via a biochemical pathway within its metabolism (ECETOC, 2003). Many biodegradative reactions are catalysed by constitutive enzymes and rely on xenobiotic molecules having structural homology to molecules that are metabolised naturally. Parent compounds or transformation products may also be degraded if a microbial species is able adapt or acquire enzymes capable of degrading particular molecules. This can occur when a gene coding for a degradative enzyme is horizontally transferred from another member of the microbial community. In terms of antibiotic biodegradation certain members of the microbial community may possess transferrable resistance genes that confer a phenotype for enzymes that can degrade a particular antibiotic (see table 1.5, this chapter). It has also been demonstrated that a microbial community is able to degrade xenobiotic molecules *sensu stricto* (alien to existing enzyme systems) by evolving new metabolic pathways (Springael and Top, 2003). This directional evolution strategy highlights the selective advantage that microorganisms gain by degrading as many substrates as possible.

1.6. Microbial Function in the Environment

Micro-organisms have an array of highly evolved biochemical pathways and physiological systems that represent broad microbial function. Many of these systems represent important stages during ecologically significant processes. A portion of these processes can be assigned to the global cycling of nutrients. Microbial action is required for the efficient cycling of carbon, nitrogen and phosphorus in their respective cycles. A healthy aquatic ecosystem relies upon major nutrients being in a delicate balance. The Redfield ratio is generally regarded as the perfect balance of carbon: nitrogen: phosphorus in an aquatic environment which is 106:16:1 (Redfield, 1934). Liebergs's law of minimum additionally states that the growth of an organism (or organisms) will be limited by the scarcest nutrient that is acting as a limiting factor (Brown, 1942). The dynamic and continuous cycling of C, N and P to make nutrients available is therefore

essential to ensure the efficient growth of primary producers and other organisms within an ecosystem.

1.6.1. The Nitrogen Cycle

The nitrogen cycle involves the interaction of microbes, plants and animals during its seven major phases of biological activity. Micro-organisms are responsible for the cycling of inorganic nitrogen during this biogeochemical process (Payne, 1981). Micro-organisms contribute to nitrogen cycling in the terrestrial, aquatic and marine environment.

The conversion of diatomic nitrogen gas to ammonia represents the major biological process by which nitrogen is made available to ecosystems. This process (called nitrogen fixation) is carried out by bacterial genera such as *Rhizobium* and *Azobacter* via nitrogenase metaloenzyme complexes (Quispel, 1974; Burns and Hardy, 1975). "Fixed" nitrogen can also enter the environment *via* lightning discharges or during the application of fertilizers that contain nitrogen that has been fixed by the Haber process. Biological fixation accounts for the majority of global nitrogen fixation however (85%). Of this, 60% occurs on land and 40% can be attributed to the aquatic environment (Brock *et al*., 1984).

In the aquatic environment (marine and freshwater) nitrogen fixation may be conducted by a range of free-living aquatic bacteria. It is cyanobacterial taxa such as *Anabaena* and *Nostoc sp.* however that are responsible for the majority of aquatic nitrogen fixation, fixing up to ten times more nitrogen than other aquatic bacteria. They are able to do this by coupling the generation of ATP (via photosynthesis) with nitrogen fixation to produce nitrogen-containing compounds. Nitrogen fixation in the terrestrial environment is conducted by free-living symbionts of leguminous plants such as *Rhizobium sp.* and *Bradyrhizobium sp*. Symbionts of trees such as *Actinomycetes* also contribute to terrestrial nitrogen fixation. The mutuality of these relationships involves the transfer of metabolites between microbe and root nodules and is extremely important to maintaining soil fertility and therefore primary production (Atlas, 1988).

Ammonia can then be used as a nitrogen source by organisms within an ecosystem. Excreted nitrogenous waste or decomposition of dead cells can then return nitrogen to the environment as ammonia, a process known as ammonification. Ammonification is an aerobic process that is conducted predominantly by soil- and sediment-dwelling micro-organisms. This source of ammonia along with ammonia already present in the environment can then undergo further biological processing. This occurs in the form of nitrification. Nitrification is the aerobic oxidation of ammonia to nitrite, then nitrite to nitrate. These processes are performed by ammonia-oxidising bacteria (such as *Nitrosomas* and *Nitrosospira*) and nitrite-oxidising bacteria (such as *Nitrobacter* and *Nitrococcus*) respectively. Nitrification can also occur anaerobically in aquatic sediments via the ANAMMOX (ANaerobic AMmonia OXidation) reaction. Such

reactions are performed by Achaea species such as *Crenarchaeota* and γ proteobacteria. These bacteria have often been shown to express specialised enzymes, such as ammonia monooxygenases (Lam *et al*., 2007).

Nitrite and nitrate formed via nitrification can then enter the assimilatory pathway, where nitrite and nitrate reductase enzymes form ammonia from nitrite and nitrate respectively. Ammonia formed in this way can then be converted into nitrogenous compounds such as amino acids by organisms such as leguminous plants. Ammonia destined for this fate can also be produced by the dissimilatory pathway, during which certain bacterial species (such as *Aeromonas* and *Enterobacter*) grow anaerobically using nitrate as a terminal electron acceptor. Nitrite is formed as a result of nitrate reduction. Ammonia, diatomic nitrogen gas or nitrous oxide can be produced from further reduction of nitrite. The formation of nitrous oxide and diatomic nitrogen gas are performed by bacterial species such as *Aquaspirillium* and *Paracoccus denitrificans* respectively (Brock *et al*., 1984).

1.6.2. The Carbon Cycle

Carbon is an essential element in biological systems, composing an integral part of cellular components such as membranes, neurotransmitters and structural components. Cleavage of high-energy carbon bonds (commonly C-H) is also an important energy production strategy for living organisms. Carbon-containing gasses such as carbon dioxide and methane are also important greenhouse gases. Cyclical concentrations of CO_2 for example have been implicated in controlling the earth's temperature over time (Berner, 1990). The carbon cycle is a biogeochemical process where carbon is exchanged between the biosphere, atmosphere and hydrosphere. This represents the process of carbon recycling on planet earth.

A number of non-biological processes contribute to the global cycling of carbon. Weathering of sedimentary rocks containing carbonate ions releases carbon dioxide gas via a hydrogen carbonate intermediate. Volcanic activity also releases carbon into the atmosphere as carbon dioxide, carbon monoxide and methane. In addition to these, carbon can be released into the atmosphere as carbon dioxide by the burning of fossil fuels and wood from forests.

In the aquatic and terrestrial environment carbon is initially fixed into simple and complex carbohydrates (CH_2O) by plants and algae that contain chlorophyll (via photosynthetic processes). Carbohydrates may then be respired by these organisms, in which case CO_2 gas is released back into the atmosphere. Alternatively, plants and algae can be eaten by organisms living on a different trophic level and carbon is passed to these animals. Carbon can again be released into the atmosphere in the form of CO_2 gas as carbohydrates are respired. Digested, partially undigested or undigested food can then be used as a carbon source by terretrrial and aquatic bacteria and fungi. Dead animals, plants or fragments of animals and plants that have been shed (detritus in aquatic

systems and humus in terrestrial sytems) can also be utilised in a similar manner. Carbon can come from autochthonous (within the ecosystem) and allochtonous (external) sources. Humus can therefore act as a carbon source for aquatic micro-organisms also (Naumann, 1918). Microbial metabolism of humus, detritus, digested or undigested food releases carbon containing gasses (such as carbon dioxide and methane) into the atmosphere. Within microbial communities interbacterial carbon cycling can also occur via viral lysing, bacterial bactivory and leakage from the carbon cycle (Cole and Caraco, 1993).

In addition to detritus and humus, SOM in soil (soil organic matter) and DOC in aquatic systems (dissolved organic carbon) can be utilized as a carbon source by micro-organisms. Both forms of carbon are interchanged with POM (particulate organic matter) and can be quantified by bacterial biomass production (BBP) rates (how much carbon has been incorporated into microbial cells over a period of time). Alternatively, SOM and DOC can be mineralized to form carbon-containing gases such as carbon dioxide and methane. SOM contains a wide array of structural and cellular components from a diversity of organisms. These substances can be humic or non-humic. Humic substances (which make up around 60-80% of SOM) are composed of a wide range of organic molecules, including carbohydrates, amino acids and other nitrogenous compounds (such as amines and amides) as well as a range of carboxylic acids. Some of the most ecologically significant humic constituents are the carboxylated fulvic and humic acids. These not only provide buffering capacity but also act as an important carbon source to microorganisms. Non-humic substances (which make up around 20-30% of SOM) present in SOM include carbohydrates, proteins and other nitrogenous compounds and a vast and diverse array of polymers, such as lignin, fats, waxes and resins. Again, many of these compounds provide valuable carbon and nitrogen sources to environmental microorganisms (Nelson and Sommers, 1982).

DOC has also been reported to contain a wide range of organic molecules. Among the most prevalent of these are carbohydrates, carboxylic acids, amines and amino acids (McDowell and Likens, 1988). DOC has been shown to contain up to 50% humic substances, highlighting the importance of carboxylated DOC constituents. Humic DOC components have been shown to be important for bacterial production in the aquatic environment (Moran and Hodson, 1990).

The ability of a wide array of bacteria and fungi to degrade (and therefore utilise) SOM components has been further demonstrated, with the soil community having enzymes such as celluases (degrade cellulose), chitinases (degrade chitin), peptidoglycan hydrolases (degrade peptidoglycan) and polyphenol oxidases (degrade polyphenols) (Ekschmitt *et al.*, 2005). In the aquatic environment, microorganisms are capable of utilising a broad range of organic molecules contained within DOC (Jankhe and Craven, 1995). Whole-lake DOC addition experiments have shown that increased DOC results in increased bacterial production, highlighting the importance

of DOC to microbial communities (Blomqvist *et al*., 2001). Tranvik and Hofle (1987) have also demonstrated the high efficiency that organic molecules (glucose and phenol) are utilized (20% of DOC) based bacterial biomass calculations.

A wide and diverse range of heterotrophic bacteria (such as bacterioplankton) play an important role in the recycling of carbon. In addition, aquatic aerobic anoxygenic phototrophic Bacteria (AAPBs) have been shown to be able to utilise sugars, tricarboxylic acids, fatty acids, amino acids, alcohols, gelatins, starch and even detergents such as tweens when light energy is unavailable to fix carbon (Shiba and Simidu, 1982; Yurkov and Gorlenko, 1990; Shiba, 1991; Yurkov and Gorlenko, 1993; Yurkov *et al*., 1994).

Microorganisms also play an important role decomposing organic molecules of anthropogenic origin. Such compounds can occur in the environment as SOM or DOC. In the last fifty years a multitude of biodegradative pathways for organic pollutants have been mapped for a range of microbial species (Pelz, 1999), revealing the ability of environmental microbes to utilize anthropogenic organic compounds as a carbon and energy source (Mishra *et al*., 2001).

1.7. Reported Effects of Antibiotics on Environmental Microbes

The occurrence of antibiotics in the environment has led to a range of studies that have explored the effects of these substances on microorganisms. Effects have been observed in several matrices such as soil (e.g.: Chander *et al* 2005) surface water (eg: Schallenberg and Armstrong, 2004), and activated sludge (e.g.: Halling Sorenson, 2000). A diverse range of effects have been found following the exposure of microbes to a wide array of antibiotic classes with reported effects for each antibiotic differing greatly depending on which end-point is being evaluated. It is likely therefore that microbial end-points respond differently depending on the antibiotic that is being tested. It is also likely that different bacterial species and strains, as well as different microbial communities will respond differentially to specific antibiotics. This may be due to the fact that different bacteria and microbial communities have variable tolerances to the antibiotic or antibiotcs that are being tested.

1.7.1. Effects of Antibiotics on Single Species

Much of the work on effects of antibiotics on environmental bacteria has utilised single species testing. A popular organism for such testing has been the marine bacterium *Vibrio fischeri* which is the test organism in the commercially available bioluminescence inhibition test system, Microtox©. The test quantifies toxicity as a decrease in chemofluorescence produced by the bacterium compared with a control over a period of 30 minutes. The reduction in chemofluorescence can be the result of either the inhibition of the growth of the *Vibrio fischeri* population or by the inhibition of the organisms' luciferase (the enzyme involved in

bioluminescence production) based biochemistry. The Microtox system has been used extensively as a standard test in recent years.

Isidori *et al.* (2005) examined the potential toxicity of six antibiotics (erythromycin, oxytetracycline, sulfamethoxazole, ofloxacin, lincomycin and clarythromycin) using the Microtox test. It was found that three of the test compounds studied exerted an EC_{50} (the concentration of a chemical required to exert an effect equal to 50% of control values) value of below 100 mg/L. These were oxytetracycline (64.5 mg/L), sulfamethoxazole (23.3 mg/L) and ofloxacin (25% inhibition at 100 mg/L). The authors considered these effect concentrations to be higher than expected. When Christensen *et al.* (2006) tested the potential toxicity of various antibiotics (oxytetracycline, erythromycin, ofloxacin and oxolinic acid) and antibiotic mixtures (oxytetracycline plus flumequine, erythromycin plus flumequine and oxytetracycline plus erythromycin) using the Microtox system, relatively high acute EC_{50} values were observed. These values ranged from 66 to 560 mg/L. Of these values oxytetracycline exerted the highest EC_{50} value (66 mg/L). Exposure to all antibiotic mixtures yielded scattered data that did not fit the analysis used by the authors. Relatively high EC_{50} values were also observed by Kim *et al.* (2007) when they used the Microtox assay to assess the toxicity of six widely used sulfonamides. In this study chemoluminescence was read after 5 and 15 minutes to determine the acute toxicity of the compounds tested. In the case of sulfamethoxazole EC_{50} values of 74.2 mg/L and 78.1 mg/L were observed after 5 and 15 minutes respectively. For sulfachlorpyridazine EC_{50} values were 53.7 and 26.4 mg/L. When Lalumera *et al.* (2004) investigated the acute toxicity (30 minutes) of flumequine and oxytetracycline towards *Vibrio fischeri* they also observed relatively high EC_{50} values of 12.1-15.3 mg/L and 121-139 mg/L respectively. The observed insensitivity of the Microtox test to the antibiotics may be a result of the short test duration (30 minutes).

Several other studies that have conducted the Microtox assay over a longer exposure period. Froehner *et al.* (2000) exposed *Vibrio fischeri* to nalidixic acid, chloramphenicol and streptomycin sulphate for 24 hours. In the case of nalidixic acid and streptomycin sulphate, no EC_{50} values could be determined after 30 minutes. However EC_{50} values observed after 24 hours were 0.21 mg/L for nalidixic acid and 21 mg/L for streptomycin sulphate. Chloramphenicol had a 30 minute EC_{50} value of 81 mg/L compared with a 24 hour EC_{50} value of 0.07 mg/L. Backhaus *et al.* (1997) also observed significantly lower EC_{50} values using a chronic bioluminescence test compared with short term testing. In the case of nalidixic acid no effects were observed in acute testing but an EC_{50} value of 0.18 mg/L was observed after a 24 hour exposure. When tetracycline was tested an EC_{50} value of 19.6 mg/L was obtained in an acute (30 mins) test compared with a chronic (24h) EC_{50} value of 0.02 mg/L. In another study Backhaus and Grimme (1999) observed a 24 hour EC_{50} value of 0.06 mg/L for chloramphenicol. They also tested a range of other antibiotics and disinfectants in the same study. 24 hour EC_{50} values were found to be relatively lower than those that were obtained during a shorter exposure period. Indeed, of the antimicrobial

compounds tested, chloramphenicol, tetracycline, nalidixic acid, norfloxacin and ofloxacin all exerted EC_{50} values of < 1 mg/L. It is interesting to note that agents that inhibit DNA/RNA were shown to be the most potent in this study, perhaps indicating the Microtox assay is suited towards testing the toxicity of compounds that are targeted to such mechanisms of action.

Work has also been conducted to evaluate the impacts of antimicrobial compounds on other microbial test species. Kummerer *et al.* (2000) demonstrated that ciprofloxacin and ofloxacin inhibited the growth of the Gram-negative species *Pseudomonas putida* with EC_{50} values of 80 μg/L (ciprofloxacin) and 10 μg/L (ofloxacin) being obtained. Since *Pseudomonas putida* is seen as being a model organism for Gram-negative environmental microbes it can be conferred these compounds may be toxic to a range of environmental microbes in general. The compounds studied were also broad-spectrum antibiotics, suggesting that wider effects may be seen with these compounds. Halling-Sorensen *et al.* (2002) also observed effects of antibiotics on pseudomonads. In this study, the growth rates of 15 pseudomonad strains (isolated from the soil microbial community) were observed in the presence of geometrical concentrations (0.25-32 mg/L) of chlortetracycline, oxytetracycline, tetracycline and tetracycline degradation products. Exposure to parent compounds resulted in MIC_{50} (minimum concentration of toxicant required to cause 50% inhibition, generally of microbial growth) values of 2 mg/L (tetracycline), 0.5 mg/L (CTC) and 1 mg/L (oxytetracycline). Degradation products of tetracycline were found to be generally less inhibitory to the growth of pseudomonads, with MIC_{50} values in the range of 12-32 mg/L. The same exposure conditions were also applied to four other soil isolates (*Agrobacterium sp.*, *Moraxella sp.* and two strains of *Bacillus sp.*). These isolates were generally more sensitive to the parent compounds (all three compounds causing an MIC_{50} of 0.25 mg/L). In addition, they were generally more sensitive to tetracycline degradation products; MIC_{50} values were in the range of 0.25-32 mg/L. These results highlight the fact that different species and strains show differential sensitivity to antibiotics. The also study highlights the fact that pseudomonads often show tolerance to a broad range of toxicants, explaining the higher MIC_{50} values that were obtained.

Other studies have looked at more specific effects of antibiotics on pseudomonads. Linares *et al.* (2006) conducted experiments that could detect specific effects of tobramycin, tetracycline and norfloxacin at sub-inhibitory concentrations (determined from minimum inhibitory concentration experiments). In these experiments, the exposure of the pathogenic *Pseudomonas aeruginosa* to all three compounds resulted in increased biofilm formation and cell motility (in the case of tobramycin). Stimulation of these phenotypes may result in the antibiotic-susceptible pathogenic *Pseudomonas aeruginosa* having a selective advantage due to their ability to swarm at a greater rate and thus exploit new heterogenous habitats in the presence of the antibiotics. In addition, exposure to sub-inhibitory concentrations of tetracycline was found to stimulate a type III secretion system (a needle-like protein appendage that is released by Gram negative bacteria)

resulting in increased cytotoxicity. Increased cytotoxicity may result in an increased chance of survival of *Pseudomonas aeruginosa* due to an increased ability to survive predation.

Single species tests have also been utilised to test the effects of tetracycline and tylosin that are bound to soil particles. Chander *et al.* (2005) inoculated two different soil types (Webster clay loam and Hubbard loamy sand) with three environmental species of bacteria (antibiotic resistant and sensitive *Salmonella* and *Escherichia coli* ATCC 25922). Dynamic and static conditions were simulated to represent effects in soils that had and hadn't been mixed with manure or nutrients. A decline in CFUs (colony forming units) of 40-60% was observed in soils spiked with tetracyclines, with the greatest decline being recorded with *Escherichia coli* ATCC 25922 CFU counts. Exposure of the three microbial species to tylosin caused a 20-30% reduction in CFU counts. Sensitive *Salmonella* strains were found to be the most sensitive species studied. This investigation further highlights the potential for different antibiotic compounds to inhibit specific processes in single microbial species, in this case the number of original cells that are seeded onto an agar plate. The study also indicated that antibiotics retain at least some of their activity when bound to soil particles and are still bioavailable in such a scenario.

The effects of antimicrobial compounds have also been tested on single species of cyanobacteria. Ando *et al* (2007) investigated the effect of seven antibiotic compounds (sulfadimethoxine, erythromycin, thiamphenicol, ampicillin, oxytetracycline, norfloxacin and trimethoprim) on the growth of eight species of cyanobacteria (*Anabe thana cylindrical* NIES-19, *Anabena flos-aquae* ATCC 29413, *Anabena variabilis* NIES-23, *Microcystis aerugenosa* NIES-44, *Microcystis wesenbergii* NIES-107, *Nostoc sp.* PCC 7120, *Synechococcus leopoldensis* IAM M-6, *Synechococcus sp.* and PCC 7002). Each species was grown in the presence and absence of antimicrobial compounds and the OD_{655} value (optical density at 655 nm) was taken as a measure of cellular growth. EC_{50} values for each exposure revealed that all nine cyanobacterial species were susceptible to most of the antimicrobial compounds tested at low concentrations (most EC_{50} values were < 1 mg/L), with the exception of sulfadimethoxine and trimethoprim. Of the different cyanobacteria tested, *Microcystis aerugenosa* and *Synechococcus sp.* were found to be the most sensitive to the compounds tested, with EC_{50} values falling well below 1 mg/L for five and four of the test compounds respectively. This study therefore illustrated the sensitivity of cyanobacteria to antimicrobial compounds in the aquatic environment. In another study by Robinson *et al.* (2004), *Microcystis aerugenosa* was shown to be sensitive to fluoroquinolone antibiotics. In five day growth tests, EC_{50} value of 7.9 μg/L (levofloxacin), 17 μg/L (ciprofloxacin), 21 μg/L (ofloxacin), 49 μg/L (enrofloxacin), 51 μg/L (levofloxacin) and 1960 μg/L (flumequine) were recorded. Again, this study highlighted the tendency for cyanobacterial processes to be inhibited by antimicrobial compounds. Halling-Sorenson *et al.* (2000) further showed the susceptibility of *Microcystis aerugenosa* to antimicrobial compounds. In growth inhibition experiments in the presence of mecillinam and ciprofloxacin, EC_{50} values of 0.06 and 0.005 mg/L (respectively) were reported. In

a second set of experiments *Microcystis aerugenosa* was shown to be extremely sensitive to a larger set of antimicrobial compounds (benzylpenecillin, CTC, olaquindox, spiramycin, streptomycin tetracycline, tiamulin and tylosin). With the exception of olaquindox, all compounds tested exerted an EC_{50} value of < 0.1 mg/L. The lowest EC_{50} value published was that of tiamulin (0.003 mg/L). The susceptibility of *Microcystis aerugenosa* to a wide range of antimicrobial compounds was therefore demonstrated.

1.7.2. Effects of Antibiotics on Microbial Respiration

Several authors have also reported toxicity of antimicrobial compounds towards microbial communities in the environment. One of the most studied effects has been on microbial respiration. Thiele-Bruhn and Beck (2005) for example conducted substrate-induced respiration (SIR) to test the effects of sulfapyridine and oxytetracycline. They conducted short-term (4 hour) and long-term (24 hour) incubations in the presence of various antibiotic concentrations and glucose (as a substrate). Experiments were conducted in two soil types (cambisol and luvisol). A significant inhibition of SIR occurred in 24-hour incubations in the case of both antibiotics tested and in both soil types. ED_{50} (dose that induces 50% inhibition) values of 6.2 and 11.5 μg/L (cambisol and lumisol respectively) were detected for sulfapyridine and 19.1 and 31.2 μg/L (cambisol and lumisol respectively) were detected for oxytetracycline. This study therefore showed the potential for various bacteriostatic agents to exert an inhibitory effect on microbes that are found in different soil types. It is interesting to note that no inhibitory effects were observed after a 4 hour incubation using SIR. These results are in agreement with variable time exposure results that have been detected with other assays (such as Microtox).

In another experiment Zielezny *et al.* (2006) tracked SIR activity over a time period in soil microcosms that had been spiked with CTC and sulfadiazine. Varying levels of respiratory inhibition were detected. When microcosms were spiked with sulfadiazine and glucose a dose-dependent decrease in microbial respiration was detected over time. It was observed that this inhibitory effect only occurred in the presence of glucose, indicating that microbial growth is a requirement for studies that track microbial activity over time in the presence of bacteriostatic antibiotics. When respiration in the presence of CTC was tracked over time no significant differences (compared with control microcosms) were observed (both with and without glucose addition). The lack of response by soil microbes to CTC was probably due to the compounds sorption to the orthic luvisol in which the experiment was conducted. In contrast to this study, Vaclavik *et al*. (2004) found that exposure to the antibiotics tylosin, tetracycline, CTC, sulfachloropyridazine and erythromycin increased SIR in soil microcosms. The increase was in the order of 1.3-1.7 times. When the tetracycline compounds were re-added into the microcosm system (days 36-100), a further increase in the soil respiration rate was observed (1.5-2.0 times the basal respiration rate). Further tylosin addition at the same time-point resulted in a reduction of respiration rate to basal levels. Addition of sulfachloropyridazine at day 36 resulted in a 0.8

fold decrease in respiration rate from day 36 to 100. These data therefore represented a variable temporal response of the microbial community to antibiotics. Schallenberg and Armstrong (2004) observed an increase in the microbial respiration rate when studying the effect of a mixture of antibiotics on microbial respiration in surface water. This significant (but undisclosed) increase in microbial respiration was observed after previous inhibition (significant but undisclosed value) was detected. The authors speculated that intermittent detoxification might explain these results. Vaclavik *et al.* (2004) theorised that three possible reasons could explain the observed increase in respiration rate. The first of these could have been that because of the specific mode of action of antibiotics, only a certain percentage of the microbial community were affected by the addition of antibiotics. Competing portions of the microbial community may therefore have been able to become more active (in terms of the measured respiration rate). In addition, dead cells may act as a carbon (and therefore energy) source for the living fraction of the microbial community and a corresponding rise in respiration rate may occur. Increased stress levels within the microbial community could also have caused an increase in respiration rate. Not all studies that have observed the effect of antibiotics on microbial respiration have amended their test system with a substrate such as glucose however. Landi *et al.* (1993) tracked CO_2 evolution in different soils (arable and forest) that had been incubated in glass mesocosms. Arable soils that been exposed to streptomycin were shown to have a significantly lower level of CO_2 evolution and therefore a lower respiration rate. In contrast, CO_2 evolution in forest soils actually increased in the presence of streptomycin. Inhibition of respiration in arable soils is believed to be caused by the antimicrobial activity of streptomycin, suggesting that the compound has antimicrobial activity in arable soils. The study did not find similar effects in forest soils however. Possible reasons for this are the utilisation of streptomycin as a carbon source (and thus respiration mediated energy source) or the ability of forest soils to utilise an increased number of dead cells (caused by biocide activity) as a carbon (energy) source. This paper once again shows the variable effects of antibiotics that have been detected when investigating microbial respiration.

1.7.3. Effects of Antibiotics on Microbial Growth

Experiments that have tested the effects of antimicrobial compounds on the growth of microbial communities have also been conducted. Growth inhibition tests have been widely applied in a number of studies (sometimes in conjunction with other methods). The effects of antimicrobial compounds on the growth of activated sludge microbes have been studied the most extensively. Kummerer *et al.* (2000) for example determined CFU (colony forming unit) counts in test vessels containing wastewater in the presence of several antibiotics. Although no EC_{50} values were obtained a slight but significant effect on CFU counts was observed in activated sludge in the presence of ciprofloxacin. No effects on CFU counts were observed in the presence of ofloxacin or metronidazole. This study therefore suggested that ciprofloxacin slightly inhibits the growth of aquatic wastewater microorganisms.

Halling Sorensen *et al.* (2002) were also able to show effects of antimicrobial compounds (tetracycline, CTC, tetracycline degradation products and CTC degradation products) on activated sludge CFU counts (acting as a representative surrogate for environmental bacteria). Using percentage inhibition (of CFUs) to calculate EC_{50} values, the authors observed widespread inhibition of CFU counts. The lowest EC_{50} values that could be observed were 0.03 mg/L (5a, 6-anhydrotetracycline hydrochloride and CTC) compared with 6 mg/L for the toxicity reference compound 3, 5-dichlorophenol. Indeed all but two of the compounds tested (iso-CTC and ter-oxytetracycline) had an EC_{50} value below that of the toxicity reference compound. The tendency for tetracyclines and tetracycline degradation products to inhibit bacterial colony formation (and therefore growth) on agar plates was therefore demonstrated. Halling-Sorenson *et al.* (2002) showed a reduction in the potency of selected antibiotics (oxytetracycline, tylosin, sulfadiazine, streptomycin, ciprofloxacin and olaquindox) in activated sludge and soil interstitial water over time in both dark and light conditions by using CFU counts as a toxicity end-point. It was observed that the potency of most antibiotics tested generally declined over time (a reduction in CFU counts were was measured) in both soil interstitial water and activated sludge, except as the result of exposure to ciprofloxacin and oxytetracycline. In the case of ciprofloxacin the potency of the compound remained unchanged over time with all treatments. The potency of oxytetracycline in this study showed an increase over time in soil interstitial water. The cause of this effect was attributed to toxicity of degradation products, as removal of parent compound alone could not explain the increase in compound potency. A reduction in potency over time with other compounds tested was thought to be the result of degradation of the parent compound, formation of less toxic transformation and degradation products or due to the possibility that portions of the microbial community had become tolerant to antimicrobial compounds. Colinas *et al.* (1994) also investigated the effect of antibiotics and mixtures of antibiotics (oxytetracycline and penicillin) on soil CFU counts. In this study active CFU counts were also taken (by staining cells). Exposure to the two compounds resulted in active CFU counts being reduced by 20% compared with the control value and total CFU counts being reduced by 29%. Not only did these results provide another example of antibiotics inhibiting soil CFU counts, but also illustrated the potential for specific antimicrobial compounds to inhibit active CFU counts. These data suggest that the cultivable portion of the microbial community as well as the total cultivable portion of the active microbial community in soil are susceptible to an oxytetracycline-penicillin mixture.

1.7.4. Effects of Antibiotics on Specific Cellular Function

Many studies that have assessed the effects of antimicrobial compounds have focused on investigating more specific cellular functions. Thiele-Bruhn (2005) for instance tested the potential effects of a range of antibiotics (CTC, sulfadimethoxine, oxytetracycline, sulfadiazine, sulfadimidine and tetracycline) on iron (III) reductase activity (an important metabolic enzyme) of bacteria from different soil types. A wide range of ED_{50} (dose of test compound required to cause a 50% inhibition in end-point) values were recorded for each compound tested. Tetracycline

values for example ranged from 2.5–580 µmol/kg. It is thought that this range could be explained by differential sorption of test compounds to different soil types. Most significantly, three of the compounds tested showed sufficient inhibitory activity to cause ED_{10} values (dose of test required to cause 10% reduction in end-point activity) of < 100 µg/kg. These compounds were the sulfonamides sulfadiazine, sulfadimidine and sulfadimethoxine. This study therefore demonstrated the sensitivity of bacterial iron (III) reductase to sulphonamide exposure in certain soil types. The tendency for different soil types to display a variable iron (III) reductase response to antibiotics was also shown.

Other effects on bacterial enzymes have also been reported. Boleas *et al.* (2005) examined the effects of oxytetracycline on dehydrogenase and phosphatase activity in a multi-species soil test systems. A significant inhibition in enzyme activity was observed at all concentrations in soils that had been amended with manure. The results from this study indicated that oxytetracycline concentrations as low as 0.01 mg/kg can cause an inhibition in phophatase activity. As these levels are close to environmentally realistic concentrations, the compound has shown the ability to interfere with phosphorus cycling in soils that have been manured.

The effect of oxytetracycline on dehydrogenase activity was found to vary, depending on whether or not manure was added to the system. In soil amended with manure a significant reduction in dehydrogenase activity was measured on day 7. Soils that had not been amended with manure however showed a significant inhibition in dehydrogenase activity after 21 days. In both cases these effects were seen at an oxytetracycline concentration of 100 mg/kg. As the dehydrogenase assay relates to carbon utilisation these results illustrated the ability of oxytetracycline to interfere with carbon cycling in soil at a relatively high oxytetracycline concentration.

Other studies have also shown effects of antibiotics on processes that occur within carbon cycling. Maul *et al*. (2006) investigated the effect of ciprofloxacin on multi-substrate carbon utilisation by leaf-bound microbial communities. Using Principal Component Analysis (PCA), it was found that a significant variation in principle component (PC) scores could be observed at 100 µg/L ciprofloxacin (compared with other treatments). In addition, utilisation of carbohydrates was shown to be 2.7-3.5 times lower at the highest exposure concentration of ciprofloxacin (100 µg/L). These data showed that ciprofloxacin significantly inhibited the ability of the microbial community (or at least part of it) to metabolise carbohydrates. The data also demonstrated that the cycling of certain macromolecules within the carbon cycle can be affected by 100 µg/L ciprofloxacin.

Kong *et al.* (2006) assessed the effect of oxytetracycline on multi substrate utilisation by soil microbial communities. They reported that oxytetracycline concentrations as low as 1 µM/L resulted in a significant reduction in total carbon utilisation. They also reported a reduced onset of

carbon utilisation and less total carbon utilisation although this was not quantified numerically. When the team looked at how different substrate types were affected they found that oxytetracycline significantly reduced substrate utilisation at 1 μM/L and above, with polymer utilisation being the most affected. Oxytetracycline concentrations of greater than 1μM/L negatively affected substrate utilisation more than 0 μM/L and 1 μM/L; this was reflected by PCA analysis, with 0 μM/L and 1 μM/L oxytetracycline clustering close to each other but far apart from higher oxytetracycline concentrations. These data therefore suggested that an oxytetracycline concentration of more then 1 μM/L results in a change in microbial community structure.

Schmitt *et al.* (2005) used multi-substrate carbon utilisation profiling to explore the effects of antibiotics (sulfachloropyridazine) on microbial function and community structure. Using a Pollution Induced Community Tolerance (PICT) approach (with Biolog plates inoculated from soil extracts) and generating community level physiological profiles (CLPP) data, they showed that sulfachloropyridazine shifted physiological fingerprints on the second PCA axis compared with controls. These results suggested that a toxicant-induced change in the manner in which the microbial community utilised various substrates occured. The authors also reported an increase in tolerance of the microbial community to sulfachloropyridazine as a result of fresh pig manure amendment. It was theorised that this could be an artefact of resistance gene transfer from microbial communities in the gastrointestinal tract of pigs.

Effects of antibiotics on the breakdown of manure itself have also been tracked (Arikan *et al.*, 2006). In this study the production of biogas (methane) was monitored in manure from cattle that had been medicated with oxytetracycline. It was observed that total biogas production (from anaerobic digestion of manure) in medicated cattle was significantly lower than biogas production in non-medicated animals; a difference of 27 % was calculated. The results from this study are in agreement with other studies. Gamal-El-Din (1986) for example observed up to 49% inhibition of biogas production due to oxytetracycline exposure. Sankvist *et al.* (1984) found that oxytetracycline also inhibits the portion of the microbial community that anaerobically digest pig slurry; a 50% inhibition was observed for six consecutive days, although this occured at a relatively high oxytetracycline concentration of 100 mg/L. This reduction represents inhibition of the part of the microbial community that liberates carbon from manure. These studies have therefore shown that tetracyclines in medicated animal manure inhibit this part of the carbon cycle. As pointed out by Arikan *et al.* (2006) in their study, there are also economic implications for farmers. Reduced biogas production will in turn result in less energy generation or natural gas being sold to the public. Farmers may in addition have to rely on other forms of fuel, possibly resulting in increased prices of produce.

In addition to effects that have been observed on the carbon and phosphorus cycles, there have also been effects seen on the nitrogen cycle as a result of antibiotic exposure. Klaver and Matthews (1994) conducted aquaria experiments (spiked with oxytetracycline); aquaria water had been inoculated with two nitrifying microbial species, *Nitrosomas* and *Nitrobacter*. They observed almost complete inhibition of nitrification within seven days of the experiment. EC_{50} values were reported to be between 8.6 and 29 mg/L during the seven-day exposure. This disruption of the nitrification process could potentially lead to a build-up of toxicants (ammonium and nitrite) within an aquatic system. The potential for the ecology of aquatic systems exposed to tetracyclines to be adversely affected was therefore demonstrated. Campos *et al.* (2001) also observed a reduction in nitrification rates. In experiments that used sludge fermenters spiked with oxytetracycline, it was found that high concentrations of oxytetracycline (EC_{50} = 250 mg/L) resulted in a significant reduction in nitrification. Although high concentrations were used in this study, the results still show the potential for tetracyclines to disrupt the nitrification process and therefore disrupt nitrogen cycling in sludge. Halling-Sorensen (2000) looked at the effects of a range of antibiotic compounds (benzyl penicillin, CTC, oxytetracycline, olaquindox, streptomycin, tiamulin, tylosin, sulfadiazine, metronidazole and oxolinic acid) on nitrification in sewage sludge. It was found that oxytetraxycline, tetracycline, CTC and streptomycin inhibited measured nitrification rates. Conversely, sulfadiazine, oxolinic acid and tylosin were shown to increase nitrification rates. Although EC_{50} values were not published for these tests, values were given for follow up experiments that looked at growth inhibition of the nitrifying bacteria *N. europaea*. The compounds that exerted the greatest inhibitory effect were the tetracyclines and oxolinic acid, with EC_{50} values in the range of 0.002–0.5 mg/L. The study therefore illustrated the inhibitory effects of broad-spectrum antibiotics on sewage sludge microbes. As such microbes are often seen as being representative of aquatic bacteria (in the OECD 209 test, for example) the potential for the compounds tested to inhibit nitrification in rivers is revealed. In addition, the presence of broad-spectrum antibiotics in sewage treatment plants could potentially inhibit the integral process of nitrification (a key set of reactions which occur in sewage treatment works).

There have also been reported effects of antibiotics on nitrification by soil microbial communities. Landi *et al.* (1993) explored the effect of streptomycin on nitrification rates in two different soil types (arable and forest). Although an initial increase in nitrification was noted (in arable soil) on day one, values decreased compared with the control value after this period. No effects on nitrification were observed in forest soils however. As nitrification is an important part of the nitrogen cycle in soils, these results presented the possible disruption of nitrogen cycling in arable soils as a result of streptomycin exposure. It should be remembered however that the dose of streptomycin that was applied to soils in this study was relatively high (3 mg/g).

Other studies have explored the potential impacts of antibiotic exposure on other nitrogen cycle processes. Costanzo *et al.* (2005) for example explored the effect of a range of antibiotics

(ciprofloxacin, amoxicillin, clarithromycin, ampicillin, amoxicillin/clavaulinic acid and erythromycin) on the process of denitrification in aquatic sediments. At concentrations of 1000 µg/L statistically significant effects were observed on denitrification due to exposure of the microbial community to clarithromycin, erythromycin and amoxicillin. The denitrification process was especially sensitive to amoxicillin exposure.

The inhibition of another process which occurs within the nitrogen cycle, ammonification, was shown to be significantly inhibited in studies which had exposed microbial communities to ampicillin under laboratory conditions (Nimenya *et al.*, 2000). In flask studies that tracked the formation of ammonium from cattle urine, ampicillin significantly inhibited the process in both the presence and absence of a urease enzyme (an enzyme that catalyses the breakdown of urea). From these results it was inferred that ampicillin inhibits the ammonification of urine by microbial communities. As this process is crucial in the environment (especially on land that supports livestock) any inhibition could lead to the build-up of toxic nitrogenous compounds (such as urea). Arikan *et al.* (2006) also studied ammonification rates from animal waste (cattle manure). A transient inhibition (but not statistically tested) in ammonification rates as a consequence of oxytetracycline exposure was observed. The inhibition was not sufficient to result in a toxic build-up of ammonia (that could potentially inhibit anaerobic digesters).

The ability of environmental microbes to incorporate (rather than breakdown) nitrogenous compounds has also been shown to be affected by exposure of environmental bacteria to antibiotics. Verma *et al.* (2007) looked at the impact of tetracycline exposure on the ability of planktonic bacteria to incorporate the amino acid leucine. They found that free (unbound) tetracycline concentrations of 5 and 1000 µg/L significantly affected leucine incorporation in river and wetland waters respectively. As leucine is an important amino acid that is required for bacterial protein production, any disruption in acquiring this nutrient could reduce the selective advantage of a (sensitive) microbe or microbial community. Protein production in planktonic bacteria was therefore shown to be extremely sensitive to tetracycline exposure in the aquatic systems that were investigated.

1.7.5. Effects of Antibiotics on Xenobiotic Degradation

The efficiency with which environmental microbes degrade xenobiotics in the presence of antibiotics has also been investigated. Chun *et al.* (2005) for example examined the effect of sulfamethazine, tylosin and CTC on the biodegradation and transformation of 17 β-Estradiol in soil (sequatchie loam). The authors reported a significant decrease in the transformation of 17 β-Estradiol (to its metabolite estrone) in the presence of all antibiotics. Interestingly, the authors noted a significant inhibition in dehydrogenase activity as a result of antibiotic exposure. This led them to the conclusion that dehydrogenases may be responsible for transformation and partial degradation of 17 β-Estradiol in the environment. Moreover, the inhibition of dehydrogenases

results in reduced transformation/degradation of 17 β-Estradiol. Allen and Walker (1988) investigated the effect of antibiotics on the degradation of pesticides. They tracked the degradation of metamitron, metazachlor and metribuzin in two soils exposed to novobiocin. Although CO_2 levels were found to be higher in both soils tested, novobiocin was found to inhibit the degradation of metazachlor and metribuzin significantly. In one soil novobiocin was also shown to inhibit the degradation of metamitron, although the extent to which this occured was minimal. The study highlighted the sensitivity of the portion of the microbial community that can degrade certain xenobiotics in different soil types. In another study by Montiero and Boxall (2009), the degradation of the anti-inflammatory drug naproxen by soil microbial communities was observed to be inhibited by the veterinary antibiotic sulfamethazine. The authors warned of the potential for mixtures of pharmaceuticals exerting a greater effect than single environmental toxicants.

1.8. Antibiotic Resistance

Antibiotic resistance is the ability of bacteria to grow and survive under normally inhibitory antibiotic concentrations (Davies, 2007). The prokaryotic kingdom has evolved specific and non-specific responses to become resistant to the effects of anti-microbial compounds. Alexander Fleming observed this during initial observations with penicillin and warned of the tendency for microorganisms to become resistant (Fleming, 1928). In the 1950s Streptomycin resistance had been observed in clinical situations with the first multi-drug resistant strains being recorded in Japan in 1955. Since then antibiotic resistance has been observed for a wide array of anti-microbial compounds (Davies, 2007). Indeed, Louise Pasture once commented, "the microbes will always have the last word," highlighting the metabolic and evolutionarily diversity of prokaryotes in response to antibiotic chemotherapy.

1.8.1. Mechanisms of Resistance

Antibiotic resistance occurs via a range of cellular biochemical mechanisms. These mechanisms are the phenotypic expression of genetic determinats called antibiotic resistance genes. Resistance genes can arise either as a result of mutated DNA (plasmid or chromosomal) or can be acquired via horizontal gene transfer or gene induction. This is the process whereby foreign DNA can be introduced into a bacterial chromosome via a bacteriophage (bacterial virus). Some mechanisms of resistance can also be an artefact of natural cellular processes (intrinsic resistance). Table 1.4 summarises the main types of resistance and how they occur. Table 1.5 summarises the specific genes and phenotypic mechanisms by which antibiotic resistance occurs. A more detailed description of the mechanism of resistance selection is provided in chapter 5.

Table 1.4: Main types of intrinsic, mutational and acquired resistance mechanisms. Mechanisms in italics indicate those mechanisms that are normally mutational (adapted from Davies, 2007).

Category of Resistance Mechanism		
Intrinsic	**Mutational**	**Acquired**
Inaccessibility of target	Target site modification	Target site modification
Multi-drug efflux systems	Target amplification	Target amplification
Drug inactivation	Repair of damaged target	Repair of damaged target
	Biofilm formation	Biofilm formation
	Increased efflux	Increased efflux
	Decreased influx	Decreased influx
	Enzyme inactivation of antibiotic	Enzyme inactivation of antibiotic
	Sequestration of antibiotic	Sequestration of antibiotic
	Target bypass	Target bypass
	Protection of target	Protection of target
	Intracellular localisation of antibiotic	Intracellular localisation of antibiotic

Table 1.5: Resistance mechanisms of major antibiotic classes.

Antibiotic Class	Phenotypic Mechanism(s)	Reference(s)
Aminoglycosides	Enzymatic modification of Aminoglycosides by acetyltransferases, nucleotidal transferases, adenylyltransferases and phosphotransferases. Modified Aminoglycoside molecules can no longer bind to the 30S ribosomal sub-unit allowing protein synthesis to occur normally.	Shaw *et al.*, 1993
Bacitracin	Cellular efflux of Bacitracin via ATP-binding cassette (ABC) transporter reducing intracellular Bacitracin concentration; increased phophorylation of C_{55}-isoprenyl phophate allowing peptidoglycan synthesis to occur; secretion of exoploysacharride, thickening Gram-positive cell wall to act as increased physical barrier to Bacitracin entry to cell.	Cain *et al.*, 1993; Pollock *et al.*, 1994; Tsuda *et al*., 2002
Beta-lactams and Cephems	>190 group 1-4 proteins and novel beta-lactamases hydrolyse beta-lactam ring structure. Modified beta-lactam molecules are then unable to act as analogues of the terminal peptide sequences of peptidoglycan precursor NAM/NAG-peptide.	Bush, 1989a; Bush, 1989b; Medeioros, 1989; Jacoby and Medeioros, 1991
Chloramphenicol	Acetylation of chloramphenicol molecules by chloramphenicol acetyltransferase enzymes. Modified chloramphenicol molecules are unable to bind to 23s ribosomal sub-unit and protein synthesis can proceed normally; reduced permeability of bacterial cells to chloramphenicol due to the loss of a 40 kDa protein.	Shaw, 1967; Shaw and Unowsky, 1968; Burns *et al.*, 1985
Glycopetides	Direction of peptidoglycan synthesis from precursors with C-terminus D-alanyl-D-lactate (VanA, VanB and Van D) or D-alanyl–D-serine (VanC, Van E and VanG). Modifications significantly reduce the affinity of peptidoglycan precursor to glycopeptides. Cell wall biosynthesis can therefore occur.	Nieto and Perkins, 1971
Macrolides	Production of Macrolide methylase enzyme that catalyses N^6-dimethylation a 23s ribosomal sub-unit adenosine. The resultant conformational change of the ribosomal P site prevents macrolide binding and protein synthesis can proceed; energy-dependent efflux of macrolides out of cytoplasm via efflux protein systems; degradation of Macrolides by EreA and EreB esterases renders Macrolides inactive.	Lai and Weisblum, 1971; Nikaido, 1998; Wondrack *et al.*, 1996

Table 1.5 (cont).

Antibiotic Class	Phenotypic Mechanism(s)	Reference(s)
Quinolones	Mutations at codons 67, 81, 82, 83, 84, 87 and 106 of *gyrA* and at positions 426, 447 and 463 of *gyrB* confer quinolone resistance. The exact mechanism is unclear but suspected to involve conformational change in Topoisomerase IV and DNA Gyrase enzymes; efflux of Quinolones from bacterial cells via efflux pumps AcrAB, MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexXY (reading frame not found), AdeABC, CmeABC, AcrAB[a], AcrEF, EmrAB, MdfA, YdhE, SmeDEF, VceAB, NorM in Gram negative micro-organisms and Blt, BmrA, Bmr3, NorA and PmrA in Gram positive micro-organisms.	Ruiz, 2003
Rifampin	Three mutations in bacterial *RpoB* (Rif^R cluster I, Rif^R cluster II and Rif^R cluster III) reduce affinity of RpoB for Rifampin; reduced permeability of microbial cells to Rifampin by as yet undescribed mechanism; energy-dependent efflux of Rifampin from intracellular matrix; enzymatic modification of Rifampin by phosphorylative enzymes and ADP-ribosylating transferase yielding inactive products.	Yazawa *et al*., 1994; Dabbs *et al.*, 1995; Abadi *et al.*, 1996; Taniguchi *et al.*, 1996; Chandrasekaran and Lalithakumari, 1998;
Sulfonamides	Production of alternative forms of DHPS (dihydropteroate synthase) with reduced affinity for sulfonamides caused by mutations in *folP* and acquisition of one of three genes *SulI*, *SulII* and *SulIII*. PABA can therefore be converted into dihydrofolate and tetrahydrofolate and normal cell function is restored.	Scholz *et al*., 1989; Vedantam *et al.*, 1998; Perreten and Boerlin, 2003
Tetracyclines	Active efflux of tetracyclines from bacterial cells via membrane-bound efflux proteins therefore Tetracycline is not allowed to confer effects within cell. Reduced; ribosomal protection proteins (highly homologous to elongation factors EF-Tu and EF-G) prevent ribosomal binding of Tetracyclines. Aminoacyl tRNA is therefore allowed to bind to ribosomal A site and protein synthesis can occur normally; enzymatic modification of Tetracycline molecules by 44kDa protein in the presence of oxygen and NADPH. Modified product does not exert ant-microbial activity	Speer *et al*., 1992

1.9. Current Testing Strategy Aimed at Protecting Environmental Microbes

Before an antibiotic can be marketed by a company it now needs to be assessed to determine its potential risk to the environment. Guidance on the environmental risk assessment of human and veterinary antibiotics is provided by the European Medicines Agency. For veterinary antibiotics, additional guidance is also provided by VICH (Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products). Current testing strategies are broadly divided into two phases, Phase I and Phase II. Phase I assessment involves an initial assessment on the likelihood and level of environmental exposure, whereas in Phase II environmental fate and effects testing of the antibiotic is performed. The battery of standard bacterial tests that are performed within these frameworks is summarised in table 1.6.

1.9.1. Testing Strategy for Human Antibiotics

The first tests to determine a Predicted No Effect Concentration for microorganisms ($PNEC_{MICROORGANISM}$) is required in Phase II Tier A: Initial environmental fate and effects assessment. EMEA (2006) recognise that the microbial community most likely to be exposed to the highest concentrations is the activated sludge community. Therefore in order to assess the impact on the microbial community the activated sludge respiration inhibition test, or ASRIT, is performed (OECD 209). The $PNEC_{MICROORGANISM}$ is then estimated from the NOEC value using an assessment factor of 10 which accounts for inter- and intra-species variability and laboratory to field extrapolation. The $PNEC_{MICROORGANISM}$ is then compared to predictions of concentrations in surface water ($PEC_{SURFACEWATER}$). If the $PEC_{SURFACEWATER:}PNEC_{MICROORGANISM}$ is > 0.1, further evaluation of the fate and effects of the medicine and/or its metabolites on microorganisms is required in Phase II Tier B. If the $PEC_{SURFACEWATER:}PNEC_{MICROORGANISM}$ is < 0.1, then the risk to microorganisms is considered to be acceptable. The risks of the antibiotic to blue green algae will also be assessed during Phase II Tier A, using a s similar approach.

Phase II Tier B of the EMEA ERA for human medicines, the risk quotient for the microorganisms to be refined. In order to achieve this, a PEC is determined for the aeration tank of the activated sludge plant. If the $PEC_{AERATION\ TANK}$: $PNEC_{MICROORGANISM}$ is > 1 then further microbial toxicity studies are required such the tests using single microbial species (e.g. *Pseudomonas putida*) as identified in the Technical Guidance Document (TGD, 2003). Finally, for medicines that are not readily biodegradable and have a K_{OC} >10000 L/kg a terrestrial environmental risk assessment is required as part of Phase II Tier B. For such medicines the impact on soil microorganisms needs to be evaluated using the OECD Nitrogen Transformation Test (OECD 216).

1.9.2. Testing Strategy for Veterinary Antibiotics

For veterinary drugs, the EMEA guideline based on VICH Phase II distinguishes between test endpoints for the aquatic compartment (for drugs used in aquaculture, and for drugs used for mass-rearing and pasture animals, if exposure of the aquatic environment is possible) and the terrestrial compartment (drugs used for intensively reared animals and pasture animals). In any case, effect testing of microbial species has only to be performed if the respective exposure triggers are exceeded. Exposure triggers are either a PEC (predicted environmental concentration) soil > 100 ug/ kg in soil, or an aquatic EIC (initial environmental concentration) > 1 ug/L).

For the freshwater environment, algae are one of the required test species in tier A. Drugs to be used in freshwater or saltwater should be tested with fresh - or saltwater algal species, respectively. In both cases, the endpoint is the EC_{50} for growth inhibition (according to OECD 201 guidelines for freshwater and ISO 10253 guidelines for salt water), and an assessment factor of 100 has to be applied to the results. The guidance notes that some agencies prefer blue-green algae (*Cyanophyta*, which are prokaryotic species) in place of green algae for freshwater testing due to their higher sensitivity for antimicrobial substances. For saltwater, no advice on the suitability of blue-green algae is included in the guideline. Tier B consists of the same algal growth inhibition testing (according to OECD 201 for freshwater and ISO 10253 for saltwater), but the NOEC has now to be used as endpoint with an assessment factor of 10. For freshwater, it is suggested to use the same species as in Tier A (e.g. testing with a blue-green algae).

With respect to the terrestrial environment, testing inhibition of nitrogen transformation (according to OECD guideline 216) is required in Tier A for veterinary drug used for intensively reared animals and pasture animals. The assay has to be conducted at two concentrations, the maximum PEC and 10 times the maximum PEC. In Tier B, the nitrification inhibition assay has to be prolonged to 100 days in case > 25% inhibition is seen after 28 days.

Table 1.6: Description of the range of standard microbial tests that are available.

Test	Description	End-point/validation	Ecological Protection Offered	Reference
Activated Sludge Respiration Inhibition Test (ASRIT) (OECD, 209)	Measures the effect of a pharmaceutical on aerobic respiration rates of activated sludge microbes after 30 minutes, 3 hours or both	% Mean respiration compared with control expressed as $EC_{50.}$ Validated against 3,5-dichlorophenol	Protects microbes in STP; provides toxicity threshold for biodegradation studies	OECD, 1984
Nitrogen Transformation Test (OECD 216)	Measures long-term effects of pharmaceuticals on nitrogen transformation rate in soils	Nitrate production at days 0, 7 14 and 28 and 100 days. Expressed as EC_x values; no validity criteria required	Protects ecologically important aspects of the nitrogen cycle performed by soil micro-organisms	OECD, 2000
Nitrification Inhibition Test (ISO 9509)	Assesses short-term inhibitory effects of pharmaceuticals on nitrifying bacteria in activated sludge over a four-hour period at 5 toxicant concentrations	% Inhibition of nitrite production compared with control. Expressed as EC_{50}; Validated against Allyl thiourea	Protects nitrifying bacteria present in activated sludge	ISO 2006
Sewage Bacteria Growth Inhibition Test (ISO 15522)	Assesses potential inhibition of growth of aerobic bacteria present in activated sludge by 5 concentrations of pharmaceutical compound	Increase in microbial cell density measured as absorbance at 530 nm; EC_x values obtained; Validated against 3,5-dichlorophenol	Offers protection to the growth of microbes found in STPs	ISO, 1999
Pseudomonas putida Cell Multiplication Inhibition Test (ISO 10712)	Assesses potential inhibition of growth of a surrogate aquatic microbial species due to a toxicant	Increase in medium turbidity at 436nm over a 16-hour period; EC_{10} and EC_{50} values are calculated by % inhibition compared with controls; validated against 3,5-dichlorophenol	Offers inferred protection to aquatic bacteria against pharmaceuticals and other toxicants	ISO, 1996
Inhibition of Light Emission of *Vibrio fischeri* (ISO 11348)	Assesses impact of chemicals on light emission from marine microbe *Vibrio fischeri*	Decrease in bioluminescence compared with controls after 15 and 30 minutes. Expressed as EC_{20} and EC_{50} values. Validated against 3,5-dichlorophenol, Zn^{2+} and Cr^{6+}	Offers inferred protection against pharmaceuticals and other toxicants to bacteria in surface waters, waste waters and soil pore waters	ISO, 1994

1.9.3. Reported Shortcomings of Microbial Standard Tests and Testing Strategy

There have been a number of reported and inherent shortcomings of current standard tests that have been reported. One such criticism relates to the recommended duration of standard tests. Kummerer *et al.* (2000) altered the test duration of the OECD 209 (activated sludge respiration) from the standard 30 minutes to 3 hours and 20 hours. They concluded that a period of 30 minutes was too short a timescale to adequately assess toxic effects based on comparison of published IC_{50} values of the same toxicants on pathogenic bacteria. After a test duration of 20 hours however IC_{50} values fell to values nearer reported IC_{50}s (concentrations which cause a 50% inhibitory effect). The study also reported differences in toxicity depending on specific properties and modes of action of individual toxicants, suggesting individual compounds or groups of compounds should be studied separately. Backhaus *et al.* (1997) also observed that the standard *Vibrio fischeri* bioluminescence assay (ISO 11348) duration was not adequate. The toxic effects of several antibiotics could not be determined after the standard 30-minute exposure period but could be determined when the assay duration was increases to 24-hours. Froehner *et al.* (2000) confirmed this, except toxic effects of antibiotics could be detected after 3 hours.

Conversely, it has been suggested that the recommended sampling days of the Nitrogen Transformation Test (OECD 216) may underestimate the impact of a toxicant. In studies that sampled at days 7 and 14 as well as the recommended 0 and 28 it was found that short-term impacts of nitrapyrin (an inhibitor of nitrification) existed at days 7 and 14. If the standardised procedure were followed these effects would have been missed. In addition, nitrate formation in control studies is extremely variable (between 1-7 mg nitrate/kg/day) (Snape *et al*., 2008). Despite the documented effects of Nitrapyrin on nitrification inhibition (eg: Abbasi *et al*., 2003) no reference toxicant is used in the OECD 216 test. As the assay is used to study chemicals that are applied directly to soil the test is also not suitable for studying chemicals that are applied to soil *via* sludge, manure or slurry. As this represents major exposure routes to soil the assay loses some relevance.

The OECD 216 has also been shown to be less sensitive than other soil-based assays (that measure carbon turnover such as substrate induced respiration) when studying effects of antibiotics on soil microbes (Schmitt *et al*., unpublished data). The sensitivity of carbon turnover based assays in the study of antibiotic toxicology has been previously demonstrated (Thiele-Bruhn and Beck, 2005; Zielezny *et al*., 2006). This may be because respiration-based assays measure a function of microbial growth, a physiological feature inhibited by the mode of action of some antibiotics (such as tetracyclines).

There are also some inherent shortcomings of the OECD 209. As the assay detects changes in respiration rate it will not be able to detect toxicity of chemical that uncouple oxidative phosphorylation or stimulate oxygen uptake. In addition, chemicals that react with the standardised medium, that are poorly soluble in water or are volatile are not suitable for testing with the OECD 209. At Phase II Tier A the $PEC_{SURFACEWATER}$: $PNEC_{MICROORGANISM}$ is calculated using data from the OECD 209. The inoculum used in the test however is activated sludge. It therefore contains a microbial population that is more likely to exist in an STP rather than being representative of an aquatic community structure. Also, the inoculum used can never be standardised due to the fact that activated sludge is collected from various STPs and will be inherently variable in, for example biomass and microbial community structure.

Single species tests aimed at the protection of aquatic microbes utilise *Vibrio fischeri*, *Pseudomonas putida* or algal species. Toxicity of a chemical to one microbe may not infer toxicity to another microbe. Chander *et al.* (2005) observed differential toxicity of tobimycin to different single soil isolates. Robinson *et al.* (2005) observed differential toxicity of a range of a range of antibiotics to various cyanobacterial species; demonstrating one cyanobacterial species may not be representative for all cyanobacterial species or strains either. In addition, *Vibrio fischeri* is a marine microorganism. It is therefore questionable if the *Vibrio fischeri* bioluminescence assay can be used as a surrogate for aquatic freshwater species as they will possess different microbial ecology.

A general criticism of the testing strategy of antibiotics (human and veterinary) in all environmental compartments (and indeed between different matrices) is the fact that there are currently no standardised approaches for testing whether a compound will act as a selective agent for antibiotic resistance. Antibiotic resistance tests would likely test the extent to which a chemical caused members of the (environmental) microbial community to develop tolerance towards that compound. Bearing in mind that antibiotic resistance can spread (mainly via genetic determinants), there is not currently a standardised test which can quantify the extent to which this may occur in the environment. Such a test would ideally be able to track the spread of antibiotic resistance both within and between various environmental matrices, as resistance may be able to spread between environmental compartments.

Another general criticism regarding the environmental risk assessment of human pharmaceuticals is that only an aquatic risk assessment is required (unless a compound has a high partitioning value). As human medicines can enter the terrestrial environment via a number of exposure routes risk assessment procedures are absent for identifying the toxicity of human medicines in the terrestrial environment.

1.10. Gaps in Knowledge and Major Concerns of Antibiotics in the Aquatic Environment

From the previous sections, it is clear that antibiotics occur across a range of environmental media, including surface waters, groundwater, soils and sediment. These compounds have the capacity to cause a range of toxic effects on microbial populations (including effects on growth, nutrient cycling, biodegradation and respiration) in the natural environment and may also play a role in the selection of antibiotic resistance. While a number of good and thorough studies have been conducted into the interactions of antibiotics in soil and activated sludge systems (see section 1.7), our understanding of the effects in aquatic systems is less well developed. The studies that have been done also have many limitations. For example, they typically focus on one or two endpoints whose environmental relevance is often questionable. The role of waterborne antibiotics in resistance selection has not been extensively studied to date. The studies that have been typically performed using standard laboratory test systems and assays also use relatively high concentrations of antibiotics. There is therefore a need to perform more rigorous studies into the impacts of antibiotics on aquatic microbial communities, especially in terms of ecologically relevant end-points such as nutrient cycling. The findings of these studies will not only allow us to better establish the risks of antibiotics to ecosystems but also help inform the development of testing approaches for use in the environmental risk assessment process which needs to be done before a company can market a new pharmaceutical product.

1.11. Choice of Test Compounds

The two antibiotics that have been chosen for study in the current project are sulfamethoxazole and CTC. They belong to the antibiotic classes called the sulfonamides and tetracyclines respectively. Both classes of antibiotic have large usage profiles both as veterinary and human antibiotics. They have also been frequently detected in several environmental matrices, including the aquatic environment. It is clear therefore that aquatic microbial communities will be exposed to both sulfamethoxazole and CTC. Previous work has also shown that both test compounds have effects on the microbial function of environmental bacteria, mainly in studies that have focused on looking at such effects on soil and sludge microbial communities. Many of the end-points that have been studied in these investigations have been ecologically relevant ones, such as nutrient cycling and xenobiotic degradation. Investigation into similar effects which may be occurring in the aquatic environment is therefore merited. Both compounds also have several mechanisms of resistance. Moreover, the genetic determinants of such resistance mechanisms (resistance genes) have been detected in several environmental matrices. The possibility for exposure of microbial communities to the two test compounds to select for antibiotic resistance is therefore a possibility and also merits investigation.

1.12. Aims and Objectives

The overall aim of this study was to explore the effects of commonly used antibiotics, with contrasting properties (mode of action, environmental fate characteristics) on aquatic microbial communities. This was achieved using the following specific objectives:

1) To develop tests and a test system that are capable of determining specific, ecologically relevant effects of antibiotics in the aquatic environment and to evaluate the developed tests using a model reference toxicant (Chapter 2).

2) To use the tests developed in 1) to determine the effects of antibiotics, with different modes of action and contrasting physico-chemical properties, on the functioning of aquatic microbial communities (Chapters 3 and 4).

3) To determine the extent to which exposure of an aquatic system to antibiotics results in selection of antibiotic resistance, both single and multidrug (Chapter 5).

Chapter 2: General Method Development

2. General Materials and Methods

2.1. Introduction

Chapter 1 outlined the fact that antibiotic compounds may enter the environment where they have been frequently detected. When in the environment, antibiotics can exert a number of ecological effects on microbial communities. The following chapter will introduce the use of specific functional assays and which can be used to test the ecological impacts of toxicants on environmental microbial communities. The use of laboratory based test systems shall also be introduced. The chapter will ultimately describe experiments that will aim to develop these techniques so that they can be used to study the effects of antibiotics on environmemtal microbial communities.

2.1.1. Environmental Microbial Function Assays

Changes in microbial function in the environment are frequently quantified via the measurement of metabolically relevant end-points. These end-points generally relate to important aspects of cellular function (such as energy liberation and protein synthesis) and can be coupled with important environmental processes such nutrient cycling. Due to the fact that enzymes catalyse many of these reactions a vast array of functional assays also quantify enzyme activity within environmental microbes and microbial communities. Functional assays have been utilised in a range of environmental matrices to study the potential effects of antibiotics in the environment (Table 2.1).

2.1.2. Multisubstrate Utilisation: The Biolog GN2 Assay

The Biolog GN2 assay is a phenotypic microarray that is capable of quantifying the metabolism of 95 distinct substrates by environmental Gram negative bacteria (shown in figure 2.1). The extent to which substrates are metabolised is gauged spectrally (at 595 nm) by the reduction of a tetrazolium dye which occurs via the gain of electrons that have been liberated from Biolog substrates (due to microbial metabolism). A blank value (water) is then subtracted from these values. The assay was first introduced as a tool to identify bacterial species and strains from pure cultures. In 1991 Garland and Mills realised the assay's potential for use in environmental science. Used in this respect, the Biolog GN2 assay is capable of tracking the catabolism of 95 distinct substrates containing a carbon source, nitrogen source or a source of both carbon and nitrogen. These substrate types can be further condensed into 6 distinct "guilds": carbohydrates, carboxylic acids, amines and amides, amino acids, polymers and other. The substrates belonging to each substrate guild are outlined in table 2.2. A Substrate guild should not be confused with an ecological guild (a group of organisms which exploit similar resources) however. The Biolog GN2 assay quantifies actual and potential microbial function meaning it also tracks the metabolism of acclimated microbial populations (Preston-Mafham *et al*., 2002).

Table 2.1: Functional assays that have been used to study the effects of antibiotics in several environmental matrices.

Assay	Environmental Matrix Studied	Assay Description	Antibiotic(s) Studied	Corresponding Environmental Process	Reference(s)
Dehydrogenase	Soil	Measures changes in reduction of a redox dye spectrally due to electron liberation from an organic substrate. Related to the Carbon Cycle	Sulfamethazine, tylosin and CTC		Chun *et al*., 2005
			Oxytetracycline		Boleas *et al*., 2005
			Sulfapyridine and oxytetracycline		Thiele-Bruhn and Beck, 2005
SIR	Soil	Measures changes in CO_2 production over time via pH change due to the oxidation of a single substrate (frequently glucose). Relates to the Carbon Cycle	Tylosin, tetracycline, CTC, sulfachloropyridazine, eryhthromycin	Carbon cycle	Vaclavik *et al*., 2004
			Antibiotic mixtures		Scallenberg and Armstrong, 2004
			Streptomycin		Landi *et al*., 2003
			CTC and sulfadiazine		Zeilezny *et al.*, 2006
Biogas production	Soil spiked with bovine manure and urine	Measurement of methane production by microbial degradation of animal waste products	Oxytetracycline		Arikan Rital *et al*., 2006
Biolog	Soil	Measures changes in redox dye reduction spectrally over time due to oxidation of 96 distinct carbon sources	Sulfadiazine	Carbon cycle, nitrogen cycle, polymer degradation	Schmitt *et al*., 2005
			Oxytetracycline		Kong *et al.*, 2006
	Surface water		Oxytetracycline		Maul *et al*., 2006

Table 2.1 (cont**).**

Assay	Environmental Matrix Studied	Assay Description	Antibiotic(s) Studied	Corresponding Environmental Process	Reference(s)
Nitrification assay	Aquaria water		Oxytetracycline		Klaver and Mathews, 1994
	Sewage sludge	Measures the conversion of nitrate to nitrite spectrally	Tylosin, sulfadiazine, streptomycin, ciprofloxacin, olaquindox		Sorensen, 2000
	Soil		Chloramphenicol and oxytetracycline		Campos *et al*., 2001
Denitrification assay	Aquatic sediment	Measures liberation of nitrogen gas or nitogen dioxide from inorganic nitrogenous compounds	Ciprofloxacin, amoxicillin, clarithromycin, ampicillin, amoxicillin / clavaulinic acid and erythromycin	Nitrogen cycle	Costanzo *et al*., 2005
Ammonification assay	Environmental urine	Measures (spectrally) ammonium production from oxidation of amino acids such as arginine	Ampicillin		Nimenja *et al.*, 2000
Leucine incorporation assay	Surface water	Measures amino acid incorporation into biomass via isotopic labelling	Tetracycline		Verma *et al*., 2007
Iron (III) reductase assay	Soil	Measures spectrally the extent to which iron compounds are reduced over time	CTC, sulfadimethoxine, oxytetracycline, sulfadiazine, sulfadimidine, tetracycline, sufadimidine	Iron cycle	Thiele-Bruhn, 2004
Phosphomonoesterase assay	Soil	Measures spectrally the conversion of phosphorus salts onto phenol	Oxytetracycline	Phosphorus cycle	Boleas *et al.*, 2005

A1 Water	A2 α-Cyclodextrin	A3 Dextrin	A4 Glycogen	A5 Tween 40	A6 Tween 80	A7 N-Acetyl-D-Galactosamine	A8 N-Acetyl-D-Glucosamine	A9 Adonitol	A10 L-Arabinose	A11 D-Arabitol	A12 D-Cellobiose
B1 i-Erythritol	B2 D-Fructose	B3 L-Fucose	B4 D-Galactose	B5 Gentiobiose	B6 α-D-Glucose	B7 m-Inositol	B8 α-D-Lactose	B9 Lactulose	B10 Maltose	B11 D-Mannitol	B12 D-Mannose
C1 D-Melibiose	C2 β-Methyl-D-Glucoside	C3 D-Psicose	C4 D-Raffinose	C5 L-Rhamnose	C6 D-Sorbitol	C7 Sucrose	C8 D-Trehalose	C9 Turanose	C10 Xylitol	C11 Pyruvic Acid Methyl Ester	C12 Succinic Acid Mono-Methyl-Ester
D1 Acetic Acid	D2 Cis-Aconitic Acid	D3 Citric Acid	D4 Formic Acid	D5 D-Galactonic Acid Lactone	D6 D-Galacturonic Acid	D7 D-Gluconic Acid	D8 D-Glucosaminic Acid	D9 D-Glucuronic Acid	D10 α-Hydroxybutyric Acid	D11 β-Hydroxybutyric Acid	D12 γ-Hydroxybutyric Acid
E1 p-Hydroxy Phenylacetic Acid	E2 Itaconic Acid	E3 α-Keto Butyric Acid	E4 α-Keto Glutaric Acid	E5 α-Keto Valeric Acid	E6 D,L-Lactic Acid	E7 Malonic Acid	E8 Propionic Acid	E9 Quinic Acid	E10 D-Saccharic Acid	E11 Sebacic Acid	E12 Succinic Acid
F1 Bromosuccinic Acid	F2 Succinamic Acid	F3 Glucuronamide	F4 L-Alaninamide	F5 D-Alanine	F6 L-Alanine	F7 L-Alanyl-glycine	F8 L-Asparagine	F9 L-Aspartic Acid	F10 L-Glutamic Acid	F11 Glycyl-L-Aspartic Acid	F12 Glycyl-L-Glutamic Acid
G1 L-Histidine	G2 Hydroxy-L-Proline	G3 L-Leucine	G4 L-Ornithine	G5 L-Phenylalanine	G6 L-Proline	G7 L-Pyroglutamic Acid	G8 D-Serine	G9 L-Serine	G10 L-Threonine	G11 D,L-Carnitine	G12 γ-Amino Butyric Acid
H1 Urocanic Acid	H2 Inosine	H3 Uridine	H4 Thymidine	H5 Phenyethyl-amine	H6 Putrescine	H7 2-Aminoethanol	H8 2,3-Butanediol	H9 Glycerol	H10 D,L-α-Glycerol Phosphate	H11 α-D-Glucose-1-Phosphate	H12 D-Glucose-6-Phosphate

Figure 2.1: The 95 distinct substrates and their schematic location on the Biolog GN2 Microplate (Biolog, 2007).

Table 2.2: Outline of substrates belonging to individual substrate guilds on the Biolog GN2 plate (Biolog, 2007).

Substrate Guild	Substrates in Guild
Amines and Amides	2-Amino ethanol, Glucuronamide, L-Alaninamide, Phenylethylamine, Putrescine, Succinamic acid
Amino Acids	γ-Amino butyric acid, D,L-Carnitine, D-Alanine, D-Serine, Glycyl-L-aspartic acid, Glycyl-L-glutamic acid, Hydroxy-L-proline, L-Alanine, L-Alanyl-glycine, L-Asparagine, L-Aspartic acid, L-Glutamic acid, L-Histidine, L-Leucine, L-Ornithine, L-Phenylalanine, L-Proline, L-Pyroglutamic acid, L-Serine, L-Threonine
Carbohydrates	α-D-Glucose, α-D-Lactose, β-Methyl-D-glucoside, Adonitol, D-Arabitol, D-Cellobiose, D-Fructose, D-Galactose, D-Mannitol, D-Mannose, D-Melibiose, D-Psicose, D-Raffinose, D-Sorbitol, D-Trehalose, Gentiobiose, *i*-Erythritol, Lactulose, L-Arabinose, L-Fucose, L-Rhamnose, L-Sorbose, Maltose, Methyl pyruvate, *m*-Inositol, Mono-methyl-succinate, *N*-Acetyl-D-galactosamine, *N*-Acetyl-D-glucosamine, Sucrose, Turanose, Xylitol,
Carboxylic Acids	α-Hydroxy butyric acid, β-Hydroxy butyric acid, γ-Hydroxy butyric acid, α-Keto butyric acid, α-Keto glutaric acid, α-Keto valeric acid, Acetic acid, Citric acid, *Cis*-aconitic acid, D,L-Lactic acid, D-Galactonic acid γ-lactone, D-Galacturonic acid, D-Gluconic acid, D-Glucosaminic acid, D-Glucuronic acid, D-Saccharic acid, Formic acid, Itaconic acid, Malonic acid, *p*-Hydroxy phenylacetic acid, Propionic acid, Quinic acid, Sebacic acid, Succinic acid
Polymers	α-Cyclodextrin, Dextrin, Glycogen, Tween 40, Tween 80

2.1.3. Advantages and Disadvantages of the Biolog GN2 Assay

A major advantage of the Biolog GN2 assay over existing risk assessment tools is that the Biolog GN2 assay can quantify the metabolism of a greater array of substrates than other assays such the ASRIT (which only quantifies glucose respiration). In addition, other types of substrate metabolism can be quantified (including glucose), such as amino acids, amines and amides. Several aspects of microbial function in the environment (such as nitrogen and carbon turnover) can therefore be studied simultaneously in one assay. The data that can be gained from the Biolog GN2 assay represent another advantage, as long as sufficient replication is applied to a study (Preston-Mafham *et al*., 2002). As well as having the ability to yield kinetic data, the Biolog GN2 assay can also be used to produce Community Level Physiological Profiles (CLPP) data by means of Principle Component Analysis (PCA). It has also been demonstrated that comparative data can be harvested for each substrate guild (eg: Kong *et al*., 2006).

There have however been a number of criticisms expressed over the Biolog GN2 assay. The first of these relates to the inoculum density that is added to each Biolog well. If the inoculum density is not equal across replicates (or individual well) then conditions will not be the same between samples. Garland and Mills (1991) showed that well colour formation was proportional to microbial density. It has been additionally demonstrated that reduced inoculum density has been correlated with a reduced lag time but not necessarily with the rate of colour development the maximum colour development possible (Haack *et al*., 1995). Garland (1996) further added that colour development also relies upon the proportion of actively respiring cells in the inoculum, so such populations must be standardised also. Another concern that has been raised has been that of nutrient carryover from environmental samples. If this is too high then the blank well may not be able to act as a control to substrate utilisation (Preston-Mafham *et al*., 2002).

Despite these drawbacks a range of precautions can be implemented to reduce any disadvantages. These include allowing as much replication as possible, taking readings at multiple time points, taking into account inoculum density and having samples of equal weight, size or volume (Preston-Mafham *et al.,* 2002).

2.1.4. Validity Criteria of Toxicity Tests

Assays that yield data that is intended to provide toxicity information for environmental microbes must be validated against a reference toxicant. The EC_{50} of the reference toxicant is of known value. Any new tests that have been developed must therefore exert an EC_{50} value that is between the stated thresholds for the particular reference toxicant that is being utilised. Table 2.2 shows the range of reference toxicants that are currently used for microbial toxicity testing and their validity criteria.

Table 2.3: Reference toxicants and their toxicity thresholds for standard microbial toxicity tests.

Test	Reference Toxicant Used	Validity Criteria / EC_{50}	Reference
Activated sludge respiration inhibition test (ASRIT) (OECD, 209)	3, 5-dichlorophenol	3–32 mg/L	OECD, 1984
Nitrification inhibition test (ISO 9509)	allyl thiourea	3–32 mg/L	ISO, 2006
Sewage bacteria growth inhibition test (ISO 15522)	3, 5-dichlorophenol	4–12 mg/L	ISO, 1999
Pseudomonas putida cell multiplication inhibition test (ISO 10712)	3, 5-dichlorophenol	10–30 mg/L	ISO, 1995
Inhibition of light emission of *Vibrio fischeri* (ISO 11348)	3, 5-dichlorophenol, Zn^{2+} and Cr^{6+}	13–26 mg/L; 2.2–11 mg/L	ISO, 1994

2.1.5. Use of Microcosms in Ecological Studies

A microcosm is a laboratory based system that reproduces a natural habitat. They have been described as being a "bridge between theory and nature" (Fraser, 1999). Indeed data yielded from microcosm studies have produced data that have supported several important ecological theories, such as the effects of changing CO_2 levels on ecosystem function (Bazzaz, 1990; Diaz *et al*., 1993). Microcosms can be composed of either a subset of a natural habitat (such as soil columns or watersheds) or can be fabricated from natural components in the laboratory. They can vary in size and complexity, spanning single test-tube experiments to entire biospheres (Fraser, 1999). The use of microcosms in ecotoxicology (in both fate and effects studies) began in the mid-1970s and their use in ecotoxicological studies have been cited as being especially widespread (Beyers and Odum, 1993).

Microcosm studies have several advantages over studying natural habitats in the field. In ecotoxicological studies the main advantage of using microcosms is the fact that microcosms can be easily manipulated in terms of contents (such as the environmental compartment that is studied and the type and concentration of a chemical that is added) and conditions (such as temperature and light intensity). Due to the ease of replicating microcosm studies (Fraser, 1999) they also provide a standardised approach; solid statistical analysis is therefore possible when data are being analysed. The use of microcosms in the study of the fate and effects of antibiotics in the environment has highlighted the added advantage that a number of end-points can be studied simultaneously in a particular environmental matrix. For example, samples can be removed and analysed chemically, biologically and physically. In practice, this allows for several aspects to be measured in a single test system. For example chemical concentrations and *in vitro* toxicity have been simultaneously studied in microcosms that had been spiked with antibiotics (e.g.: Zeilezny *et al*., 2006). For this reason a microcosm can potentially be seen as being a "one system fits all" solution.

One of the main drawbacks of microcosm studies is the inherent lack of space afforded for study compared to a natural environment. Ingerslev *et al.* (2000) demonstrated the importance of test volume. They observed an increased lag time with regard to the biodegradation of 2, 4-dichlorophenoxyacetic acid and *p*-nitrophenol (PNP). These data suggest that test volume is an important parameter to take into consideration when studying biological systems in microcosm studies and that test volume should be maximised as much as possible. The authors suggested that an increased microbial biodiversity is linked to higher test volumes.

Microcosms have also been criticised for being too simplistic, due to the fact that only a subset of species are typically included. Another common criticism is the fact that microcosms do not reflect the complexity of natural ecosystems. As such, many believe that microcosms cannot provide reliable models for the ecosystems which they are attempting to study (Drake and Kramer, 2011). Other critics of microcosms point out that they are not subject to the variability of natural

conditions that real ecosystems are subject to, such as temporal aspects. These include diurnal and seasonal periodicities (such as weather and temperature changes) and environmental stochasticity (differences in growth rates of populations). In addition, physical processes are difficult to recreate in microcosms. Examples of how these can affect the realism of microcosm data include the effects of including a water-sediment interface and the mixing of a water column by wind (Carpenter, 1996; Schindler, 1998). Bulling *et al*. (2006) point out that real ecosystems are subject to variable conditions that are extremely difficult to control in microcosm studies. From an ecotoxicological point of view, many microcosm systems also use artificially high concentrations of chemicals (compared with those found in natural ecosystems) so that the fate and effects of chemicals can be quantified.

As such, microcosms which attempt to study microbial communities are unlikely to truly reflect natural populations of bacteria due to the fact that natural populations are subject to a huge array of variables and parameters. For these reasons, aquatic microbial microcosms will only provide a "snapshot" of what is occurring in natural environments. In addition, the increased complexity of natural environments mean that results of microcosm studies may be difficult to interpret in terms of what is actually occurring in nature.

2.1.6. Rationale for Assay Choice

One of the aims of this project was to track the effects of antibiotics on ecologically important end-points. Many of the end-points that have been studied in the available literature have been those related to nutrient cycling, especially those that can linked to carbon and nitrogen turnover. As such, the bioassays that will be utilized in the current investigation will also aim to link the exposure of microbial communities to antibiotics with effects on nutrient cycling. Most assays that have been conducted with respect to looking at the effects of antibiotics on microbial communities rely on looking at a single end-point, as has been performed with ammonification, nitrification and denitrification assays. It was noted however that the Biolog GN2 assay could simultaneously track the utilization of a wide range of ecologically important substrates over time. The range of substrates that are potentially utilized also correlate to either the carbon or nitrogen cycle, or both in the case of substrates that have both carbon and nitrogen in their structures (such as amino acids). Additional information can also be collected regarding the microbial metabolism of polymers. This additional information may potentially give insights into how polymer degradation (in the aquatic environment) may be affected by antibiotics. Many pollutants are polymeric structures. As such, polymer degradation within a Biolog plate may provide clues as to how the exposure of a microbial community to antibiotics affects, for example, pollutant biodegradation. The fact that the Biolog GN2 assay can also yield information on how microbial communities may respond to antibiotics in terms of changes in community structure is also an attractive feature. As such, it was decided that all available time would be dedicated to developing a system that utilizes the Biolog GN2 system, including the development of suitable data analysis techniques.

2.1.7. Choice of Statistical Analyses for Project

2.1.7.1 Biolog Kinetic Data

Biolog kinetic data was chosen to be based upon a logistic growth model. This was in turn based upon a microbial Zwietering growth equation (Lee *et al*., 1992). The model can be programmed into statistical software (for example Sigmaplot) and a non-linear regression analysis can be performed. This allows for the visualisation of data over time via substrate utilization curves. This approach also allows for various parameters associated with microbial growth to be modelled, allowing for the analysis of how a toxicant may affect different aspects of substrate utilisation. The first modelled parameter which can be studied is the lag phase during the growth of a microbial community. During the lag phase, before substrate utilisation (analogous to the lag phase before microbial growth), bacteria adapt themselves to growth conditions. It is the period during which bacteria are maturing and not yet able to divide. During the lag phase of the bacterial growth cycle, synthesis of RNA, enzymes and other molecules occurs. The lag phase is therefore important for the microbial community as it prepares bacteria for the next phases of growth (exponential and stationary) and allows them to adapt to their environment. The second modelled parameter is the maximum rate of substrate utilisation (analogous to the maximum rate of growth of the microbial population). This parameter allows for the quantification of the maximum rate at which the microbial community is utilizing substrates. Using sample size and the margin of variation between samples, a one way analysis of variation (ANOVA) can be used to determine statistically significant differences between maximum rates and lag phases between different toxicant treatments compared with control values.

2.1.7.2. Multivariate Analysis and Ordination of Biolog Data

Biolog data can be thought of as having 95 different variables (representing individual substrates). These variables can be reduced into a smaller number of principle components by PCA (Principal Component Analysis). Principal components (PCs) are identified by taking eigenvalues of a covariance matrix, which is calculated from original data values (which are mean subtracted). Higher eigenvalues represent a greater degree of variation. The resulting numbers which are calculated account for a diminishing degree of variation within the dataset as the PC number increases. As such the first two PCs usually describe the greatest (if not all) degree of variation within a data set. The result of this analysis is to reduce the dimensionality of large datasets which can then be compared to each other more easily based on how similar they are to each other. This is achieved by comparing resultant PC scores. This process is called multidimensional scaling and is useful for analysing large, multidimensional datasets. As such, PCA can often show structure in such datasets (Randerson, 1993; Podani, 2000).

When a PCA analysis of a data set has been completed, a visual representation of a dataset can be presented as a scatter plot. This is achieved by plotting the scores of the first two PCs for each

replicate of a treatment group on an *x-y* axis. Data points (replicates) that are ordinated on a scatter plot will appear to cluster together if their PC scores are similar on both axis of the scatter plot. Due to the fact that PCA followed by ordination of PC scores on a scatter plot can be used to visualise large datasets, this method of analysis is useful for visualising how similar large datasets are across different treatments. The method is also a useful tool for reducing the dimensionality of multidimensional datasets. However, PCA analysis cannot be considered as being a quantitative approach to analysing a dataset, but rather a useful exploratory tool for data mining.

With regard to using PCA and scatter plot ordination to visualise Biolog data, the approach has been successfully used to form CLPPs (Community Level Physiological Profiles) of microbial communities. The concept behind this idea is that microbial communities which are more similar to each other will utilise a substrate utilisation profile which is more similar to each other as calculated by similarities in the first two PC scores between treatment groups. This approach has been utilised to visualise changes in the structure of microbial communities based upon how similar their substrate utilisation profiles are (Schmitt *et al*., 2005; Kong *et al.*, 2006). In the context of this project PCA analyses of substrate utilisation profiles will therefore be used to visualise how changes in microbial community structure may result from the exposure of bacterial populations to antibiotics.

2.1.7.3. Functional Analysis of Biolog Data

As well providing kinetic and multivariate data, multi substrate utilisation data can be analysed to give insights into changes in the functional diversity of microbial communities (Preston-Mafham *et al*., 2002). A useful approach when discovering how the functional diversity of a microbial community has been altered is to employ the use of a diversity index, such as the Shannon-Weaver Diversity index (SWDI). The SWDI has previously been used as a measure of biodiversity, with a higher diversity index indicating a higher biodiversity. When studying changes in the functional diversity of substrate utilisation by a microbial community, an individual substrate acts as a surrogate for an individual species. Changes in the diversity of substrates that are utilised by the microbial community can therefore be tracked. The results of these analyses will therefore give an indication as to how the exposure of a microbial community to antibiotics may affect its ability to utilize a diversity of substrates.

2.1.7.4. Data Transformation and Analysis of CFU (Colony Forming Unit) Data

It is the aim of this project to generate relevant CFU data. Due to the non-parametric nature of these data a parametric statistical analysis of variation cannot be used to analyse this data due to the fact that the data are not normally distributed. As such, CFU data shall be log naturally transformed. This will result in the data being normally distributed. Once this has been completed

then the data shall be analysed using a parametric analysis of variation. This approach has the advantage of allowing a more statistically powerful analysis to be performed, rather than a non-parametric test which would represent a more conservative approach. This would possibly underestimate statistically significant changes between treatment groups.

2.2. Materials and Methods

2.2.1. Chemicals and Equipment

All chemicals were purchased from Sigma Aldrich (Poole, Dorset, UK). 5 litre microcosm vessels, Quickfit precision glassware, HEPA Filters and general laboratory equipment were purchased from Fisher Scientific (Loughbourgh, UK). Spectral analysis was performed by a Spectramax spectrophotometer (Molecular Solutions, MA, USA). Biolog GN2 Plates were purchased from Technopath (Limerick, Eire; on license from BIOLOG, CA, USA). Glass rods were prepared by glass-cutting equipment and smoothed by oxyacetylene flaming.

2.2.2. Surface Water

Surface water was collected from the River Dart, Buckfastleigh, Devon (grid reference SX7366). Weather conditions on the day of sampling were dry and temperatures were average for the time of year (15˚C). Temperature and pH readings were taken upon arrival to Brixham Environmental Laboratory. Surface water was sieved through a 1 mm mesh to remove large particles and stored at 4°C in the site cold store until required.

2.2.3. Activated Sludge and Preparation of Treated Sewage

Activated sludge (return line) was collected from Buckland Sewage Treatment Plant, Newton Abbott, Devon, UK. Upon arrival to the laboratory activated sludge was aerated at 100 ml O_2/min. Activated sludge was allowed to settle and the supernatant was siphoned off using a syringe. The supernatant represented treated sewage.

2.2.4. Preparation of OECD Synthetic Sewage

OECD synthetic sewage was prepared according to OECD 303a guidelines (Simulation Test-Aerobic Sewage Treatment: Activated Sludge Units). A 100 fold concentrate was prepared by dissolving the following components in 1 litre of water- 16 g peptone; 11 g meat extract; 3 g urea; 0.7 g NaCl; 0.4 g $CaCl_2.2H_2O$; 0.2 g $MgSO_4.7H_2O$; 2.8 g K_2HPO_4. The synthetic sewage concentrate was then autoclaved for 15 mins at 121°C and stored in the dark at 0-4°C. 100 fold dilutions were prepared from the stock concentrate by mixing 10 ml concentrate with 1 litre of sterile water.

2.2.5. Microcosm Development

2.2.5.1. Inoculum Type and Density

2.2.5.1.1. Procedure

An initial dilution of surface water was prepared by diluting 10% v/v river water with 90% v/v sterile water. Further 1 in 10 serial dilutions were prepared to obtain a river water dilution series of 0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. A river water / treated sewage suspension was prepared by mixing 10% v/v treated sewage with 90% v/v river water. An initial dilution of this suspension was prepared by diluting 10% v/v river water with 90% sterile water v/v. Further 1 in 10 serial dilutions were prepared to obtain river water / treated sewage dilutions of 0, 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}. Triplicate Biolog GN2 plates were then inoculated with 150 µl of each dilution and shaken on a micromixer in the dark for 10 minutes. Plates were then incubated at 20°C for 80 hours. The liquid in each well of the Biolog plate was periodically read spectrally at 595 nm.

2.2.5.1.2. Initial Data Analysis

Values in all wells were autoblanked using SoftMax Pro (Molecular devices). All data were then transferred to a Microsoft Excel spreadsheet where AWCD (average well colour development) and standard deviation values were calculated. All graphs for these experiments were compiled using Microsoft Excel.

2.2.6. Initial Microcosm Studies

Initial microcosm studies were conducted to examine how control microcosm data behaved over time using the Biolog GN2 assay. Microcosm contents were chosen based on previous experiments that had examined the type and density of inoculum Biolog GN2 plates should inoculated with.

2.2.6.1. Microcosm Set-up

It was concluded that a neat mixture of river water and treated sewage (9:1 v/v) was the optimum inoculum for functional studies and was therefore chosen as the microcosm contents. Advice from external communication (personal communication with Jose Tarazona, March 2006) had raised issues regarding the detergent concentration in 10% treated sewage however, so microcosm contents were amended to contain a final concentration of river water, treated sewage and OECD synthetic sewage (90:5:5 v/v). It was also concluded that this amendment would have the added advantage of providing a nutrient and energy source for the microbial community while also giving the environmental realism that treated sewage addition would bring.

The final microcosm set-up is illustrated by figure 2.1. Individual microcosms were composed of 5 litre fermentation vessels. Microcosms were sealed with Quickfit flanges and glass stoppers to

prevent external contamination but also allow access to the system as required for sampling. Aeration of the microcosms was achieved by the use of Quickfit aeration adapters that were connected to glass rods and PTFE tubing. Air flow under negative pressure was provided by a vacuum pump to achieve a nominal air flow rate of ~100 ml O_2 / min. 0.22 µm HEPA filters were attached to glass tubing receiving external air to prevent infection from out-with microcosm vessels. All microcosms were incubated at 20°C +/- 2°C.

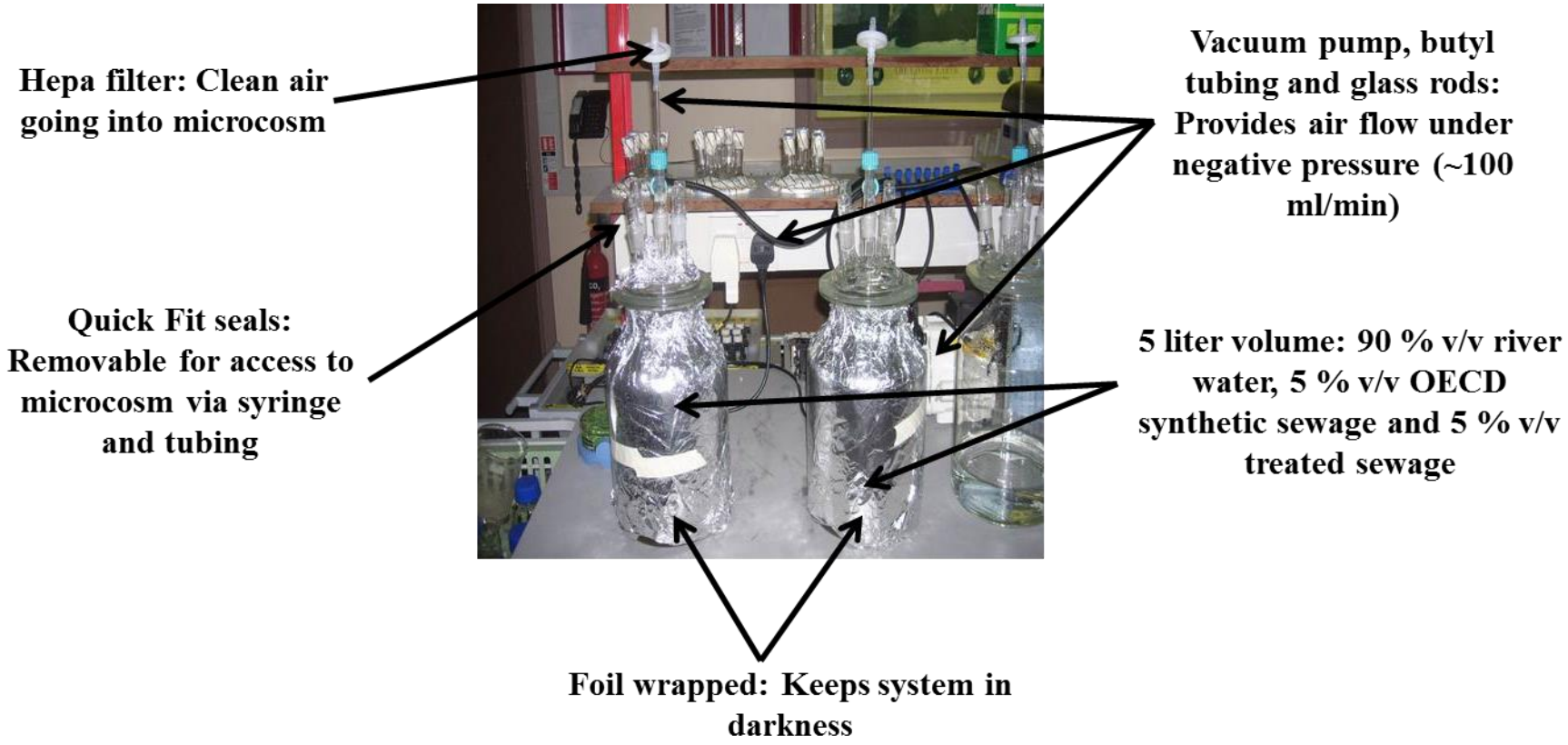


Figure 2.2: Annotated diagram of the final microcosm set-up.

2.2.7. Validation of the Microcosm-Biolog GN2 system

2.2.7.1. Experimental Procedure

Twelve microcosms were set-up as described in section 2.8.2.1. Nine microcosms were spiked with the OECD reference toxicant 3, 5-DCP. Three microcosms were spiked at 32 mg/L 3, 5-DCP, three were spiked at 3.2 mg/L 3, 5-DCP and three were spiked at 0.32 mg/L 3, 5-DCP. Three control microcosms were not spiked with any reference toxicant (0 mg/L).

Following addition of the DCP, samples were taken from each microcosm every day for a week. Biolog GN2 plates were inoculated with 150 µl of these microcosm liquid samples (as described by section 2.9.1) and shaken in the dark for 10 minutes using a micromixer. Triplicate Biolog GN2 plates were inoculated for samples obtained on days 1, 4 and 7 and single Biolog GN2 plates were inoculated for samples taken on days 2, 3, 5 and 6. This strategy was seen being a compromise between practicality (time taken to read plates and inoculate more plates), statistical rigour and financial viability. Each plate was then incubated in the dark at 20°C for at least 80 hours with periodic readings taken spectrally at 595 nm using a spectrophotometer.

2.2.8. Data Analysis

2.2.8.1. Kinetic Analysis

Biolog data were initially treated as described in section 2.6.1.2. A three parameter non-linear Zweitering logistic growth model (Lee *et al*., 1995) was then applied to control-normalised AWCD values for each 3,5-DCP treatment at each time point using Microsoft Excel and the Microsoft Excel solver function, such that a kinetic response over time could be plotted. The three parameter logistic growth function used was as follows-

$$A = A_{max} / 1 + \exp [4\mu_m / A_{max} (\lambda - t) + 2]$$

Where A= Absorbance at time, t

A_{max}= Maximum absorbance recorded in Biolog GN2 plate wells

μ_m= Maximum rate of colour development in Biolog GN2 plate wells

λ= Lag phase duration before onset of substrate utilisation

Non-linear regressions were then performed on AWCD data using Sigmaplot (SYSTAT, IL, USA). Coefficient and standard error values for λ and μ_m were then compared for statistical significance versus control values via a one way ANOVA ($P < 0.05$). This was performed by specifically designed Astrazeneca software (Alan Sharpe, Brixham Environmental Laboratories).

2.2.8.2. Principal Component Analysis (PCA) and Ordination of PCA Data

Principal Component Analyses were performed on $AWCD_{MAX}$ values for each day of the exposure period. PCA analysis was performed using Minitab 15 (PA, USA). Data was ordinated by plotting the first principal components (PCs) on the *x* axis and the second set of PCs on the *y* axis (also using Minitab 15). This decision was taken due to the fact that the first two PCs frequently describe the majority of variation within a dataset, with PC 1 accounting for the vast majority of this (Randerson, 1993; Podani, 2000). Using this analysis, replicates with similar PC scores on each axis of the scatter plot (PC1 and PC2, or *x* and *y*) will appear to cluster together as they vary less from each other. Replicates which have been shown to vary greatly from each other will have different PC scores and will not cluster on a PC scatter plot. Indeed, the greater the variation between unrelated replicates, the greater the distance replicates will appear in the two dimensional space of the scatter plot. Replicates from different antibiotic treatments are represented by shapes of different colours. Replicates which appear to cluster were considered to have similar substrate utilisation profiles and therefore similar CLPPs (Community Level Physiological Profiles). Within the context of the microbial community, similar CLPPs will be used to tentatively indicate a change in microbial community structure.

2.2.8.3. Analysis of the Functional Diversity of Substrate Utilisation

Functional diversity of substrate utilisation was analysed by calculating the richness of substrate utilisation using the Shannon-Weaver Diversity Index (SWDI) according to the equation-

$$H' = -\sum pi \ln (pi)$$

Where H' = the SWDI

Pi = the relative abundance of each substrate given by the formula-

$$Pi = A_{INDIVIDUAL\ SUBSTRATE} / AWCD_{MAX}$$

Where $A_{INDIVIDUAL\ SUBSTRATE}$ = the control-corrected absorbance value for each well on the Biolog GN2 plate for each replicate in a treatment group

$AWCD_{MAX}$ = the maximum average well colour development for each replicate in a treatment group

The SWDI is then expressed as exponential raised to the power of the SWDI value, such that-

$$e^{H'} = \text{EXP } H'$$

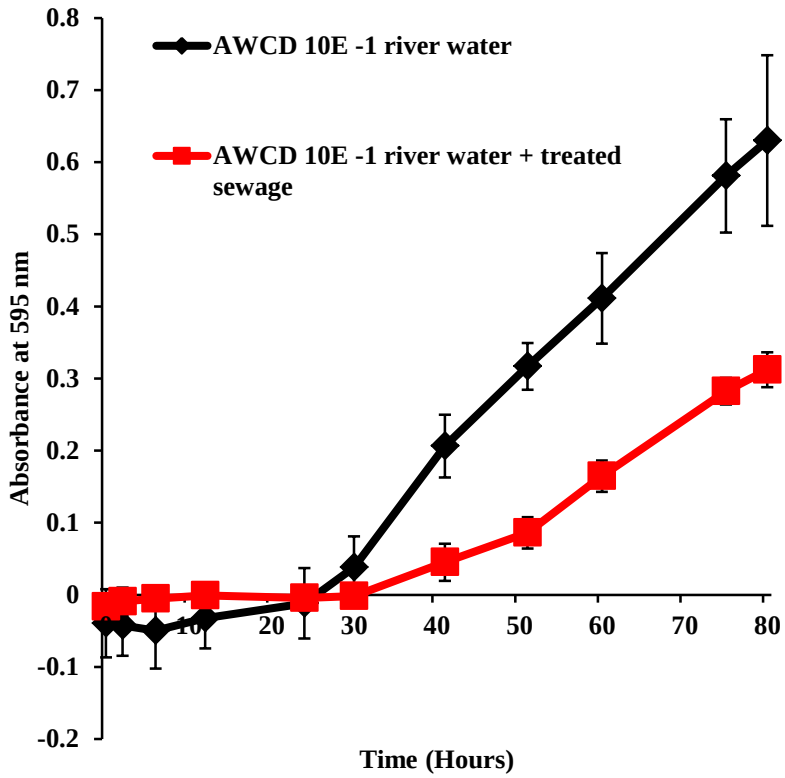
All calculations were performed using Microsoft Excel. Templates were designed and utilised which were capable of calculating the SWDI for exposure days which used three and nine replicate Biolog plates per dosing group. Values were then graphed using Microsoft Excel. Student's *t* Tests were then performed (using Sigmaplot) between $e^{H'}$ values from between each treatment group and the corresponding control value.

2.3. Results

2.3.1. Inoculum Type and Density

Results from the studies into effects of inoculum density and inoculum type (figure 2.3) show that when river water and a river water/treated sewage suspension were diluted, the maximum colour development after 80 hours was markedly reduced. At all dilutions the colour production in plates containing river water only was visibly less than the maximum colour production in plates containing a river water/treated sewage suspension. In more dilute samples (10^{-4}) there was a higher degree of variation between replicate Biolog plates. Conversely, where there had been no dilution there was very little variation between replicate plates. Due to the formation of suitable colour development across all treatments, there was no evidence of nutrient carryover posing a threat to the resolution of colour development. There did however seem to be an effect of inoculum dilution and type on lag phase time however; in general, colour development at lower dilutions and plates treated with river water only took visibly longer to develop. In all cases, an incubation period of $\geq$ 75 hours was adequate to resolve a clear trend. For future Biolog GN2 inoculations an exposure period of at least 75 hours was therefore selected.

a)



b)

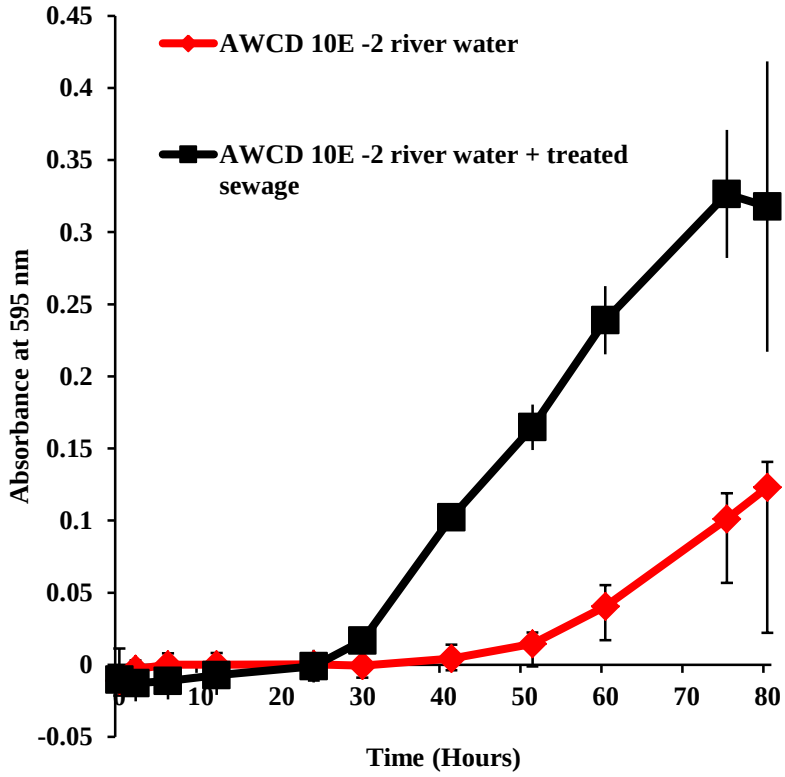
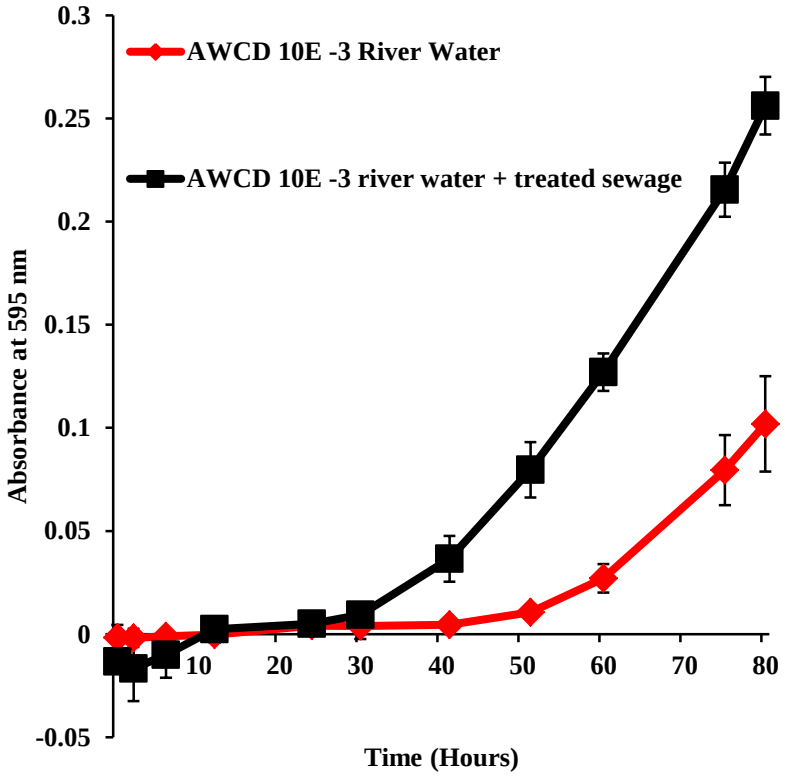


Figure 2.3: Graphs with data illustrating the effect of inoculum type and density on total colour development (AWCD) over time in Biolog GN2 plates. a) 10^{-1} dilution b) 10^{-2} dilution c) 10^{-3} dilution d) 10^{-4} dilution e) No dilution.

c)



d)

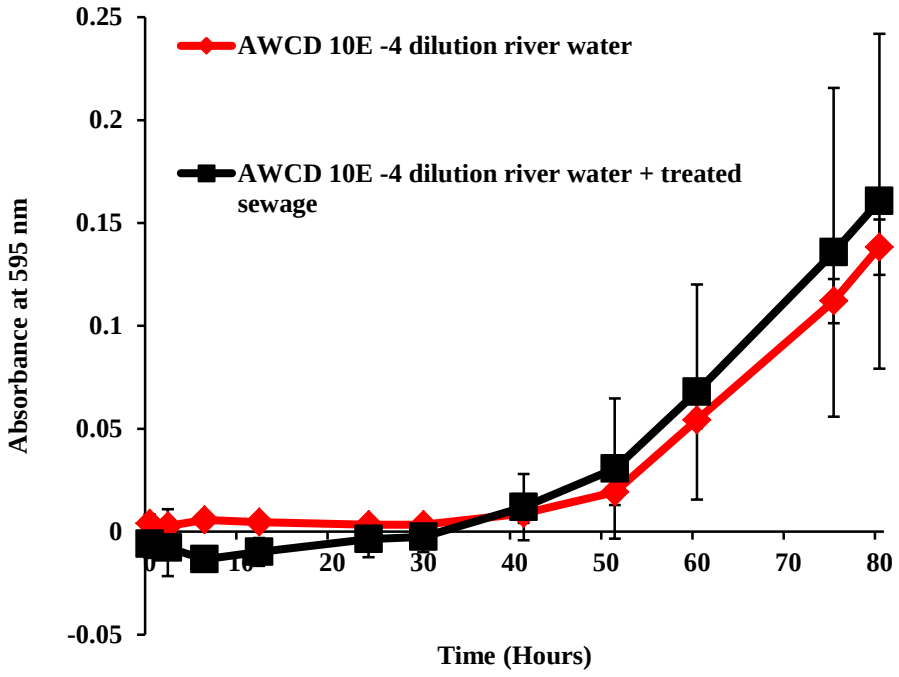


Figure 2.3 (continued).

e)

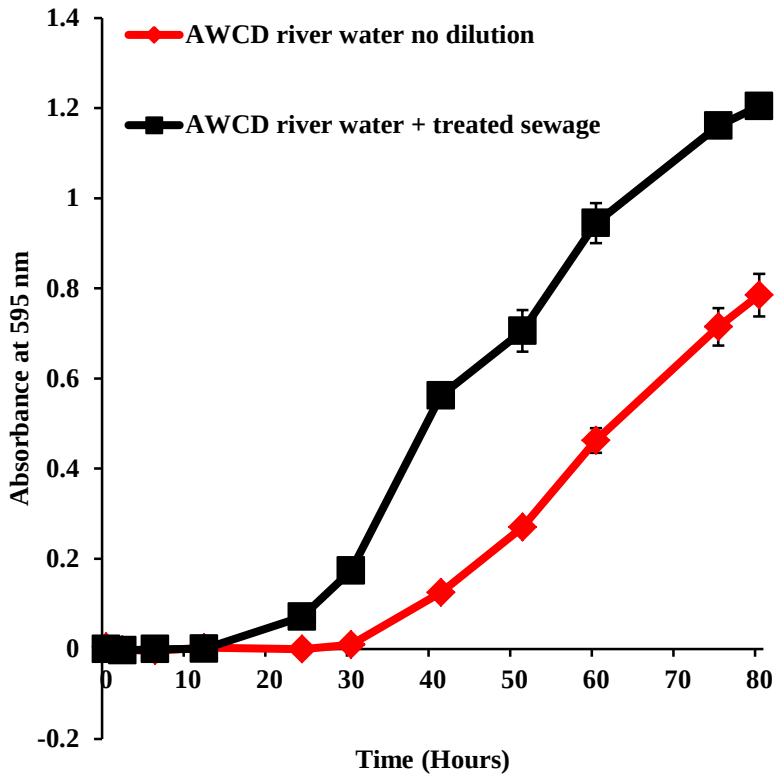


Figure 2.3 (continued).

2.3.2. Validation of the Biolog GN2/Microcosm System with 3, 5-DCP

The data in figure 2.4 show kinetic plots of total substrate utilisation on days 1, 4 and 6 for the different 3, 5-DCP treatments and the controls. PCA analysis of A_{MAX} data is represented by score plots on days 1 and 4 in figure 2.5. The data in figure 2.6 represent maximum rate and lag phase duration versus exposure period duration. The information in table 2.4 shows significant and non-significant changes in maximum rate and lag phase duration for the validation exposure duration. The data in figure 2.7 represent SWDI of substrate utilisation showing statistical significance over all days of the exposure.

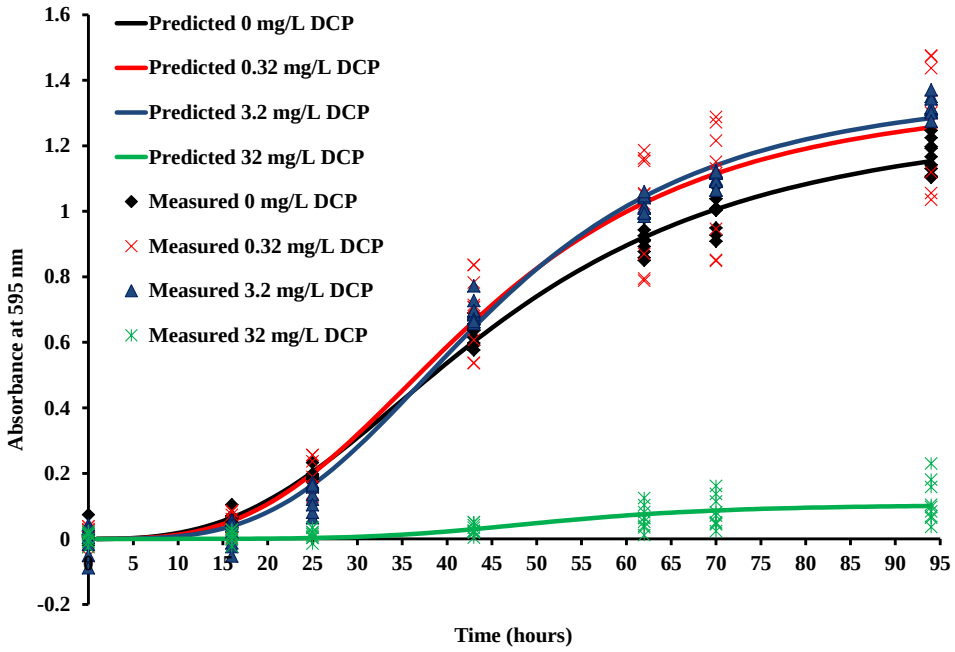
The data represented by kinetic model plots for days 1, 4 and 6 indicate an inhibition of total substrate utilisation as a result of exposing the microbial communities to 32 mg/L 3, 5-DCP. A significant increase in lag phase duration on days 1-4 could also be detected. When the max rate of substrate utilisation was analysed a significant reduction was detected (compared with control data) at the highest 3, 5-DCP concentration on all days of the exposure period. On day 1 of the exposure period a significant increase in lag phase duration could also be detected in microcosms exposed to 3.2 mg/L 3, 5-DCP. At the same concentration a significant increase in the max rate of substrate utilisation was also detected. On day 6 of the exposure period a significant reduction in lag phase duration suggested the onset of a recovery at the highest 3, 5-DCP concentration.

PCA analyses of total substrate utilisation confirm that the multi substrate utilisation profile of the microbial community in microcosms exposed to 32 mg/L was different to the control community. This was suggested by the unique clustering of data from control values. The data represented by scatter plots from all other days also suggested differential substrate utilisation profiles for replicates exposed to all other 3, 5-DCP concentrations. These data suggest that the multi substrate utilisation profile of dosed microbial communities was different to the control profile. It can be tentatively concluded therefore that the microbial community structure in all dosed microcosms was different to those that were present in control microcosms. There was some overlap between clustering of 0 and 0.32 mg/L 3, 5–DCP exposed replicates on days 1 and 4 however. This would suggest that there was a small degree of similarity between the multi substrate utilisation profile and thus the microbial community structure.

Analysis of multi substrate utilisation data using the SWDI shows that the functional diversity of substrate utilisation was significantly reduced by 32 mg/L 3, 5-DCP on all days of the exposure period. The diversity of substrates that the microbial community was metabolising was also significantly inhibited by 3.2 mg/L 3, 5-DCP on day one of the exposure period. On all other days there were not any significant changes in the diversity of substrates that were being utilised. It is likely therefore that the microbial community that was present in microcosms that were spiked with this concentration of 3, 5–DCP had recovered by day 2 of the exposure period. In contrast, microbial communities that were exposed to 32 mg/L 3, 5-DCP did not show any signs of recovery during the exposure period.

The results therefore suggest that 3, 5-DCP can exert an effect on total substrate utilisation down to a 3, 5-DCP concentration of 0.32 mg/L when analysed by multivariate statistics on all days of the exposure period. When looking at the effects of 3, 5-DCP as detected by kinetic data however acute effects were only seen at a lower concentration of 3.2 mg/L at day. More chronic kinetic effects of the reference toxicant (after day 1) were observed at the highest dose of 3, 5-DCP.

a)



b)

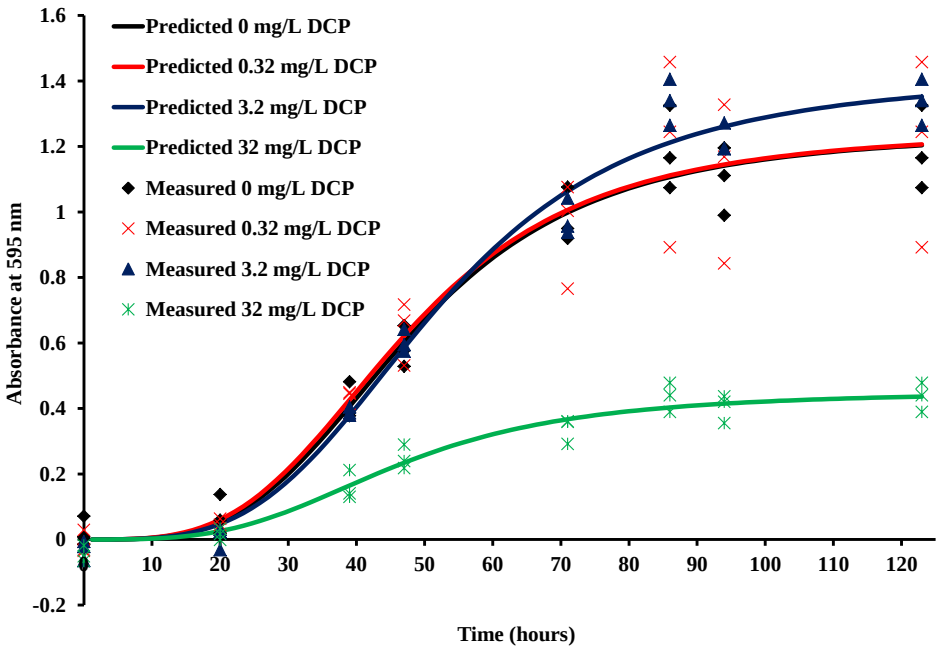


Figure 2.4: Kinetic plots showing total substrate utilisation in control and 3,5-DCP exposed microcosms on a) Day 1, b) Day 2 and c) Day 4.

c)

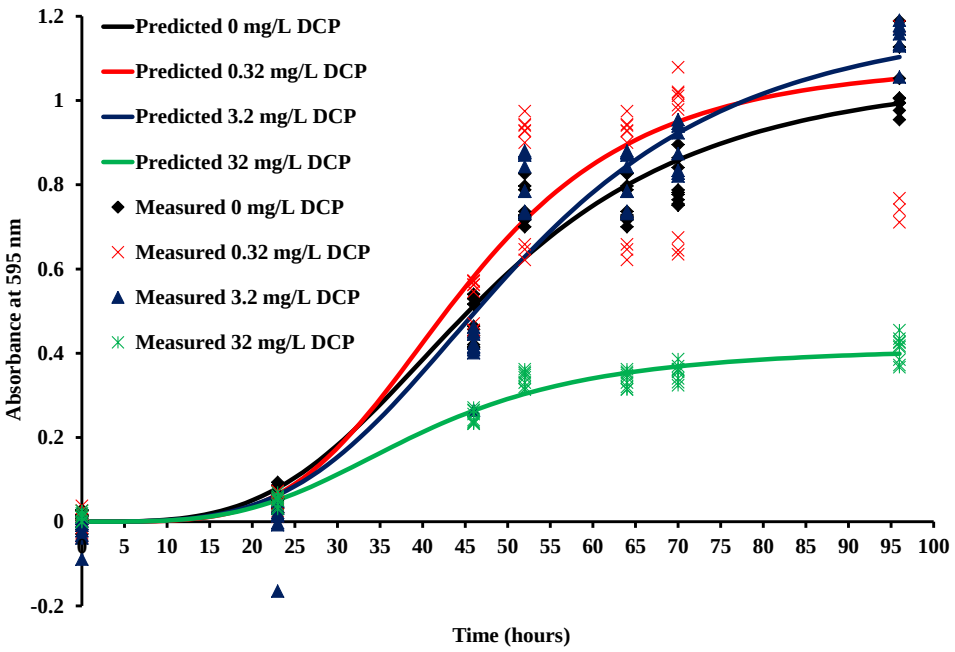
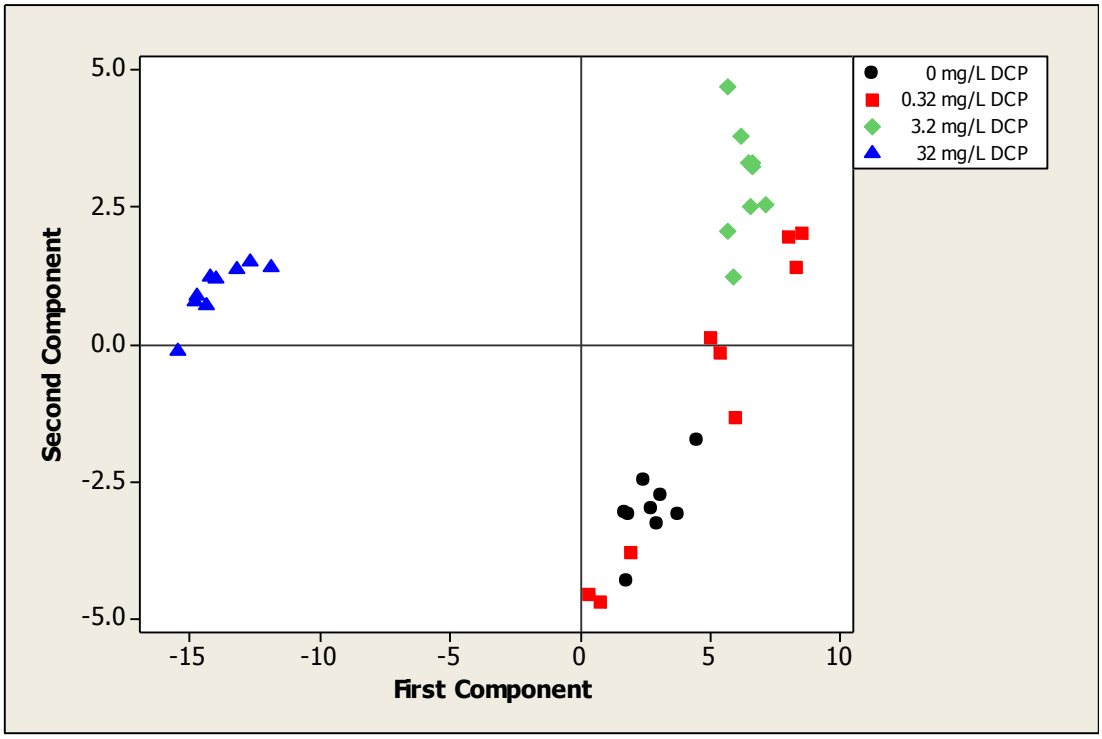


Figure 2.4 (cont).

a)



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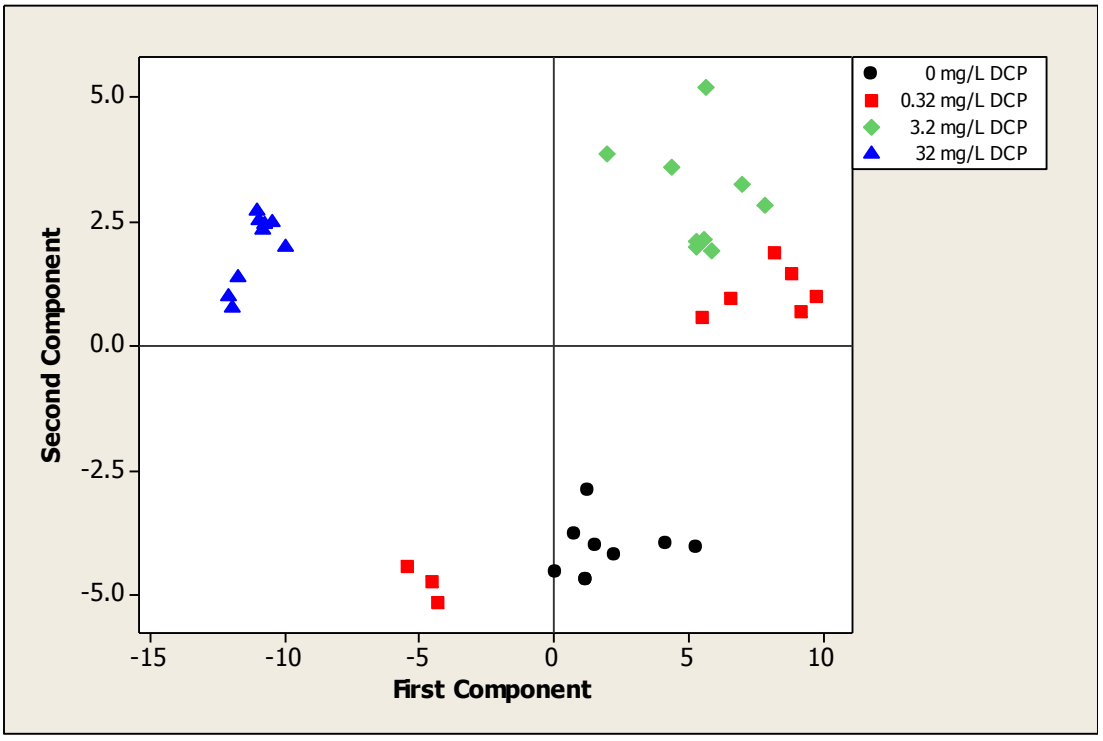
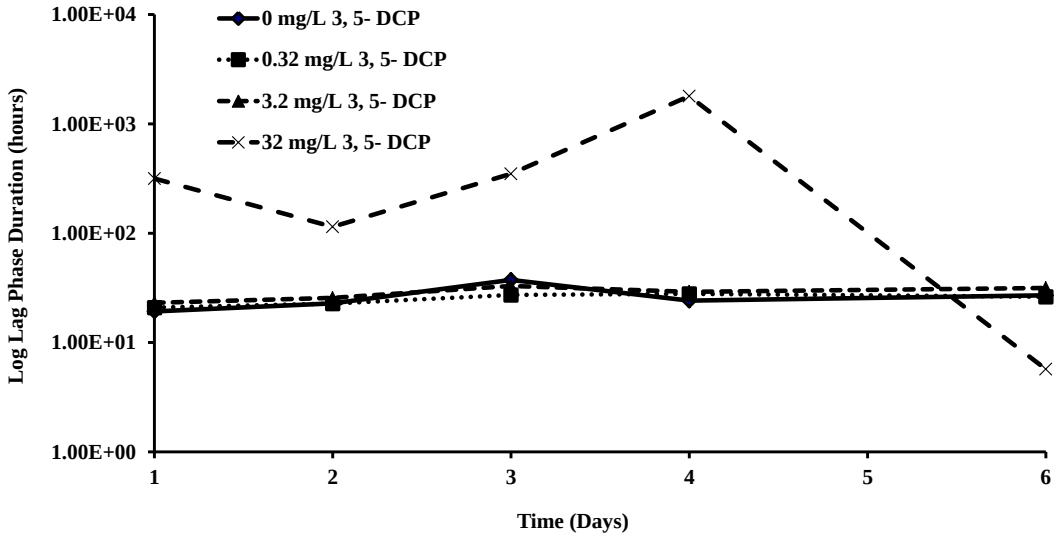


Figure 2.5: Scatter plots of PC scores for the first two principle components for individual replicates from each 3, 5-DCP treatment group on a) Day 1 and b) Day 4. Day 1 (a) proportion of variability: PC1 = 75.1%, PC2 = 7.6%; Day 4 (b) proportion of variability: PC1 = 57.8%, PC2 = 10.8%.

a)



b)

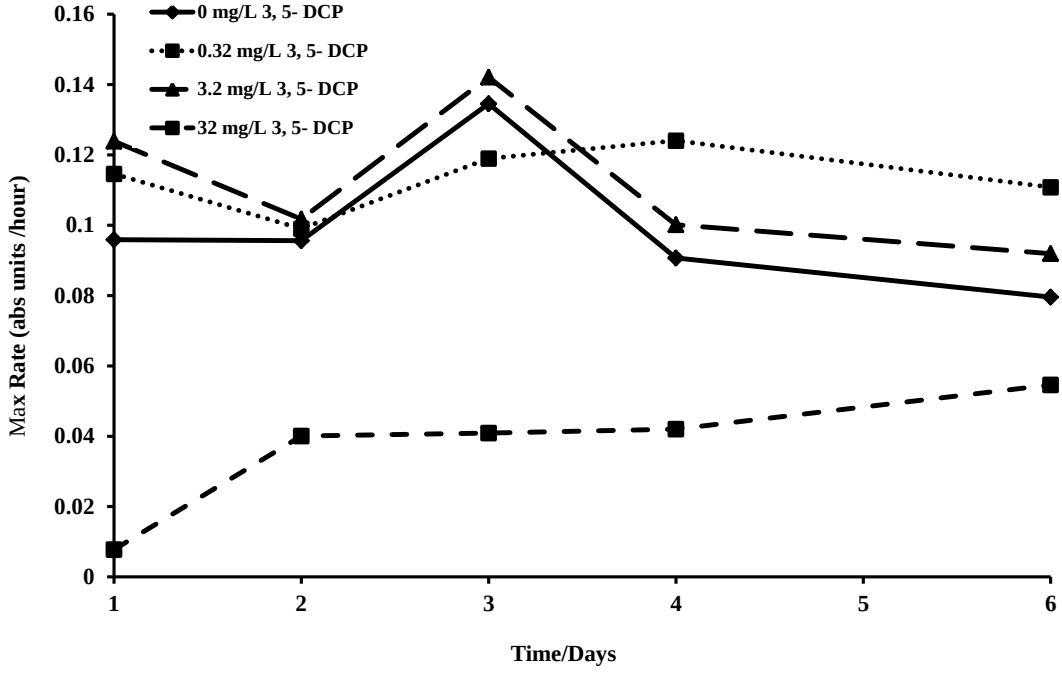


Figure 2.6: a) Log lag phase versus exposure period time for total substrate utilisation and b) Max rate of total substrate utilisation versus exposure period time following 3, 5-DCP exposure.

Table 2.4: Summary of one-way ANOVA results for lag phase values from Biolog data for inocula from microcosms following different exposure times to 3, 5-DCP in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[3 , 5-DCP] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.32	**> ***	< ns	< ns	> ns	n/a	> ns
3.2	**> ***	> ns	< ns	> ns	n/a	> ns
32	**> ***	**> ***	**> ***	**> ***	n/a	**< ***

Table 2.5: Summary of one-way ANOVA results for maximum rate of substrate utilization values from Biolog data for inocula from microcosms following different exposure times to 3, 5-DCP in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[3 , 5-DCP] / mg/L	**1**	**2**	**3**	4	**5**	**6**
0.32	> ns	> ns	> ns	> ns	n/a	> ns
3.2	**> ***	> ns	> ns	> ns	n/a	> ns
32	**< ***	**< ***	**< ***	**< ***	n/a	**< ***

[1] <: Value was less than the control value, but statistical significance was not shown; >: Value was greater than the control value, but statistical significance was not shown; =: Value was equal to the control value; <*: Value was less than the control value and $p < 0.05$; >*: Value was greater than the control value and $p < 0.05$.

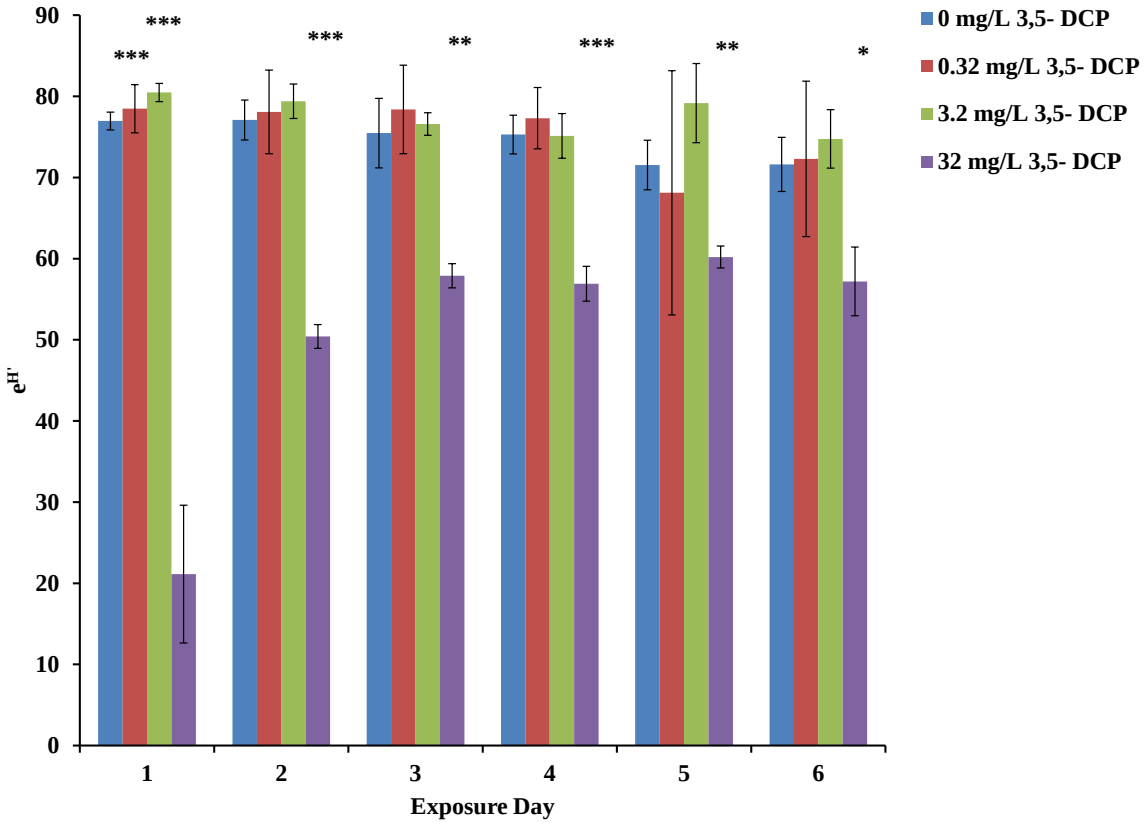


Figure 2.7: Bar graph illustrating richness of substrate of utilisation ($e^{H'}$) versus exposure day at various [3, 5-DCP] compared with untreated data as analysed by the SWDI. Statistically significant changes in species richness are depicted by asterisk notation (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

2.4. Discussion

An aquatic assay and microcosm system were developed to quantify the effects of a toxicant on the ecological functioning of aquatic microbial communities. The system had to act as a surrogate for the aquatic environment. To achieve this, natural river water and treated sewage were used in the microcosms. As such the microcosm system would act as a "bridge between theory and nature" (Fraser, 1999). The addition of treated sewage to the microcosm system posed the potential threat of nutrient carryover, possibly reducing the magnitude of the spectral response that could be detected in Biolog GN2 plates (Preston-Mafham *et al*., 2002). As such inoculum density experiments were conducted. The results of these studies revealed that natural waters amended with treated sewage gave a greater spectral response than river water alone. This suggested that the activity of the microbial community (in terms of substrate utilisation) was greater. Due to the reduced variation in undiluted samples and the greater functional response that was observed in a neat mixture of 9:1 river water: treated sewage, this was seen as being the optimum matrix for microcosm/Biolog GN2 studies. Due to concerns over detergent levels in treated sewage (and the toxicological effects they may exert on microbial communities) the system was amended with 5% treated sewage and 5% OECD synthetic sewage. The addition of OECD synthetic sewage had the dual function of acting as a nutrient source as it does in other regulatory test such as the OECD 303A test for example. The final microcosm system was also given a maximal volume in relation to available lab space and equipment (5 litre fermentation vessels). This allowed the most diverse microbial community to inhabit the microcosm system as possible. Ingerslev *et al.* (2000) had previously noted that a larger volume within a microcosm system increases the diversity of the microbial community in biodegradation studies.

With the microcosm/Biolog system now developed it was then determined if the microbial community (within the microcosm system) could be inhibited by the OECD reference toxicant 3,5-DCP in terms of a kinetic response. The effect of 3, 5-DCP on the community structure and functional diversity of the microbial community were also investigated. Time series experiments were therefore conducted at 3 different 3, 5-DCP concentrations and one control concentration (0 mg/L 3, 5-DCP). Statistical analyses of kinetic data revealed that significant effects on lag phase duration and the max the rate of total substrate utilisation could be determined on all exposure days at the highest toxicant concentration that was tested (32 mg/L). The same data analysis of microcosms exposed to 3.2 mg/L 3, 5-DCP revealed that significant changes in lag phase duration and max rate could also be detected on day 1 of the exposure period. Kinetic plots and statistical analyses appeared to indicate that the current test system fell within the validity criteria of the ASRIT assay (EC_{50} value of between 3-32 mg/L) (OECD, 1984) on all days of the exposure period. PCA analysis could resolve the effects of 3, 5-DCP down to a concentration of 0.32 mg/L up to day 6 of the exposure period. The mechanism of these effects remains unclear, so further analysis is necessary to ascertain this. These results infer that that the composition of the microbial community

is likely to have been altered by exposure to 0.32 mg/L 3, 5-DCP. The exact changes in microbial community structure could be confirmed by molecular techniques such as DGGE (Denaturing Gradient Gel Electrophoresis), TRFLP (Terminal Fragment Length Polymorphism) or DNA sequencing. It is clear though that multivariate statistics were able to resolve the effects of lower 3, 5-DCP concentrations than was possible by a kinetic analysis. Kinetic analyses remain more specific however as both lag phase duration and the max rate of substrate utilisation can be tracked. Both analyses therefore exhibit strengths and weaknesses. As such it would be prudent to analyse data with both sets of statistical techniques in future studies. As such, a quantitative and semi-quantitative approach can be taken.

Functional diversity analysis (of multisubstrate utilisation) demonstrated that the microbial community was significantly inhibited by 32 mg/L 3, 5-DCP on all exposure days and was also inhibited by 3.2 mg/L 3, 5-DCP on day 1 of the experiment. These data suggest that the microbial community (or certain parts of the microbial community) were unable to utilise the same diversity (or range) of substrates that microbial communities exposed to 0 and 0.32 mg/L 3, 5-DCP were able to utilise. These results suggest that the microcosm/Biolog GN2 system may be able to identify similar effects when the microbial community is exposed to other toxicants, such as antibiotics. In terms of studying the effects of antimicrobial toxicants on the diversity of substrate utilisation therefore, the data from the present investigation suggest that the reference compound 3, 5-DCP can be used a positive control for studies that have a duration similar to the one used in this study (6 days). The microcosms/Biolog GN2 system has therefore been shown to detect both acute and chronic effects on the functional diversity of substrate utilisation.

Due to the small concentration range an exact EC_{50} value was difficult to derive for the present study. As such, future experiments of this nature could repeat current experiments with a greater number of concentrations. It is likely however that the EC_{50} of 3, 5-DCP in the current investigation lies between the range of 3 and 32 mg/L. For a regulatory test such as the ASRIT, the results of any test must show that the reference compound (3, 5-DCP) exerts an EC_{50} of between 3 and 32 mg/L for the study to be considered valid. The current investigation is therefore likely to be considered valid from a regulatory viewpoint. Other future work could compare the EC_{50} value of a repeated study (using more exposure concentrations) with some EC_{50} values that have been observed in a range of regulatory studies using standard tests such as the ASRIT. This would likely involve close collaboration with an institution such as a CRO (Contract Research Organisation). Such laboratories would have access to a huge array of such data as regulatory tests are conducted there on a daily basis.

The results of this chapter point to the current microcosm/Biolog GN2 system showing promise as a risk assessment tool. The test could be used in place of the ASRIT assay in phase II, tier A of the environmental risk assessment procedure. Alternatively, the system could also act as a higher tier study, in phase II, tier B for example. In any case, the system has several advantages over existing

methods. The main advantage is that the system tests changes in microbial function in a simulated river water environment. No standard test currently offers protection to complex aquatic microbial communities. The current system also tracks the utilisation of a large array of substrates from a range of substrate types (guilds). Standard tests such as the ASRIT use only glucose as a metabolic substrate. But natural environments contain a much wider array of substrates. In addition, in many cases the specific mode of action of toxicity of a compound may be able to be related to a specific substrate utilisation profile. Kong *et al*. (2006) for example showed a decrease in utilisation of all substrate guilds as a result of exposing soil microbial communities to oxytetracycline. This could be attributed to the inhibition of protein synthesis and subsequent reduction in production of essential cellular proteins (such as enzymes and metabolic co-factors).

The current system also offers a larger time scale to study the temporal effects of a toxicant, as Biolog plates are inoculated on each day of the exposure period. This allows for the effects of a toxicant (on microbial communities) to be tracked over a greater time period than is possible with other tests such as the ASRIT. This offers the advantage that the effects of a toxicant can be identified over a longer exposure period. In addition, this provides the opportunity to study any potential recovery of the microbial community which may occur. Also, as Biolog plates are incubated for ≥ 75 hours, a kinetic response can be calculated for each day of the exposure period. This is not possible using tests such as the ASRIT, which only measures oxygen consumption (of the microbial community) over a ~15 minute period at the end of a 3 hour exposure period. Several authors have raised concerns over the brevity of standard test such as the ASRIT and Microtox (Backhaus and Grimme, 1997; Kummerer *et al*., 2004). A longer exposure period may be required when studying the effects of certain compounds that inhibit the growth of aquatic microorganisms such as bacteriostatic antibiotics (e.g.: tetracyclines).

Due to the potential for guild (related to specific substrate types) data to be analysed, the microcosm/Biolog GN2 system can also be used a tool to study distinct aspects of the carbon and nitrogen cycle. Specifically, amine/amide and amino acid utilisation can be linked to the nitrogen cycle and carbohydrate/carboxylic acid utilisation can be linked to the carbon cycle. Analysis of polymer utilisation data can also be used to quantify how polymers are degraded in the environment in the presence of a toxicant. With a lack of standardised systems/assays to quantify the potential toxicity of antibiotics (or other toxicants) in the aquatic compartment in terms of specific microbial function the current results show promise of this being achieved with the methods that have been developed within this chapter. In the next two Chapters, the developed methods are applied to two 'model' antibiotics: chlortetracycline and sulfamethoxazole.

Chapter 3: Effects of Chlortetracycline on Microbial Functioning in Aquatic Systems

3. Effects of CTC on Microbial Function

3.1. Tetracyclines

Benjamin M. Duggar first isolated chlortetracycline from the soil-dwelling bacterium *Streptomyces aureofaciens* in 1945 The discovery of CTC heralded the discovery of the first broad-spectrum antibiotic, with the compound capable of treating infections from both Gram positive and Gram negative bacteria, as well as well as atypical infections such as chlamydiae and rickettsiae (Chopra and Roberts, 2001). It is thought to be effective against approximately fifty diseases. The discovery of the next tetracycline, oxytetracycline soon followed. The compound was isolated from another soil bacterium, *Streptomyces rimosus*, in 1950 (Finlay *et al*., 1950). The structure of oxytetracycline was determined shortly after by Robert Burns Woodward, paving the way for Lloyd H. Conover to produce the first synthetic tetracyline in 1953 (Bhattacharya, 2003). A number of natural and semi-synthetic tetracyclines have been discovered since, with the newest group (the tigilcyclines) having recently gone through phase II clinical trials (Chopra and Roberts, 2001). The chronology of tetracycline discovery is summarised in table 3.1.

Table 3.1: Chronology of tetracycline discovery (modified from Chopra and Roberts, 2001).

Chemical Name	Generic Name	Trade Name	Year of Discovery
7-CTC	CTC	Aureomycin	1948
5- hydroxytetracycline tetracycline	oxytetracycline	Terramycin	1950
tetracycline	tetracycline	Achromycin	1953
6-demethyl-7-CTC	demethylchlortetracycline	Declomycin	1957
2- *N*-pyrrolidinomethyltetracycline	rolitetracycline	Reverin	1958
2-*N*-lysinomethyltetracycline	limecycline	Tetralysal	1961
N-methylol-7-CTC	clomocycline	Megaclor	1963
6- methylene-5-hydroxytetracycline	methacycline	Rondomycin	1965
6-deoxy-5-hydroxytetracycline	doxycycline	Vibramycin	1967
7-dimethylamino-6-demethyl-6-deoxytetracycline	minocycline	Minocin	1972
9-(t-butylglycylamido)-minocycline	tertiary-butylglycylamidominocycline	Tigilcycline	1993

3.1.2. Chemistry of Tetracyclines

Having an octahydrotetracene-2-carboxamide skeleton, tetracyclines are a sub-class of the polyketides and are known collectively as derivatives of polycyclic naphthacene carboxamide. Tetracyclines are composed of four linearly fused tetracyclic rings (named A, B, C and D). A number of functional groups can be covalently bonded to the 4 tetracycline rings to form specific members of the tetracycline group. The structure of the linear fused tetracycline ring is subject to the naturally occurring conversion of keto and enol forms on positions 11, 12 and 12a of the D ring and stereochemical configurations at the ring A/ring B interface at positions 4a and 12a. An additional stereochemical conformation can also be found at position 4. Tetracylines are zwitterionic. In addition, tetracyclines can exist in an extended or folded form (Jin *et al*., 2007).

Tetracyline, oxytetracycline and CTC exhibit reversible epimerisation at the C4 position. 4-epimers (epi-CTC, epi-oxytetracycline and epi-tetracycline) of these compounds are formed as a result of epimerisation reactions (Doershuck *et al*., 1955). Epimerisation of tetracyclines has been shown to occur between pH 2 and 6 although epimerisation has been found to occur at pH 7 in the case of epi-tetracycline. A molar ratio of between 0.4 and 0.6 exists for the epi-tetracycline: tetracycline equilibrium (McCornick *et al.,* 1957). Hussar *et al*. (1968) have reported that CTC and tetracycline tend to epimerise to a greater extent than oxytetracycline. This has been attributed to the binding of the C5 hydroxyl and dimethylamino groups.

The chemical behaviour of tetracyclines is pH dependent. Respective iso-tetracyclines may be formed by cleavage of the C6 hydroxyl group under alkaline conditions (Mitscher, 1978). CTC is especially vulnerable to C6 hydroxyl cleavage and is irreversibly transformed into iso-CTC under alkaline conditions (Waller *et al.,* 1952; Stephens *et al.,* 1954). If oxygen is present under alkaline conditions desmethyl or didesmethyl analogs of tetracyclines can be formed, such as *N*-desmethyl-tetracycline (Waller *et al.,* 1952; Stephens *et al*., 1954).

Tetracycline chemistry is also affected by lower pH. Dehydration of a hydrogen atom at position C5a and of the hydroxyl group at position C6 occurs under acidic conditions with CTC, oxytetracycline and tetracycline. Respective epimers of each compound are subject to the same reactions. In the case of oxytetracycline the process is irreversible. The resultant products of these reactions are apooxytetracycline, apoepioxytetracycline, apochlorterracycline, apo-epi-CTC, apotetracycline and apo-epitetracycline. Due to their relative instability, apooxytetracycline and apo-epioxytetracycline can be further transformed into α and β phtalides due to scission of ring B at the C5 hydroxyl position (Hochstein *et al*., 1953).

Another important aspect of tetracycline (and epi-tetracycline) chemistry is their tendency to form keto and enol tautomers in aqueous solution. Under alkaline conditions, CTC and 4-epi-CTC can undergo keto/enol-tautomerism between position C11a and C12 (Noidong *et al*., 1990). There are a reported 64 tautomer permeations existing for tetracycline considering the five possible

protonations (H_4Tc^+, H_3Tc, H_2Tc^-, $HTc^{2-}$and Tc^{3-}) and four acid dissociation constants (pK_as at pH 3.2, 7.6, 9.6, and 12) that can occur (Duarte *et al*., 1998; Jin *et al.*, 2007). The specific combination of tautomers depends on physiochemical factors such pH and the dieclctric constant of a solution. There has not however been keto/enol tautomerism reported for oxytetracycline (Halling-Sørensen *et al*., 2002).

The tendency for tetracyclines to chelate with divalent and trivalent metal ions at positions C11 and C12 represent another important aspect of tetracycline chemistry. Most work in this area has focused on chelation with Ca^{2+} and Mg^{2+}, due to their physiological significance (Jin *et al*., 2007). Binding stoichoimetries of 1:1, 1:2, and 2:1 bound ions per tetracycline molecule have been calculated for both ions between pH 6.5 and 6.8 (Lambs *et al*., 1984; Berthon *et al*., 1983; Newman and Frank, 1976; Martin, 1979; Wessels *et al*., 1998; Schmitt and Schneider, 2000). The binding of tetracyclines with minerals, such as aluminium and iron hydrous oxides has also been demonstrated (Cheng and Karthikeyan, 2005). Several chelation sites can be found on the tetracycline molecule. These include the β-diketone system on positions C11 and C12 and at the enol systems of C1 and C3. A chelating carboxamide group can also be found at position C2 of the tetracycline A-ring (Blackwood, 1985).

3.1.3. Tetracycline Mode of Action

The first aspect of tetracycline mode of action is the mechanisms by which they enter the Gram-positive and Gram-negative cell (Chopra and Roberts, 2001). In Gram-negative enteric species tetracycline crosses the outer membrane by passive diffusion. The molecule does so by passing through the porin membrane channel proteins OmpF and OmpC. Tetracycline molecules enter porin channels as positively charged co-ordination complexes, probably with Mg^{2+} ions (Chopra and Ball, 1982; Chopra, 1985; Schnappinger and Hillen, 1996). The Donnan potential (the formation of electric potential between two solutions) of the outer membrane draws the cationic tetracycline-Mg^{2+} into the cellular periplasm. It is thought that tetraycline molecules accumulate in the cell as uncharged, weakly hydrophilic molecules (having now dissociated with Mg^{2+} ions). It is this species that can then be transported across the inner cytoplasmic membrane. In Gram-positive bacteria it is an electroneutral lipophillic tetracycline species that passes through the outer cytoplasmic membrane. The passing of tetracyclines into microbial cells is energy dependent. The energy is supplied by a ΔpH-dependent proton motive force (Yamaguchi *et al*., 1991a; 1991b; Nikaido and Thanassi, 1993; Chnappinger and Hillen, 1996). Due to the divalent metal concentration and pH within the cytoplasm it is likely that the tetracycline molecule becomes chelated once more before exerting its mode of action (Chnappinger and Hillen, 1996).

Once in the cytoplasm tetracyclines exert their bacteriostatic (growth is prevented, but cells are not killed) action primarily by transient inhibition of protein synthesis at the ribosomal level (Tritton, 1977; Gale *et al.*, 1981; Chopra, 1985; Rasmussen *et al.*, 1991). Inhibition of protein synthesis

occurs mainly when tetracycline molecules preferentially bind to the ribosomal A (acceptor) site of prokaryotic 70S ribosomes, reducing tRNA binding efficiency to 20% of normal levels (Epe *et al*., 1987). Since the A site binds aminoacyl tRNA during the translation stage of protein synthesis, codon-anticodon matching is disrupted. Tetracyclines exert this effect by binding to six binding sites on the 30S sub-unit of the 70S ribosome (Pioletti *et al*., 2001). Tet-1 represents the aminoacyl tRNA docking site and is the major (or "primary") tetracycline-binding site (Epe *et al*., 1987).

Tet-1 is located between the distorted minor groove of H34 and the stem–loop of H31 (Cate *et al*., 1999). Tetracycline molecules are held in place by a clamp formed by bases 1196 and 1054 of 30S sub-unit rRNA (ribosomal RNA). The binding of tetracyclines to this region is Mg^{2+} - dependent (Pioletti *et al*., 2001). Tetracyclines are also known to inhibit the binding of release factors RF1 and RF2 to the ribosomal small sub-unit A-site. Release factors are responsible for the binding to ribosomes when certain stop signals appear at the A site (UAG and UAA (for RF-1) or UGA and UAA (for RF-2). Peptides are released from the A site when these sequences are recognized (Brown *et al.*, 1993). If a tetracycline is bound to these sites then peptidyl formation will be greatly disadvantaged. A further five tetracycline binding sites (Tet-2–Tet-6) have also been identified. With the exception of Tet-2, all binding sites are located within the 16S rRNA structure.

The binding of tetracyclines at loop 970 of the ribosomal P site has consequences for correct positioning of tRNA molecules during protein synthesis, since this site is thought to be essential for correctly positioning tRNA (Saraiya *et al*., 2008). The high affinity binding of tetracyclines to the ribosomal protein S7 (near the anti-codon loop at the ribosomal P site) represents another mechanism by which tetracyclines inhibit protein synthesis. Protein S7 has been shown to be in close contact with the anticodon loop of P site-bound tRNA and with mRNA upstream of crucial decoding regions (Wower *et al.*, 1993; Doring *et al*., 1994). Protein S7 also has important cross-linking properties with 16S rRNA (Moller *et al.*, 1978; Urlaub *et al*., 1997). The interaction of tetracyclines with protein S7 therefore has effects on ribosomal codon binding and on the conformational structure of the ribosomal small sub-unit.

Another binding site (Tet-6) is located on the ribosomal E (or "exit") site, where deacylated peptides leave the ribosome. Although the exact function of the E site has remained controversial recent studies have shown that it is essential for avoiding frameshift mutations and for efficient translocation of polypeptide chains. It has also been shown to have a role in GTPase turnover activity (Sergiev *et al*., 2005). Binding of tetracyclines to the E site might also further affect 30S ribosomal functioning. The binding of tetracyclines to ribosomal sites A, P therefore has several direct effects on protein synthesis with inferred effects on the ribosomal E-site.

Tetracyclines have also been shown to have other binding targets that help explain its mode of action. One of these is the binding of tetracyclines to elongation factor Tu (Ef-Tu) (Gordon, 1969; Lucas-Lenard and Raenni, 1969; Ravel *et al.*, 1969; Shorey *et al.*, 1969; Skoultchi *et al*., 1970;

Spirin and Asatryan, 1976; Semenkov *et al*., 1982). EF-Tu-GTP is the most abundant protein in *E. coli* cells and is responsible for the binding and delivery of an aminoacyl-tRNA complex to the A site on the ribosome. The process is dependent on the binding of GTPase and ATPase. The binding of EF-Tu-Tetracycline was recently crystalised, revealing Mg^{2+} dependent binding domains that are shared by all GTPases and many ATPases. The binding of tetracyclines to Ef-Tu therefore represents a pre-translational process that inhibits microbial protein synthesis (Heffron *et al*., 2006). Interestingly, Werner *et al.* (1975) had previously reported the inhibition of the translocation process by tetracylines. It is thought this may occur via the targeting of an elongation factor that is homologous to Ef-Tu, Eg-G (Heffron *et al*., 2006).

Several tetracycline analogs also exist that can act as bactericidal (i.e they kill cells) agents. These compounds (also termed "atypical tetracyclines") include anhydrotetracycline, anhydroCTC, 6-thiatetracycline, chelocardin and 4-epi-anhydro-CTC. Anhydro-CTC and 6-thiatetracycline do still possess some ribosomal inhibition properties however (protection of base A892) (Chopra, 1994). Enzyme leakage studies have revealed that tetracycline analogs target the cytoplasmic membrane. Cell lysis occurs and cells subsequently die (Oliva *et al*., 1992). It has also been shown that atypical tetracyclines prevent nucleic acids being incorporated into DNA and RNA (Rasmussen *et al.*, 1991). Although the exact mechanism of action of atypical tetracyclines is not fully understood, it is believed that the lipophillic compounds become trapped in the hydrophobic cytoplasmic membrane where they exert their effect (Rasmussen *et al.*, 1991). This theory is galvanised by studies that have shown that atypical tetracyclines can still exert an anti-microbial effect in the presence of tetracycline efflux pumps and ribosomal protection proteins (Oliva and Chopra, 1992). Due to side effects in clinical trials (possibly due to indiscriminate mode of action in prokaryotes and eukaryotes) atypical tetracyclines are not used therapeutically (Chopra and Roberts, 2001).

3.1.4. Tetracycline Usage

Tetracyclines are broad-spectrum antibiotics and are therefore used to treat a wide range of microbial infections (Chopra and Roberts, 2001). Tetracyclines can be applied as either a topical, oral or intravenous application, both in human and veterinary medical practice. Tetracyclines are also used for non-anti-infective purposes. In some countries for example tetracyclines are used as growth-promoters in husbandry, cattle swine and poultry (Sanderson *et al*., 2005). However, the use of tetracyclines as growth promoters was phased out in the EU in 2000. Tetracyclines have also been utilised as prophylactics in fish farming (Primavera *et al*., 1993). Tetracyclines also have a number of alternative uses in human medicine. The compounds have been utilised clinically as chelating ionophores, inhibitors of inflammation, in proteolysis, angiogenesis and as anti-apoptotic agents (Ross *et al*., 1998; Sapadin and Fleischmajer, 2006).

Despite the use of tetracyclines as growth promoters in Denmark and the Netherlands being reduced by 50% between 1997 and 2004 they were still among the most widely used antibiotics in the EU (KTBL, 2005; MANRAN, 2004).

3.1.5. Occurrence of Tetracyclines in the Environment

Tetracyclines have been detected in several environmental matrices such as surface water (e.g.: Calamari *et al*., 2003), soil (e.g.: Hamscher *et al*., 2002), groundwater (e.g.: Karthikeyan and. Bleam, 2003) and marine sediment (e.g.: Lalumera *et al*., 2004). Reported occurrences of tetracycline residues in the environment are summarised in table 3.1. Table 3.1 illustrates the fact that tetracyclines have been found mainly in water bodies. The data also suggest that tetracyclines have been detected in soils that have been amended with liquid manure. This would make sense as manure represents a major exposure route of antibiotics into the soil environment.

Table 3.2: Reported occurrences of tetracyclines in several environmental matrices. Data in italics indicates limit of quantification.

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference
CTC	Soil amended with liquid manure	Denmark	0.6 x 10^{-3} mg/kg *1.1x 10^{-3} mg/kg*	nd - 15.5 x 10^{-3} mg/kg	Jacobsen *et al.*, 2004
	Soil amended with liquid manure	Germany	2 x 10^{-3} mg/kg *5x 10^{-3} mg/kg*	4.6 - 7.3 x 10^{-3} mg/kg	Hamsher *et al.*, 2002
	Stream water	USA	0.05 mg/kg	0.49 - 0.69 mg/kg	Yang and Carlson, 2004b
oxytetracycline	Stream water	USA	*0.10* µg/L	0.16 µg/L	Kolpin *et al.*, 2002
	Surface water	Italy	*<0.0003 µg/L*	0.01435 µg/L	Calamari *et al.*, 2003
	Surface water	Italy	*<0.0003 µg/L*	nd – 0.01920 µg/L	Calamari *et al.*, 2003
	Stream water	USA	*0.10 µg/L*	0.34 µg/L	Kolpin *et al.*, 2002
	Lake water (near agricultural land)	USA	*0.05 µg/L*	0.13 µg/L	Yang and Carlson, 2004b
	Lake water	USA	0.05	0.09	Yang and Carlson, 2004b
tetracycline	Marine sediment	Italy	0.061 µg /lg	246.3 µg /kg	Lalumera *et al.*, 2004
	Soil amended with liquid manure	Germany	1 x 10^{-3} mg/kg *(5x 10^{-3}) mg/kg*	(86.2 - 198.7) x 10^{-3} mg/kg	Hamsher *et al.*, 2002
	Surface water	USA	0.05 µg/L	0.14 µg/L	Yang and Carlson, 2004b
	Surface water	USA	0.05 µg/L	0.06 and 0.12 µg/L	Yang and Carlson, 2004b

Table 3.2 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentration	Reference
tetracycline (cont)	Groundwater	USA	*0.05 μg/L*	0.5 μg/L	Karthikeyan and. Bleam, 2003
doxycycline	Lake water	USA	0.05 μg/L	0.05 μg/L	Yang and Carlson, 2004b
	Surface water	USA	0.05 μg/L	0.08 μg/L	Yang and Carlson, 2004b
	Surface water	USA	0.05 μg/L	nd and 0.05	Yang and Carlson, 2004b
democlocycline	Surface water	USA	0.05 μg/L	0.12 and 0.32 μg/L	Yang and Carlson, 2004b
	Surface water	USA	0.05 μg/L	0.44 μg/L	Yang and Carlson, 2004b

LOD = Limit of detection; LOQ = Limit of quantification; nd = not detected.

3.1.6. Fate and Behavior of Tetracyclines in the Environment

The behavior of tetracyclines in soil, manure, sewage treatment plants and the aquatic environment (surface water and sediment) have been reported. Such is the complexity of tetracycline behavior in the environment it has been suggested that the traditional approach of linking log Kow and organic carbon content with sorption alone are not sufficient (Tolls, 2001). Due to three environmentally relevant pKa values, tetracyclines can exist as cations, zwitterions or as net negatively charged anions (Sassman and Lee, 2005). Additional reported aspects of tetracycline behavior can be attributed to the chelation of tetracycline residues with metallic ions that are present in the environment, such as metal percholates (Mikulski *et al*., 1988), aluminium (De Almeida *et al*., 1998; Dos Santos *et al*., 2000; Gu and Karthikeyan, 2005), magnesium (Wessels *et al*., 1998; Schmitt and Schneider, 2000) and iron oxides (Gu and Karthikeyan, 2005). It is noteworthy that quite often contradictory data can be found with regard to the fate of tetracyclines in the environment. It is likely that specific environmental conditions (such as soil organic matter content for example) govern the fate of tetracyclines in the environment. As such, each environmental matrix and its specific physiochemical and biological properties should be taken into consideration when studying the fate of tetracylines in the environment. The same assertion is also likely to be true for other complex and ionisable antibiotics that are present in the environment, such as sulfonamides, which will also be investigated during this study.

3.1.6.1. Fate of Tetracyclines in Soil

Sorption of tetracyclines to a range of soil types has been investigated. Pils and Laird (2007) reported 96% sorption of CTC and tetracycline to clay components in soil and whole soils that contain high proportions of clay in laboratory based studies. Allaire *et al*. (2006) also observed sorption of CTC to clay and sandy loam soils in laboratory studies. They concluded that CTC sorption was significantly greater in clay soils than in sandy loam soils. This data is in agreement with another study that reported CTC binding twice as much to clay soils than sandy loam soils (Chander *et al*., 2005). Rabolle and Spliid (2000) reported that oxytetracycline sorbed almost twice as much to sandy loam soils than it did to sandy soils, with observed Kd values of 1026 and 417 respectively. Allaire *et al*. (2006) additionally showed that 95% of CTC sorption occurs within the first ten minutes following CTC addition. Kulshrestha *et al*. (2004) investigated the sorption of oxytetracycline to clay soils as a function of pH. They found that oxytetracycline sorption decreases with increasing pH, revealing a pH dependent sorption to clay soils. Laboratory studies have also revealed that sorption of oxytetracycline to clay soils can be reduced by the presence of humic substances, suggesting organic matter in soils interferes with tetracycline binding (Kulshrestha *et al*., 2004). Sithole and Guy (1987) had previously demonstrated biphasic tetracycline sorption to humic acid and peat, suggesting the presence of two distinct tetracycline binding sites on organic matter. Jones *et al*. (2005) observed higher oxytetracycline sorption to

soils with an organic matter content of between 4 and 9% than those with less than 4%, suggesting tetracyclines sorb to soils with higher organic matter content or different types of organic content.

Modeling the sorption of three tetracyclines (tetracycline, oxytetracycline and CTC) to clay soils has revealed a proton uptake mechanism at acidic pH and a surface binding mechanism at alkaline pH (Figueroa *et al*., 2004). Jones *et al*. (2005) further suggested that cation exchange may explain sorption between soils and the positively charged tertiary amine group of oxytetracycline occur at pH 5.5. The sorption of tetracyclines to soil organic matter (such as humic substances) is likely to occur via H-bonding between phenolic and carboxyl groups (Sithole and Guy, 1987).

Laboratory and field studies have been conducted to investigate the movement of tetracyclines through soil horizons. Kay *et al*. (2004) tracked the movement of oxytetracycline through a macroporous tile drained clay soil over a two-year period. Oxytetracycline was found mainly in the topsoil, with some movement to deeper soil layers and drainage water. In another soil column study, tetracycline could be detected up to 6 feet belowthe soil surface. Tetracycline could also be detected in a river adjacent to spiked soil columns (2% of tetracycline in applied manure). The soil type in this study was lower in clay and higher in organic matter, indicating that soils lower in clay matter and higher in organic matter can facilitate the movement of tetracycline through soil and into surface water (Aga *et al*., 2003). A laboratory-based study by Kay *et al*. (2005) found that the breakage of macropores (by tilling) significantly reduced the concentration of oxytetracycline found in soil drainage water, implicating the role of preferential macropore flow in oxytetracycline movement through soil where it has been shown occur.

Several studies that have investigated the movement of tetracyclines through soil have only detected the compounds in upper soil layers. Two studies conducted could not detect oxytetracycline or CTC transport below 30 cm soil depth over a two-year period. Although one soil had high clay content, the other had relatively low clay content, suggesting tetracyclines can sorb to lower clay content or to other soil components. Both studies therefore suggested that tetracyclines are not transported to surface water in certain soils (Kay *et al.*, 2005; Hamscher *et al*., 2002; Hamscher *et al*., 2005). These findings are in agreement with an earlier study that did not detect oxytetracycline in the leachate of a soil column (Rabolle and Spliit, 2000). In general however the movement of tetracyclines through soils has yet to be fully elucidated (Pils and Laird, 2007).

The impact of run-off on tetracyline movement from soil to surface water has also been investigated. Yang and Carlson (2003) reported higher levels of tetracyclines in streams near agricultural land compared to pristine locations. The authors attributed this to run-off from soil due to the fact that the areas studied were known to be prone to run-off. Another study reported extremely low levels of tetracyclines in surface water due to run-off compared with other veterinary medicines however (Davis *et al*., 2006), suggesting such events are dependent on

individual areas. Kim and Carlson (2007) also found higher concentrations of CTC in water bodies adjacent to agricultural activity, suggesting the role of surface run-off.

In terms of tetracycline degradation in soils, studies have revealed varying results. Hamscher *et al*. (2002) for example observed no significant change in tetracycline concentration during a seven-month period of a field study in which tetracycline was applied to soil from manure. Another study however reported significant losses of tetracycline from soil columns that had also received tetracyclines via manure application. The authors attributed some tetracycline loss to degradation processes (Aga *et al*., 2003). Soeberg *et al*. (2004) investigated the degradation of CTC and its transformation products and epimers at various pHs, temperatures and light conditions in soil interstitial water. It was observed that the presence of solar irradiation and higher temperatures shortened half-lives of all compounds at all temperatures and pHs tested, with the exception of 4-epi-CTC (solar irradiation did not affect its degradation). It was also observed that all compounds tested were generally more persistent under acidic conditions compared with neutral conditions suggesting that CTC, epimers of CTC and transformation products of CTC will be more persistent in acidic soils.

The degradation of oxytetracycline has also been investigated in soil. In clay soil column studies, oxytetracycline could not be detected after 52 days after application of oxytetracycline-spiked manure (Kay *et al*., 2005). In another investigation, oxytetracycline had been fully removed from a clay loam soil lysimeter 4 months after application of oxytetracycline (Kay *et al.*, 2005). Conversely, Aga *et al*. (2005) could not detect any significant removal of oxytetracycline in manure-amended soil five months after treatment (using ELISA analysis), but could detect 50% removal of parent compound after three-months using LC-MS analysis. As ELISA detects total tetracyclines, tetracycline epimers and tetracycline tautomers (Aga *et al.*, 2003) it is possible that these were being detected in soils five-months after amendment and were not detected by LC-MS. Halling Sorensen *et al*. (2003) investigated the abiotic formation and degradation of oxytetracyclines in soil interstitial water. The formation of epi-oxytetracycline, *N*-DM-oxytetracycline and *N*-DDM-oxytetracycline were shown to initially increase, suggesting abiotic processes (such as hydrolysis and oxidation) transform oxytetracycline in the soil environment. In terms of degradation, half-lives of between 2.3 days (epi-oxytetracycline) and 270 days (β-apo-oxytetracycline) were observed. It was also found that α- apo- oxytetracycline degraded faster under light conditions and epi- oxytetracycline degraded faster under dark conditions. Thiele-Bruhn and Peters (2007) observed two fractions of oxytetracycline and CTC with differing (biphasic) photodegradability, agreeing with Halling Sorensen *et al*. (2003) that only some tetracyclines (or tetracycline species) undergo photodegradation. Oka *et al*. (1989) had previously identified two-membered ring structures as a result of tetracycline photodegradation. The literature therefore suggests some aspects of tetracycline degradation, but not all, are significantly influenced by the presence of light.

3.1.6.2. Fate of Tetracyclines in the Aquatic Environment

The behavior of tetracyclines has also been investigated in aquatic systems. Just like in soil systems, tetracyclines have been reported to adsorb to organic matter (such as humic acids and peat) in different types of clay and sediments. Tetracyclines form polar interactions with divalent cations in clay as well as H bonds present in tannic acids in clay. Tetracycline sorption to clays in aquatic systems has been shown to decrease as ionic strength and pH increase (Sithole and Guy, 1987). Tetracyclines have also been shown to adsorb to both freshwater and marine sediments. Pouliquen and Le Bris (1996) observed that oxytetracycline sorption was greater in marine sediments with higher mineral content and higher organic matter content. They also found that smaller particle size (< 63μm) resulted in increased tetracycline sorption due to a larger surface area being available. Tetracyclines have also been shown desorb from aquatic sediment. Simon (2005) conducted oxytetracycline desorption studies in various rivers. It was reported that between 0.6-3.3 μg/g oxytetracycline (per dry sediment weight) was easily desorbed. Higher concentrations of easily desorbed oxytetracycline were found upstream of a sewage treatment plant. Smith and Samuelsen (1996) also demonstrated that a small fraction of oxytetracycline (between 0.04 and 3.82%) is lost from marine sediment as a result of washout into the aqueous phase (seawater).

The movement of tetracyclines in the aquatic environment has also been investigated. Rose and Pederson (2005) conducted computer simulations to predict oxytetracycline (from aquacultural discharge) movement through rivers. Higher oxytetracycline concentrations were expected to occur in sediment that were downstream (4 mg/kg) than were found in sediments that were upstream of aquacultural activity (≤ 0.2 mg/kg). Kerry *et al*. (1996) directly measured oxytetracycline concentrations below fish farms and in areas adjacent to fish farm cages. They detected oxytetracycline concentrations of between 0.65 and 1.26 μg/kg (directly under cages) and 4.2 μg/kg in areas that were 10 m from cages (in the direction of the water current). These results suggested that oxytetracycline distribution was confined to a small area of sediment under or adjacent to cage areas in the direction of the prevailing water current.

The dissipation of tetracyclines in the aquatic environment has also been studied. Verma *et al.* (2007) measured the dissipation of tetracycline in river and wetland waters. They observed half-lives of 2 and 3 days for river and wetland waters respectively in the presence of light. When the experiment was conducted in the absence of light significantly higher half- lives of 18 and 13 days (for river and wetland waters respectively) were observed. The results of this study therefore implicate the role of photodegradation in the rapid dissipation of tetracyclines. The results also indicated the role of microbial degradation, hydrolysis or a mixture of both in the dissipation of tetracyclines in natural water systems. Sanderson *et al*. (2005) investigated the dissipation of tetracycline, oxytetracycline, CTC and doxycycline in outdoor aquatic mesocosms. Half-lives of between 1 and 4 days were observed. When the dissipation of higher concentrations of tetracyclines were tested, it was observed that the parent compounds could still be detected in

mesocosms 4 weeks into the exposure period, with doxycycline dissipating fastest followed by tetracycline, oxytetracycline then CTC.

3.1.7. Reported Effects of Tetracyclines on Environmental Microbes

There have been several reported effects of tetracyclines on various aspects of microbial growth and cellular function. Much of this work has focused on single species testing. The Microtox test has yielded significantly different results depending on the test duration. Short-term testing using oxytetracycline as a test compound has yielded EC_{50} values of between 64.5 and 139 mg/l (Lalumera *et al.*, 2004; Isidori *et al.*, 2004; Christensen *et al*., 2006). Backhaus and Grimme (1999) extended the Microtox test duration to 24 hours using Tetracycline as a test compound. They observed an EC_{50} value of < 1 mg/l suggesting tetracyclines may exert differential toxicity on *Vibrio fischeri* during prolonged exposures.

Halling-Sorenson *et al.* (2002) tested a variety of tetracyclines and tetracycline degradation products on the growth rates of 15 pseudomonad strains. EC_{50} values of between 0.5 and 32 mg/l were observed, with degradation products of tetracyclines exerting a lesser toxic effect. Linares *et al.* (2006) investigated the effects of tetracycline on the pathogenic environmental bacterium *Pseudomonas aeruginosa*. At sub-inhibitory concentrations of tetracycline exposure they observed increased biofilm formation and an increase in a type III secretion system. This causes elevated cytotoxicity. These observations suggest that tetracycline exposure may result in an increase in swarming behaviour a response to avoid predation.

Other tests have shown the effects of tetracyclines on the growth of other single species. Halling - Sorenson *et al*. (2002) tested the effect of various tetracyclines and tetracycline degradation products on the growth rate of three soil isolates (*Agrobacterium sp.*, *Moraxella sp.* and two strains of *Bacillus sp.*). EC_{50} values ranged from 0.25–32 mg/l, with the lowest EC_{50} values being observed for the parent compounds (all were 0.25 mg/l). In another study Chander *et al.* (2005) observed declines in CFU counts of soil bacteria of between 40-60% as a result of exposure to tetracyclines, with the isolate *Escherichia coli* ATCC 25922 being affected the most.

The most significant effects exerted on single species by tetracyclines have been observed using cyanobacteria as single test species. Both Robinson (2007) and Halling-Sorensen (2000) observed low EC_{50} values when cyanobacterial growth was measured optically. Halling Sorensen (2000) observed an EC_{50} value of < 0.1 mg/l due to tetracycline exposure while Robinson *et al*. (2007) observed an EC_{50} of < 1 mg/l as a result of oxytetracycline exposure. These studies highlight the sensitivity of aquatic cyanobacteria to tetracycline exposure.

Tetracyclines have also been reported to effects the growth of whole microbial communities. Halling Sorensen *et al*. (2002) observed inhibition to growth of activated slugged bacteria as a result of exposure to tetracycline, CTC, tetracycline degradation products and CTC degradation

products. EC_{50} values as low as 0.03 mg/l (for 5a, 6-anhydrotetracycline hydrochloride and CTC respectively) were observed, with all but two compounds tested having EC_{50} values less than that of the reference toxicant. Halling-Sorensen *et al*. (2002) showed that the toxicity of tetracyclines to activated sludge bacteria decreases over time however, as suggested by results obtained using oxytetracycline as test compound. In the same study, the antibiotic activity of oxytetracycline increased over time in soil interstitial water studies, suggesting a possible toxic effect of oxytetracycline degradation products over time

Several authors have also reported changes to microbial respiration as a result of exposure to tetracyclines. Thiele-Bruhn and Beck (2005) observed inhibitory ED_{50} values of 19.1 and 31.2 µg/l for oxytetracycline in two different soil types. In contrast, other studies have reported no significant changes in microbial respiration rates as a result of tetracycline exposure. Zielezny *et al*. (2006) concluded CTC had no effect on respiration substrate induced respiration. Vaclavik *et al*. (2004) observed a 1.5-2 fold increase in substrate induced respiration rates as a result of exposure to CTC in soil microcosms. These conflicting results suggest that factors other than the presence of tetracyclines may be having an effect on the results of these experiments. Vaklivic *et al*. (2004) for example theorised that dead microbial cells may being degraded by the microbial community, which had potentially resulted in higher respiration rates due to cellular material being respired.

Tetracyclines have also been shown to have significant effects on specific microbial function. Thiele-Bruhn demonstrated lower end ED_{50} values of 1.2 mg/kg for CTC, 5.3 mg/kg for oxytetracycline and 3 mg/kg for tetracycline. Boleas *et al*. (2005) tested dehydrogenase activity of microorganisms from manured and non-manured soils following oxytetracycline addition. They observed a significant reduction in dehydrogenase activity after 7 days in manured systems and after 21 days in non-manured microcosms. In the same study oxytetracycline concentrations as low as 100 mg/kg caused a significant decrease in phosphomonoesterase activity in soils that had fertilised with manure.

Kong *et al*. (2006) examined the effects of oxytetracycline on multisubstrate carbon utilization (the ability of microbes to degrade a range of organic substrates). A significant reduction in substrate utilisation could be observed at oxytetracycline concentrations as low as 0.46 mg/l. When the authors looked at effects on specific substrate utilisation they observed a significant reduction in the utilization of all substrate guilds at an oxytetracycline concentration of above 0.46 mg/l. These results were also reflected multivariate analysis, with oxytetracycline concentrations of above 0.46 mg/l clustering distinctly from control data.

Several studies have investigated the effects of tetracyclines on biogas production (from the degradation of manure). Arikan *et al*. (2006) and Gamal-El-Din (1986) demonstrated reductions in biogas production of 27% and 49% respectively in fields inhabited by medicated animals. Sankvist

et al. (1984) tracked the effects of oxytetracycline on the anaerobic digestion of manure. They observed a 50% reduction in carbon cycling over a period of six consecutive days.

Other studies have reported effects of tetracyclines on processes which occur within the nitrogen cycle. In aquaria experiments for example, Klaver and Matthews (1994) reported significant effects of oxytetracycline on the growth of two nitrifying microbial species *Nitrosomas* and *Nitrobacter*; EC_{50} values of between 8.6 and 29 mg/l were observed during the seven-day exposure period.

3.1.8. Aims and Objectives of Chapter

The overall aim of the work described in this Chapter was to assess the potential effects of chlortetracycline on aquatic microbial communities. This was achieved using the following specific objectives;

1) To utilize the methods developed in Chapter 2 as well as measures of colony forming units to assess the effects of CTC on microbial population numbers and the ecological functioning of microbial communities in aquatic systems.

2) To explore the potential for recovery of microbial communities following CTC exposure in terms of ecological functioning.

3) To link functional data to effects of CTC in terms of the likely effect CTC may be exerting and to link these data to how these effects may be affecting wider ecological processes in the aquatic environment.

3.2. Materials and Methods

3.2.1. Chemicals

All materials and chemicals were purchased and prepared according to section 2.7.1. R2A agar was purchased from Oxoid (Cambridge, UK). CTC hydrochloride was purchased from Sigma Aldrich (Poole, UK).

3.2.2. Surface water

Surface water was collected and prepared according to section 2.2.2.

3.2.3. Activated Sludge and Preparation of Treated Sewage

Activated sludge was collected and prepared according to section 2.2.3.

3.2.4. Preparation of OECD Synthetic Sewage

OECD synthetic sewage was prepared according to section 2.2.4.

3.2.5. Preparation of R2A Plates

11.5g R2A agar was weighed on a calibrated balance and mixed with 1 litre of deionised water. The R2A agars / deionised water suspension was then slowly heated while being stirred on a magnetic infrared hotplate. When the mixture was completely melted it was autoclaved at 121°C for 15 minutes. Melted R2A agar was then poured into sterile Petri dishes leaving an air space of ~0.25 cm.

3.2.6. CTC Exposure Experiments

Twelve microcosms were set-up as described in section 2.2.6.1. Nine microcosms were spiked with an aqueous CTC solution. Three microcosms were spiked to a nominal CTC concentration of 1 mg/l, three to a nominal CTC concentration of 0.32 mg/l and three to a nominal CTC concentration of 0.1 mg/l. Three control microcosms received no CTC solution. A time series experiment was then conducted as described in sections 2.2.7.1.

3.2.7. CFU (Colony Forming Unit) Counts

Serial dilutions (0, 10^{-1}, 10^{-2} and 10^{-3}) of microcosm contents were prepared using sterile water. Triplicate R2A and R2A-CTC agar plates were spread-plated with each 0.1 ml of each dilution factor using a sterile plate spreader. Plates were then incubated at 20°C for 72 hours. After incubation, the numbers of individual CFUs on each plate were enumerated by eye on each agar plate. Only CFU counts of between 30 and 300 were accepted for further analysis.

3.2.8. Data Analysis

Initial Biolog A_{MAX} values were treated as in section 2.2.5.1.2. A kinetic model was applied to $AWCD_{MAX}$ values as described in section 2.2.8.1. Max rate and lag phase duration values were plotted using Microsoft Excel.

Non-linear regression of AWCD data was performed by SigmaPlot 4.01 (SYSTAT, IL, USA). Co-efficient and standard error values for λ and μm were then compared for significance ($P < 0.05$) using an Excel macro (Alan Sharpe, Brixham Environmental Laboratory).

Principle Component Analysis (PCA) was performed using Minitab 15 (PA, USA). PCA was performed on maximum absorbance (A_{MAX}) values from each corresponding time point. All data treatment was then performed on A_{MAX} AWCD values from Biolog guild data (amines and amides, amino acids, carbohydrates, carboxylic acids and polymers). Analysis of the functional diversity of total substrate utilisation was conducted as described in section 2.2.8.3.

CFU count data was transferred onto an Excel spread sheet that had been previously manipulated to calculate CFU dilution data. On the same spread sheet, data were then log-transformed so that a parametric analysis could be performed. Data were then tested for analysis of variance using a one way analysis of variance (ANOVA). Statistical significance was at the $P < 0.05$ level. This was performed using Sigmaplot.

3.3. Chapter 3 Results

3.3.1. Effects of CTC on Organic Multi-Substrate Utilisation

The data in figure 3.1 illustrate total organic substrate utilisation as kinetic model plots on days one, two and three of the six day exposure period. Figure 3.2 shows PCA score plots for the first two principle components (normally associated with the greatest variation) on exposure days one and four. The data in figure 3.3 illustrate lag phase duration and the maximum rate of substrate utilisation on exposure days 1-6. The information in tables 3.3 and 3.4 show the statistical significance of lag phase and max rate values compared with control data.

3.3.1.1. Kinetic Model Plots

Total substrate utilisation plots for day 1 (fig 3.1a) show that the highest absorbance occurred in control microcosms, followed by microcosms containing 0.32, 0.1 and 1 mg/l CTC. Although this trend continued in microcosms containing 1 mg/l on exposure day 2 (fig 3.1b) the magnitude of trend was not as great, with the plot becoming more similar to that of the control. Other CTC treatment plots became more similar to control curves. In addition, a higher maximum absorbance value was observed for microcosms containing 0.1 mg/l, with the two treatments above this approaching the maximum absorbance level of the control microcosm; day 2 plots therefore suggest the beginning of a recovery in microcosms that contained 0.1 and 0.32 mg/l CTC with reduced effects at the upper CTC concentration. Kinetic model plots therefore seem to illustrate an acute effect of CTC, with an apparent recovery immediately after an acute effect has been exerted.

3.3.1.2. Lag Phase versus Exposure Time

When lag phase duration was plotted against exposure time plots showed that all CTC concentrations resulted in a longer lag phase duration; this increase was statistically significant across all CTC treatments (table 3.3). On day two of the exposure, the lag phase duration decreased at all treatments and in control microcosms also. The lag phase was still significantly higher than the control treatment in microcosms containing 1 mg/l CTC however. 48 hours after exposure saw no significant increase or decrease in lag phase for any CTC treatment. After 72 hours however a statistically significant decrease in lag phase was recorded for all CTC treatments. This trend continued for the duration of the exposure period (until 120 hours) in microcosms containing 1 mg/l CTC. In microcosms that had been exposed to 0.32 mg/l CTC a significant decrease in lag phase was also observed 120 hours after exposure.

3.3.1.3. Max Rate versus Exposure Time

On days 1 and 2 of the exposure a reduction in the max rate of total organic substrate utilisation was seen with increasing CTC concentration. On day 1 the difference were statistically significant

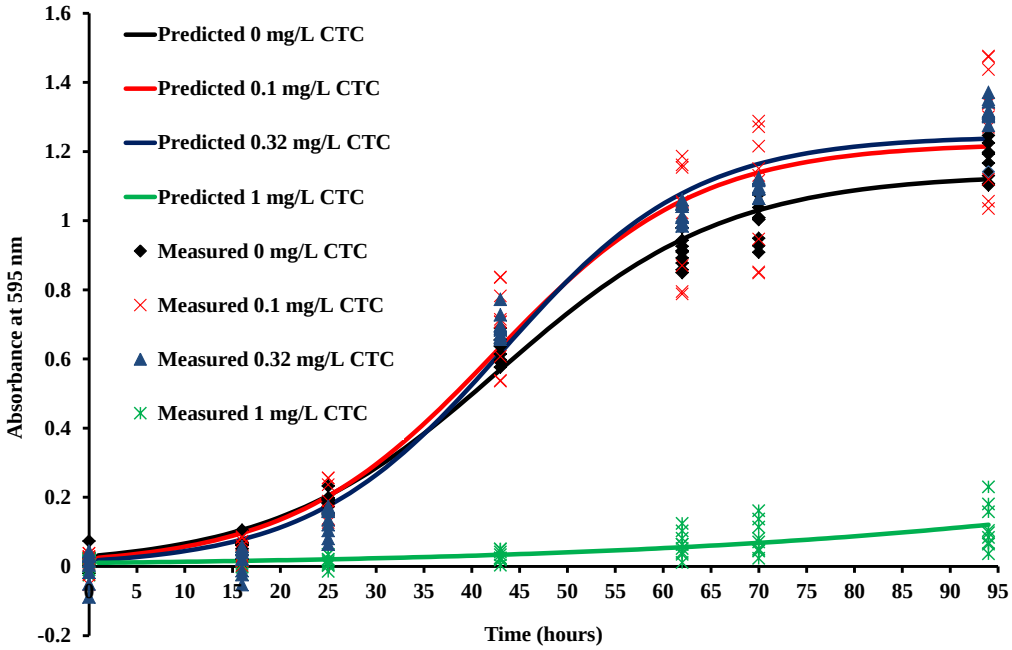
at all CTC treatments (table 3.4). On day 2 max rate values were again lower than control values at all CTC treatments, although values were higher than day 1 readings for all CTC concentrations. A statistically significant reduction in max rate values was only observed in microcosms containing 1 mg/l however. 72 hours after CTC exposure max rate values at the highest two CTC concentrations rose above the control value, although not significantly. For the rest of the exposure period max rate values remained lower than control readings. On the final day after exposure this decrease was statistically significant in microcosms treated with 1 mg/l.

3.3.1.4. PCA Analysis

Data shown as PCA plots of the second versus the first principle components 0 hours after exposure (day 1) showed that control replicates and 0.1 mg/l replicates clustered closely together, suggesting that the microbial community was similar in microcosms exposed to these CTC doses. Replicates from microcosms containing 0.32 mg/l and 1mg/l clustered independently from both each other and the control-0.1 mg/l cluster. These data suggests that higher CTC concentrations caused differences in mulisubstrate utilisation profiles in microcosms spiked with the two highest CTC concentrations. Microbial communities exposed to higher doses were therefore likely to be different than communities that were present at lower and control doses of CTC. These data also suggest that microbial communities present in microcosms exposed to higher CTC doses tended to be less similar to both each other and control and communities, as well as to communities that had been exposed to a lower CTC dose. It is likely that the microbial community structure was also different in microcosms that had been exposed to these doses of CTC.

Day 4 PCA score plots also show clustering of 0.1 mg/l and control microcosm (0 mg/l) replicates, again suggesting carbon utilisation profiles (and therefore microbial communities) were similar. Distinct clustering occurred in replicates from the top two CTC concentrations. In addition, these clusters were completely separate from any other clusters in terms of distance. This suggests that 0.32 and 1 mg/l CTC treatment alter the carbon utilisation profile of the microbial community. Day 4 PCA results also suggest that higher CTC doses result in changes in microbial community structure.

a)



b)

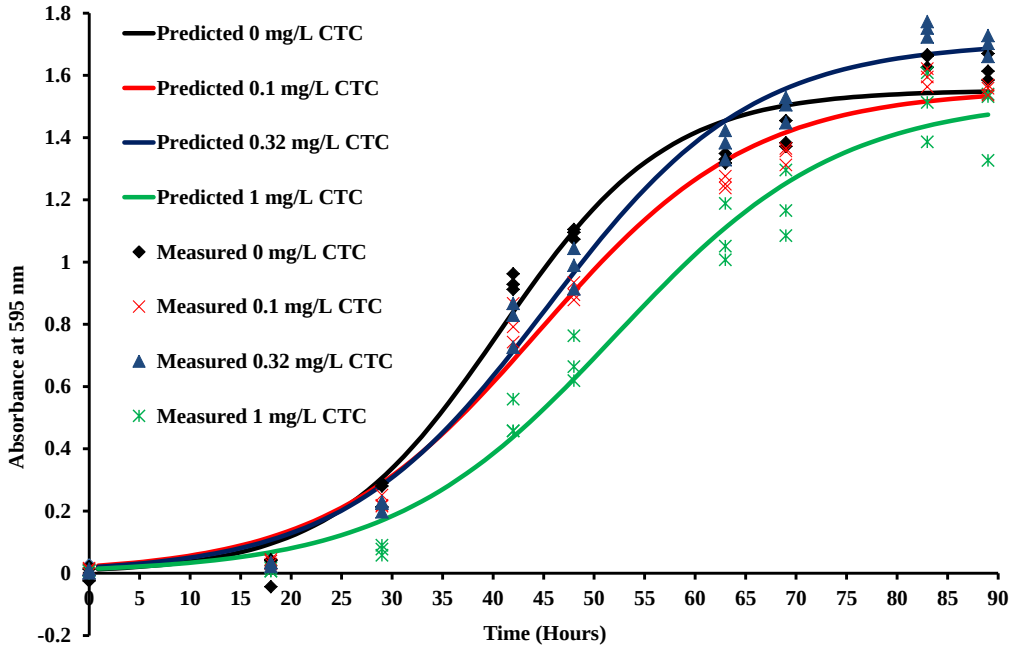


Figure 3.1: Kinetic plots of total substrate utilisation following CTC exposure on a) day 1, b) day 2 and c) day 3.

c)

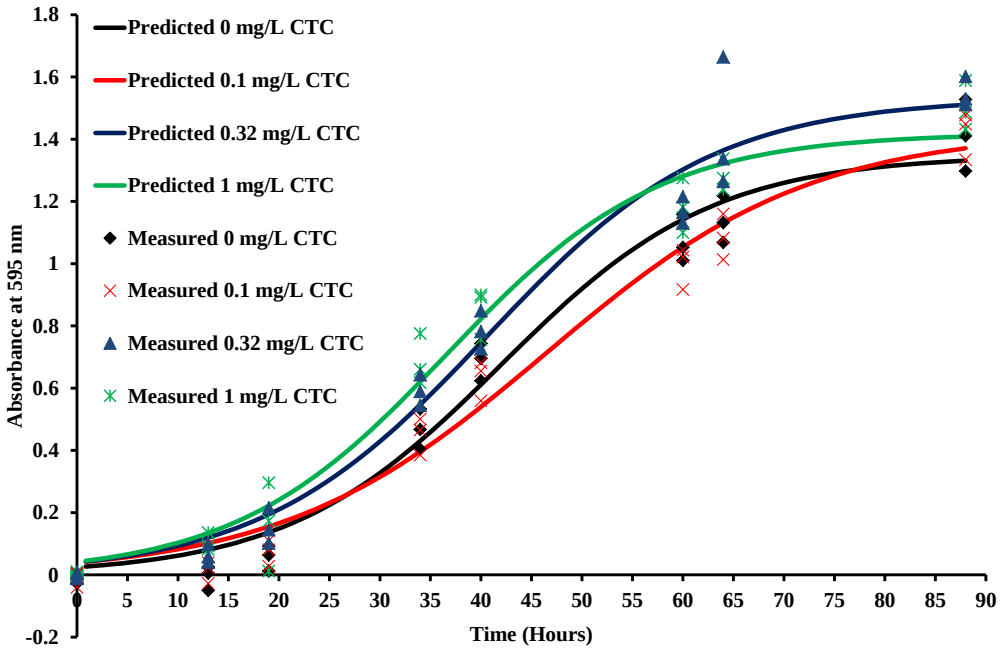
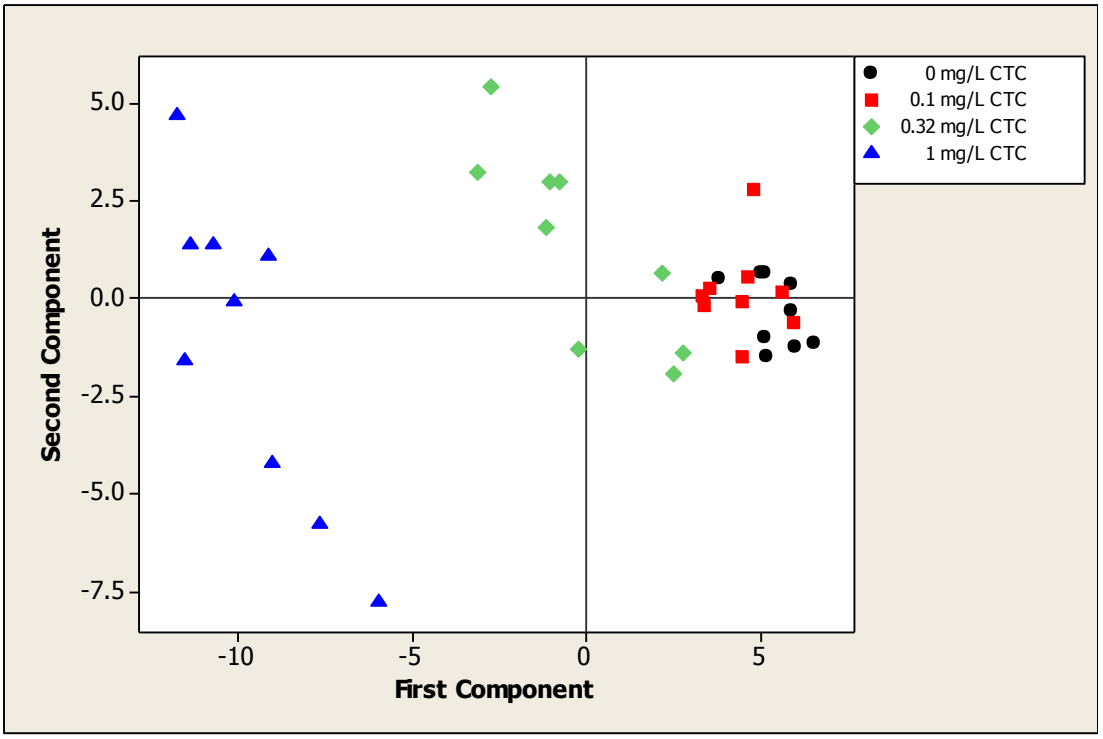


Figure 3.1 (cont).

a)



b)

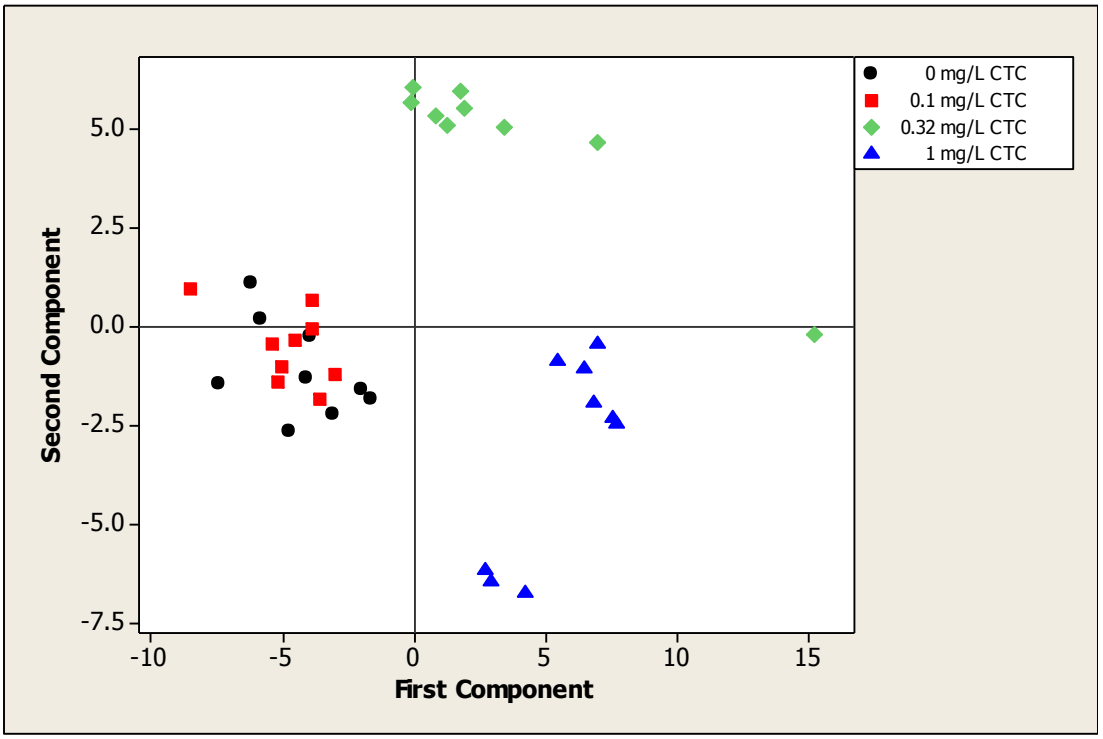
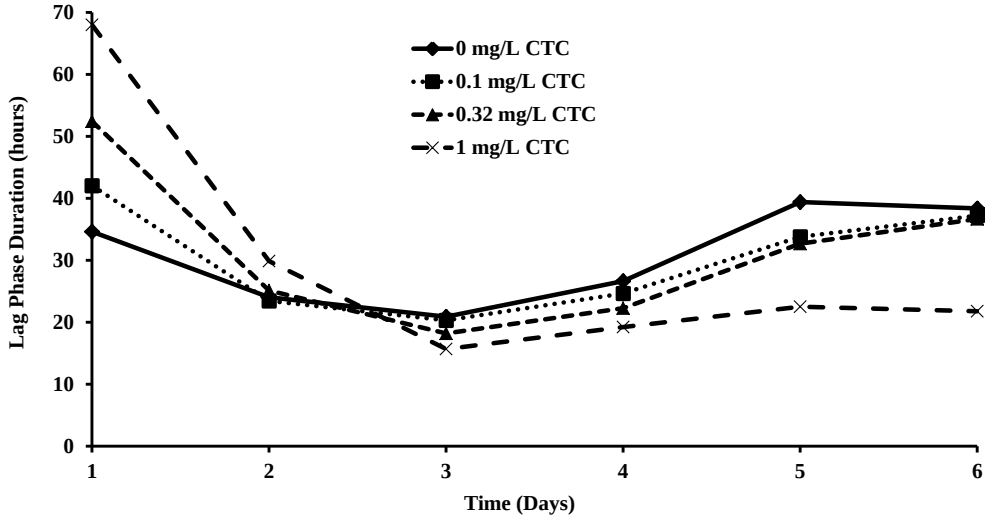


Figure 3.2: Scatter plots of PC scores for the first two principle components for individual replicates from each CTC treatment groups representing total substrate utilisation on a) day 1 and b) day 4. Day 1 (a) proportion of variation: PC1 = 41.3%, PC2 = 6.9%; Day 2 (b) proportion of variation: PC1 = 31.4%, PC2 = 12.4%.

a)



b)

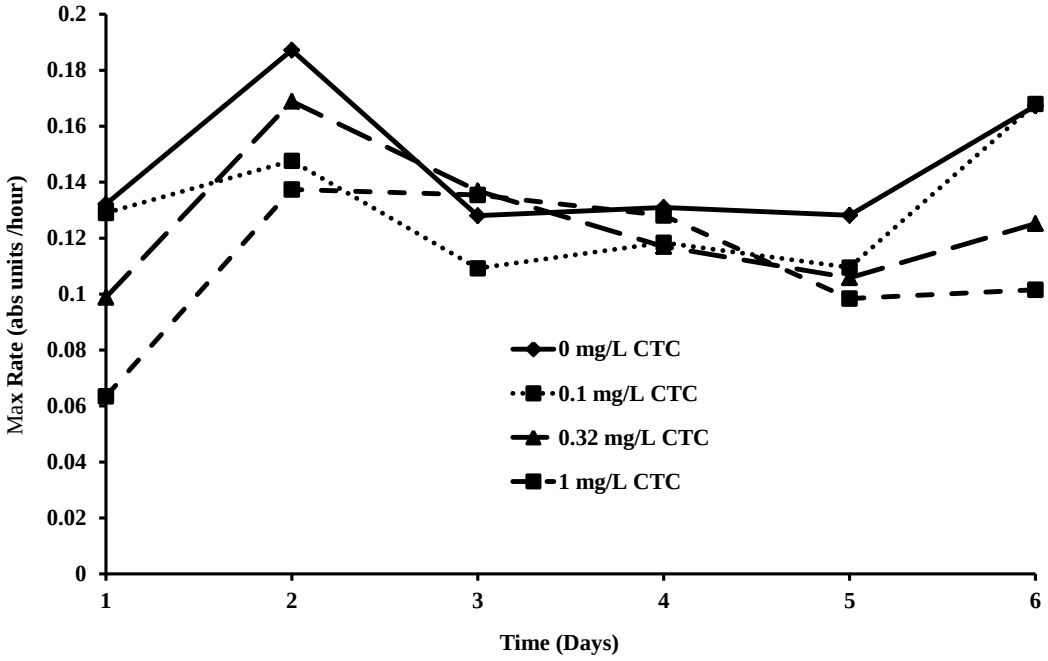


Figure 3.3: a) Lag phase duration versus exposure period time for total substrate utilisation and b) Max rate of total substrate utilisation versus exposure period time following exposure to CTC.

Table 3.3: Summary of one-way ANOVA results for lag phase values before total substrate utilization from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	>*	< ns	< ns	< *	< ns	< ns
0.32	>*	> ns	< ns	< *	< ns	< *
1	>*	>*	< ns	< *	< *	< *

Table 3.4: Summary of one-way ANOVA results for maximum rate of total substrate utilization values from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	< ns	<ns	<ns	<ns	<ns	>ns
0.32	<*	<ns	>ns	<ns	<ns	<ns
1	<*	<*	>ns	<ns	<ns	<*

3.3.2. Effect of CTC on Amine and Amide Utilisation

The data shown in figure 3.4 illustrate amide and amine utilisation on exposure days 1, 2 and 3 that were plotted using a kinetic model. Data in figure 3.5 show PCA plots for the first two principle components scores on days 1 and 4. The information in figure 3.6 illustrates lag phase and maximum rate data on exposure days 1-6. Tables 3.5 and 3.6 show the statistical significance of lag phase and max rate values compared with control data.

3.3.2.1. Kinetic Model Plots of Amine and Amide Utilisation

The data in kinetic plots on day 1 (0 hours) show a negative correlation between amine and amide utilisation and increasing CTC concentration. At all concentrations a longer incubation period was required to produce colour development and colour development levels was not as high as levels that were seen in the control plot. The magnitude of colour that had been developed at the end of the incubation period in treated Biolog plates was lower than control plates. In addition, control plots followed a steeper gradient.

In plots that represent data for 24 hours after exposure to CTC, a similar trend continued at all CTC concentrations. An increase in slope and maximum colour formation were observed at CTC concentrations of 1 mg/L and 0.32 mg/L and the onset of colour formation appeared shorter than in day 1 plots of the same treatments. In plots illustrating data of a 0.1 mg/L CTC treatment, the onset of colour development appeared to occur faster than was shown by day 1 plots.

The data shown by day 3 kinetic plots show fewer differences between exposure concentrations. The data in all plots show similar times of colour production onset. The slope of each curve also appeared similar across the concentration range. The maximum level of colour production appeared to be either equal to or greater than control values with the exception of the highest CTC treatment (1 mg/L).

3.3.2.2. Lag Phase of Amine and Amide Utilisation versus Exposure Time

On day 1 lag phase duration was higher than control values for all CTC treatments. A (statistically) significantly higher lag phase duration was only observed in microcosms spiked with 0.1 mg/L CTC however (Table 3.5). A similar trend was observed 24 hours after exposure to CTC, although lag phases had shortened compared with day 1 values. A statistically significant increase in lag phase duration was observed in microcosms containing 1 mg/L CTC on day 2 however. From days 3 to 5 no significant difference in lag phase duration was observed at any dose compared with control data, although values for all CTC concentrations gradually decreased to values that were less than that of the control value. On day 6 of the exposure period lag phase values were significantly lower than those of control microcosms at all CTC treatments.

3.3.2.3. Max Rate of Amine and Amide Utilisation versus Exposure Time

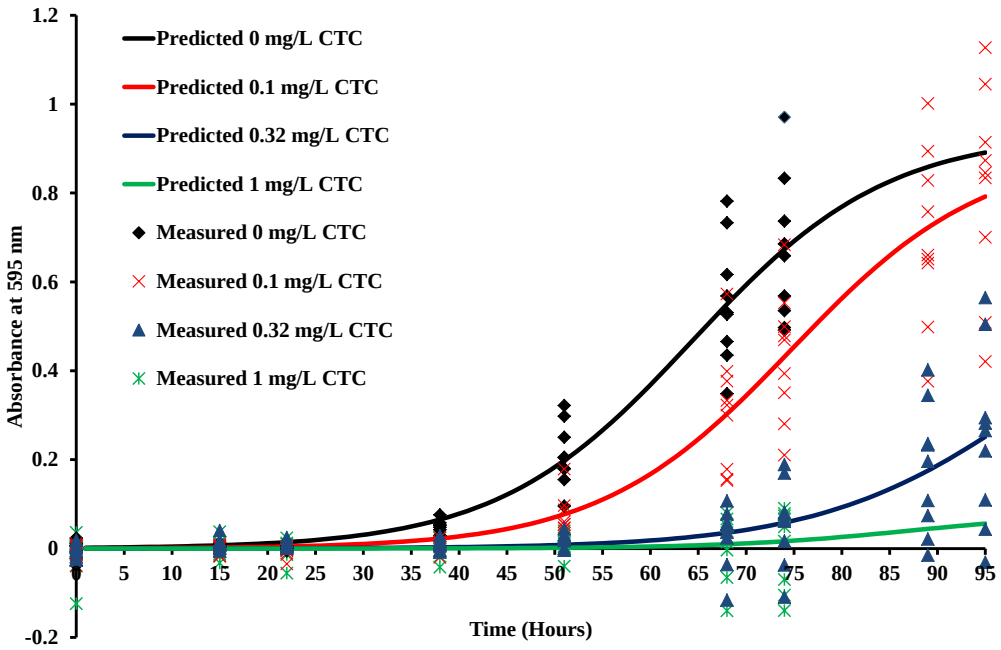
Max rate values on day 1 were generally less than the control value for all concentrations of CTC (3.3b). A significantly lower max rate value compared with control data was observed at 0.1 mg/L CTC (Table 3.6). 24 hours after exposure to CTC max rates values had increased compared to day 0 values, although they were still below control levels. No statistical differences were observed compared with control data however. There was no significant statistical difference between the control max rate and the max rate of any CTC treatments until day 5 after exposure. At this point the max rate value in microcosms containing 1 mg/L CTC was significantly less than the max rate value that was recorded in control microcosms. A similar but not statistically significant trend was seen with max rate values at all other CTC treatments.

3.3.2.4. PCA Analysis of Amine and Amide Utilisation

Data shown by PCA score plots of the first two principle components on day 1 show that replicates treated with 0.32 and 1 mg/L cluster together, suggesting that amine and amide utilisation had a similar metabolic fingerprint. These data also suggest that the portions of the microbial community that were utilising amines and amides (likely to be microbes involved with nitrogen cycling) had a similar community structure in microcosms that had been spiked with these CTC concentrations. There was also a slight overlap of replicate clustering between the 0.32 and 0.1 mg/L groups suggesting that a small degree of similarity between these two treatments. These data suggest that these portions of the microbial community that were utilising amines and amides had some structural similarity. Replicates from microcosms treated with 0.1 mg/L CTC generally clustered with control replicates however, suggesting that amine and amide utilisation followed a similar pattern in these microcosms. This points to the likelihood that members of the microbial community that were utilising amines and amides at these doses were likely to have had a similar community structure.

On day 4 of the exposure a similar trend to day 1 was apparent in terms of replicate groups that had clustered. The two clusters (0/ 0.1 mg/L and 0.32 /1 mg/L) were however closer together on day 4, suggesting that all treatments were becoming more similar to each other. These data suggest that the community structure of the portion of the microbial community that was utilising amines and amides in these microcosms was becoming more similar on day 4. In addition, a greater degree of clustering had occurred between the control / 0.1 mg/L cluster and replicates from microcosms spiked with 0.32 mg/L CTC. This would suggest that the portion of the microbial community that was utilising amines and amides at these CTC concentrations were becoming more structurally similar.

a)



b)

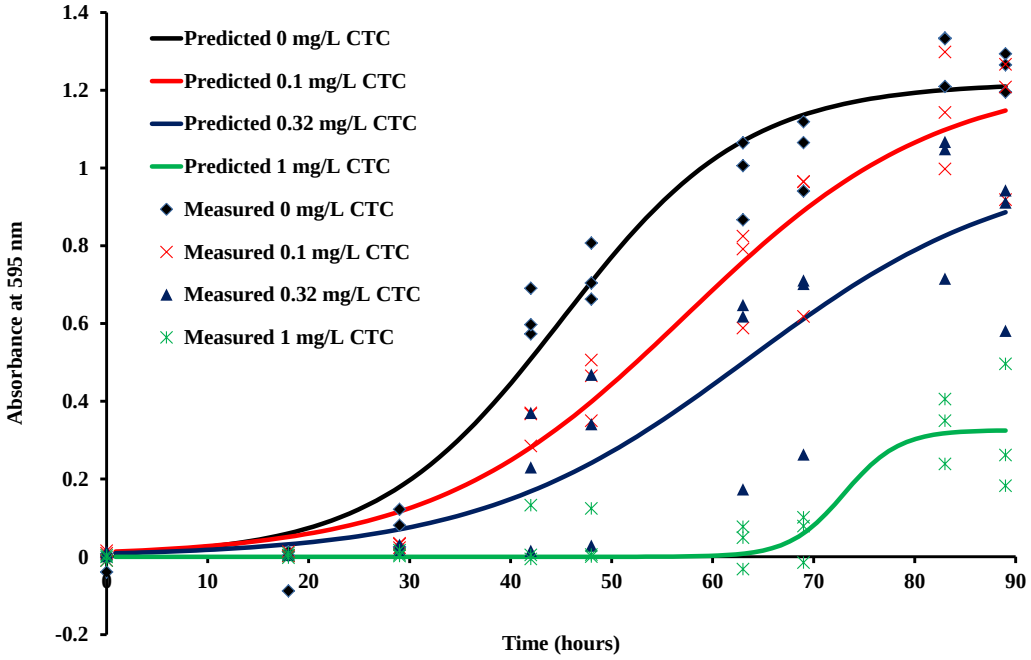


Figure 3.4: Kinetic plots of amine and amide utilisation following exposure to CTC on a) day 1, b) day 2 and c) day 3.

c)

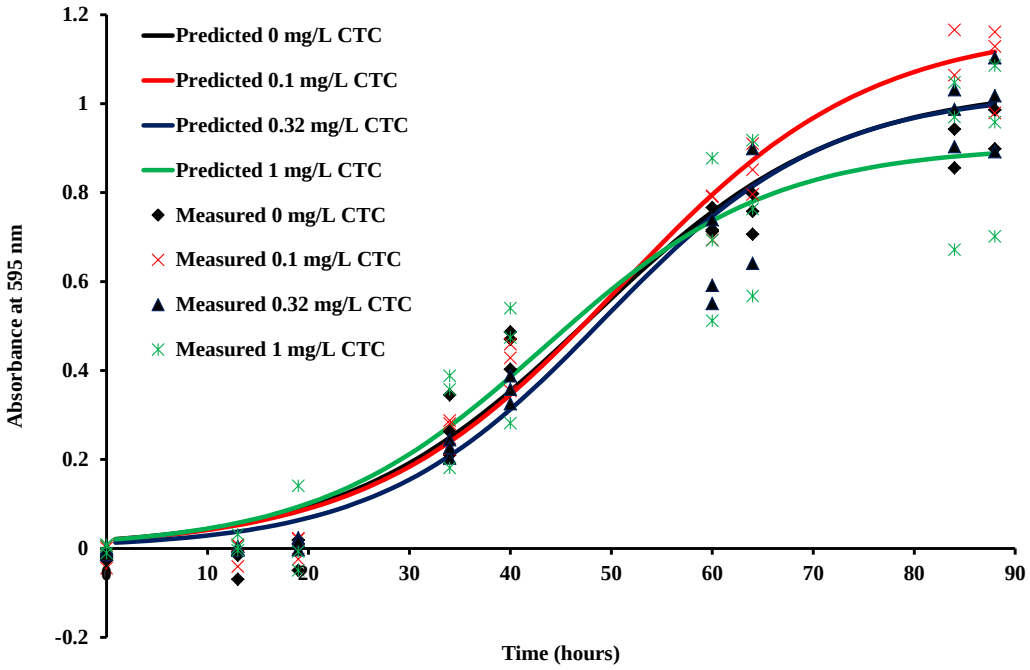
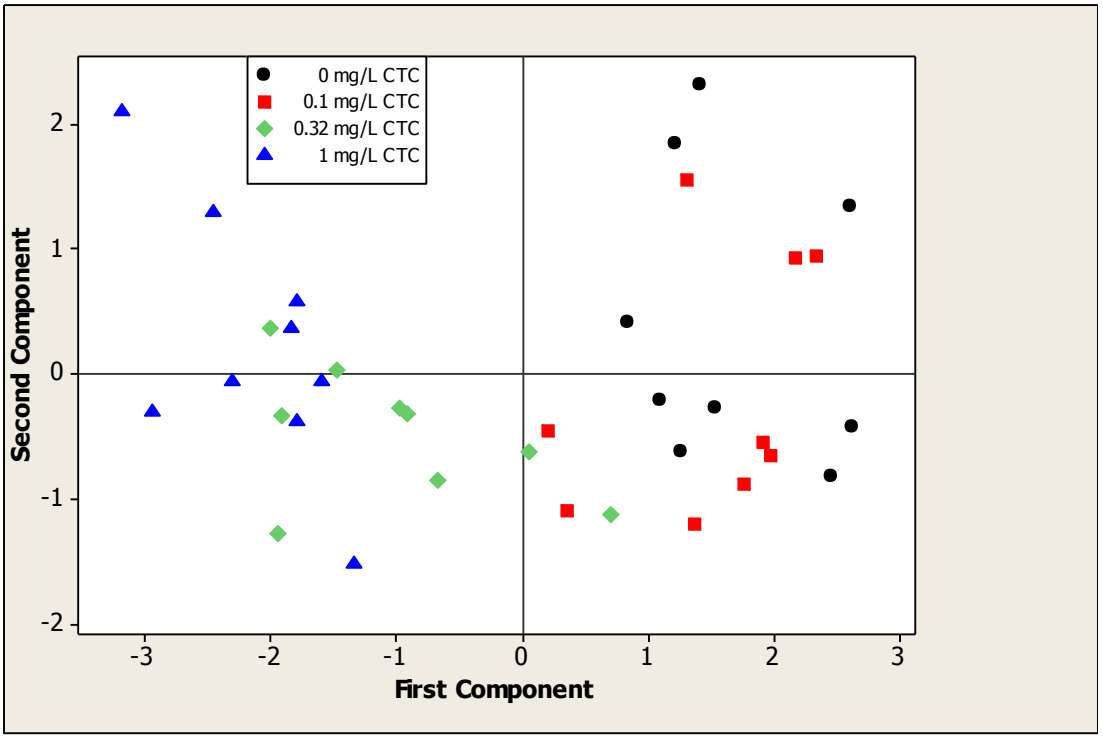


Figure 3.4 (cont).

a)



b)

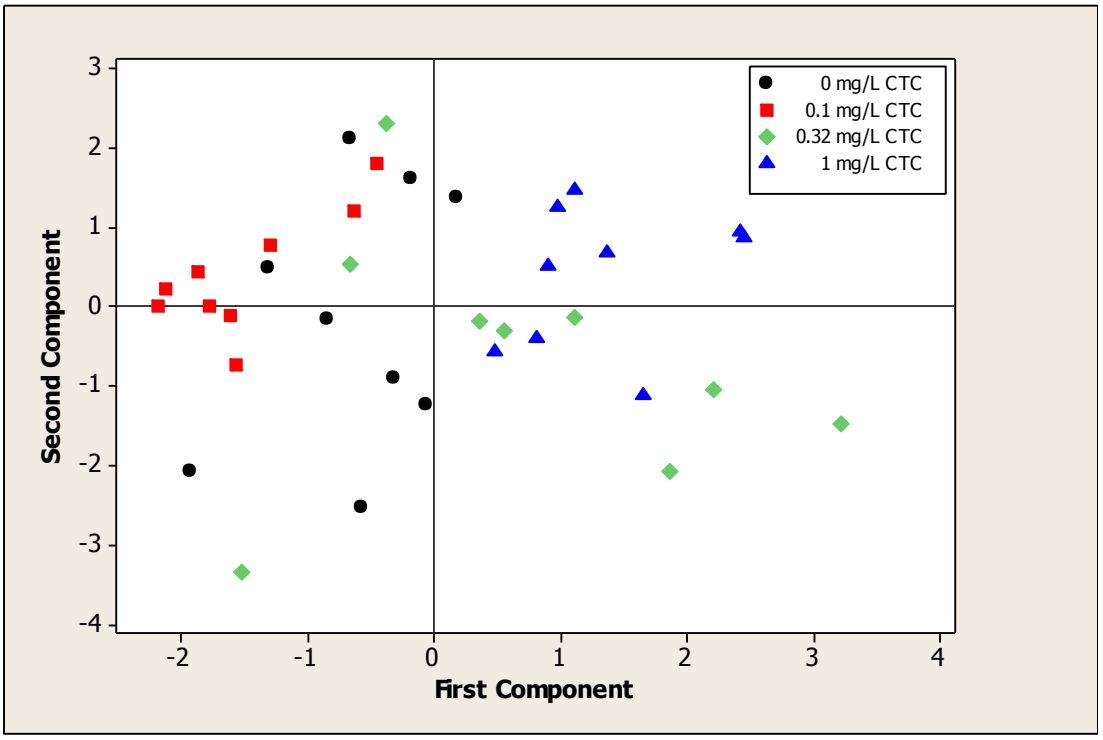
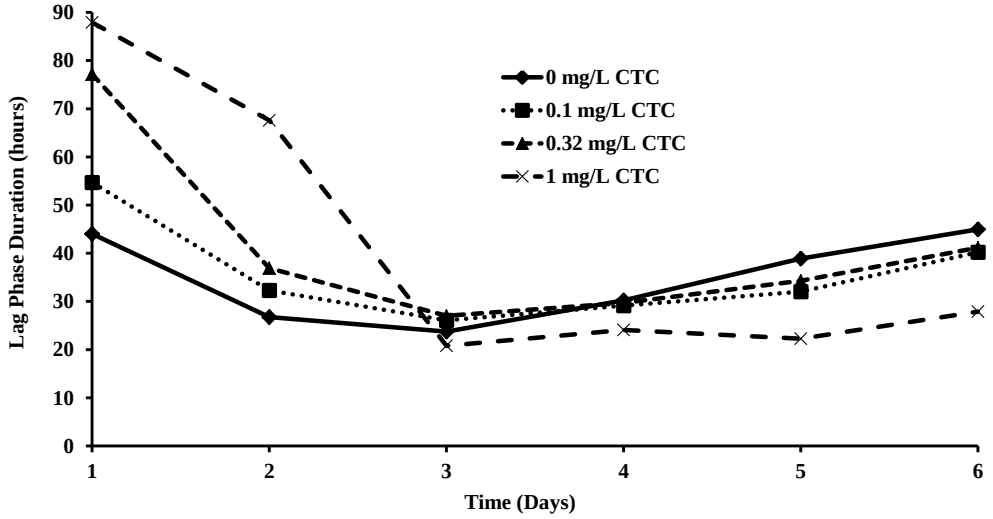


Figure 3.5: Scatter plots of PC scores for the first two principle components for individual replicates from each CTC treatment group representing amine and amide utilisation on a) Day 1 and b) Day 4. Day 1 (a) proportion of variation: PC1 = 54.2%, PC2 = 16.2%; Day 4 (b) proportion of variation: PC1 = 35.2%, PC2 = 28.9%.

a)



b)

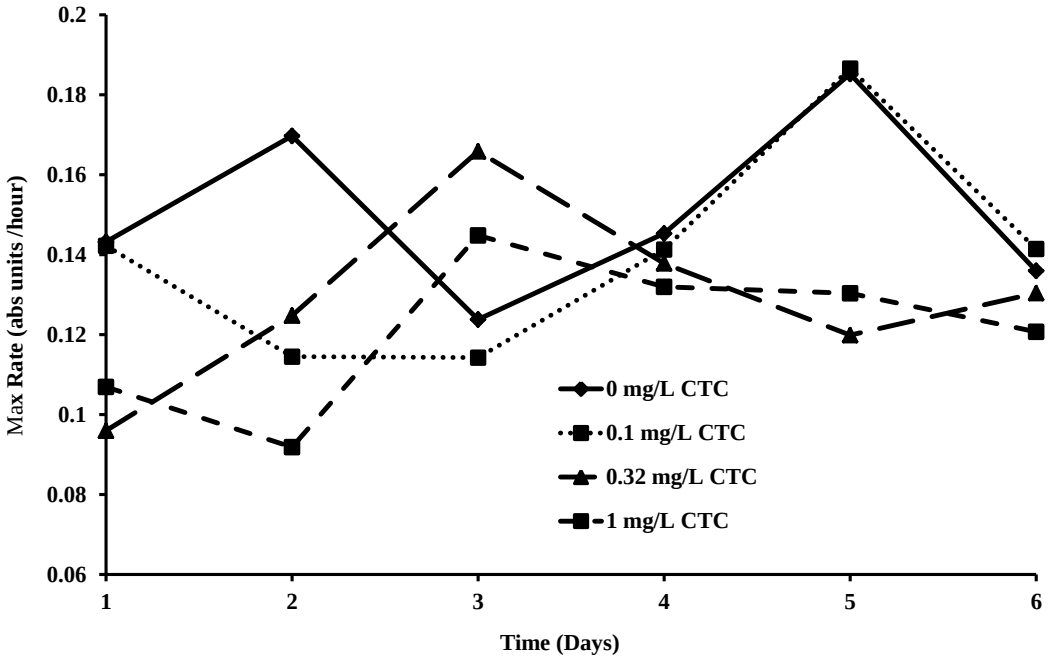


Figure 3.6: a) Lag phase duration versus exposure period time for amine and amide substrate utilisation and b) Max rate of amine and amide utilisation versus exposure period time following exposure to CTC.

Table 3.5: Summary of one-way ANOVA results for lag phase values before amine and amide utilization from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	>*	>ns	>ns	<ns	<ns	<ns
0.32	>ns	>ns	<ns	<ns	<ns	<*
1	>ns	>*	<ns	<*	<*	<*

Table 3.6: Summary of one-way ANOVA results for maximum rate of amine and amide utilization values from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	<ns	<ns	>ns	>ns	<ns	<ns
0.32	<ns	<*	<ns	<ns	<ns	<ns
1	<ns	<ns	<ns	>ns	<ns	<*

3.3.3. Effect of CTC on Amino Acid Utilisation

The data that are shown in figure 3.7 illustrates amino acid utilisation on exposure days 1, 2 and 3 plotted using a kinetic model. The data that are shown by figure 3.8 shows PCA plots for the first two principle components scores on days 1 and 4. The data in figure 3.9 illustrate lag phase and maximum rate on exposure days 1-6. The information in tables 3.7 and 3.8 show the statistical significance of lag phase and max rate values compared with control data.

3.3.3.1. Kinetic Model Plots of Amino Acid Utilisation

Amino acid utilisation, in terms of slope, onset of colour development and maximum colour development was inhibited by all CTC doses on day one of the exposure (figure 3.7a).

On day 2 (24 hours after exposure) the time taken for onset of colour development was slower in all treated microcosms than in control microcosms. Although it is difficult to distinguish the slopes the kinetic plots merely by eye, it is clear that the maximum absorbance was greater than control values at all CTC concentrations (figure 3.7b).

Day 3 kinetic plots (figure 3.7c) show a reduction in the onset of colour formation for all CTC treatments compared to control kinetic plots. In addition, treated and control slopes appeared to be similar. The maximum level of colour development was also comparable for all plots, with the exception of those representing an exposure of 0.32 mg/L CTC; a greater final absorbance was reached in Biolog plates that were seeded from these microcosms.

3.3.3.2. Lag Phase of Amino Acid Utilisation versus Exposure Time

On day 1 lag phase durations were higher than control values at all concentrations of CTC. Significantly higher lag phase durations were observed at CTC concentrations of 0.1 and 0.32 mg/L CTC.

Lag phase values then dropped sharply between days 1 and 2, with no lag phase being observed in microcosms that had been exposed to 1 mg/L CTC. Lag phases for all CTC treatments on day 2 were statistically significant compared with control values (table 3.7). This trend continued on day 3. A rise in lag phase duration was observed compared with day 2 values, although no statistical significance was observed. A significant decrease in lag phase duration did occur however for the rest of the exposure period in microcosms containing 1 mg/L CTC. This trend was mirrored at all other concentrations, with a significant decrease in lag phase duration being observed 120 hours after exposure at a CTC concentration of 0.32mg/L (figure 3.9a and table 3.7).

3.3.3.3. Max Rate of Amino Acid Utilisation versus Exposure Time

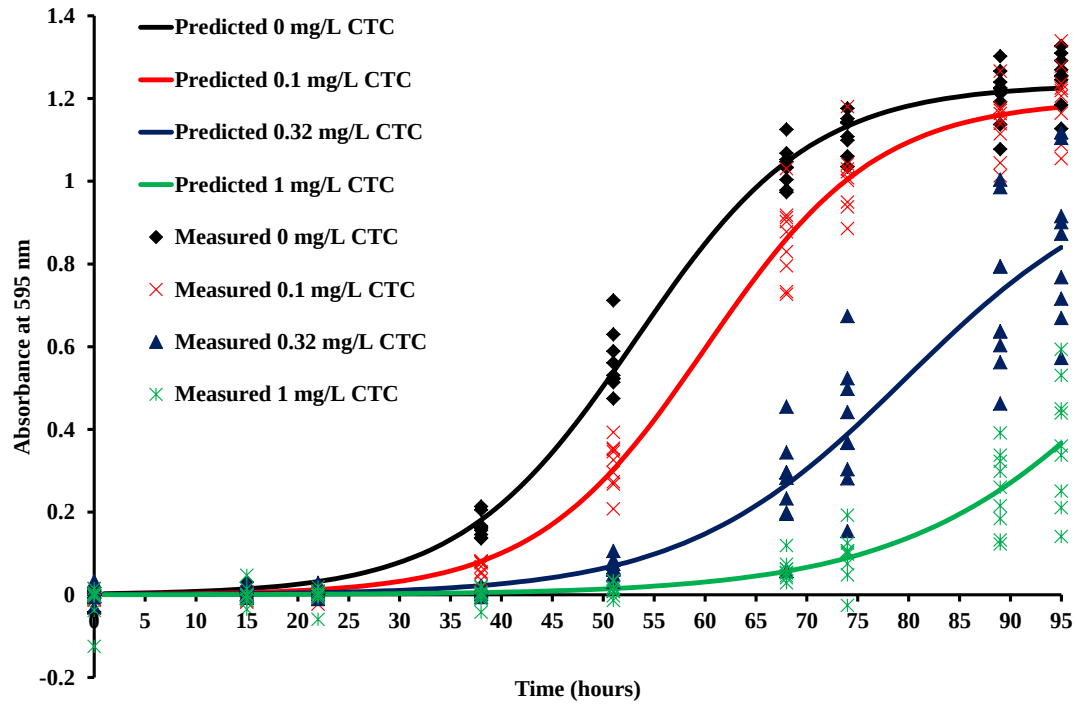
On day 1 of the exposure period treatments of 0.32 and 1 mg/L CTC caused a decrease in max rate values. A statistically significant decrease in max rate was only observed at 0.32 mg/L CTC. On exposure day 2 all treatments had resulted in a max rate value that was significantly lower than that of the control. In the case of the highest two CTC concentrations max rate values had dropped since day 1. Max rate values at a CTC concentration of 0.32 mg/L had increased since day 1 however. This trend continued until day 4 of the exposure, after which max rate values dropped below control readings; no statistically significant changes were observed after day 1. A rise in the max rate of colour production was also observed between days 2 and 3 and days 3 and 5 for CTC concentrations of 1 and 0.1 mg/L respectively. After these periods, max rate values fell to levels nearer control readings. On the final day of the exposure a significant decrease in the max rate of colour production was observed at 1 mg/L CTC (figure 3.9b and table 3.8).

3.3.3.4. PCA Analysis of Amino Acid Utilisation

At 0 hours after CTC exposure (day 1) replicates from the 1 mg/L exposure clustered in a manner that was distinct from all other exposure concentrations. This suggests that the amino acid utilisation profile at the highest test concentration was distinct from the profile of other treatment groups. This finding supports the theory that exposure to 1 mg/L CTC alters the community structure of microbes that are utilising amino acids. The majority of replicates from the 0.32 mg/L exposure also clustered separately. These data suggest that the portion of the microbial community that was utilising amino acids was altered by 0.32 mg/L CTC. A separate cluster was formed by replicates from the control and 0.1 mg/L groups, suggesting that these treatments formed a similar amino acid utilisation profile. These data suggest that the portion of the microbial community that was utilising amino acids was not altered structurally by 0.1 mg/L CTC. There was also some limited overlap between this cluster and the 0.32 mg/L cluster, suggesting that some replicates from the 0.32 mg/L group had a similar amino acid utilisation profile (figure 3.8a). These data point the fact that the community structure of amino acid utilising bacteria had a degree of similarity.

On day 4 (72 hours after exposure) control and 0.1 mg/L replicates again clustered together, suggesting that a similar amino acid utilisation profile had resulted from these two treatments. These data again suggest that the microbial community structure in these microcosms was similar to those present in microcosm treated with other CTC concentration. Two distinct clusters were formed by replicates from the 0.32 and 1mg/L groups, suggesting distinct amino acid utilisation profiles (figure 3.8b). Day 4 PCA data therefore suggests that the respective portions of the microbial community that were utilising amino acids had a similar community structure.

a)



b)

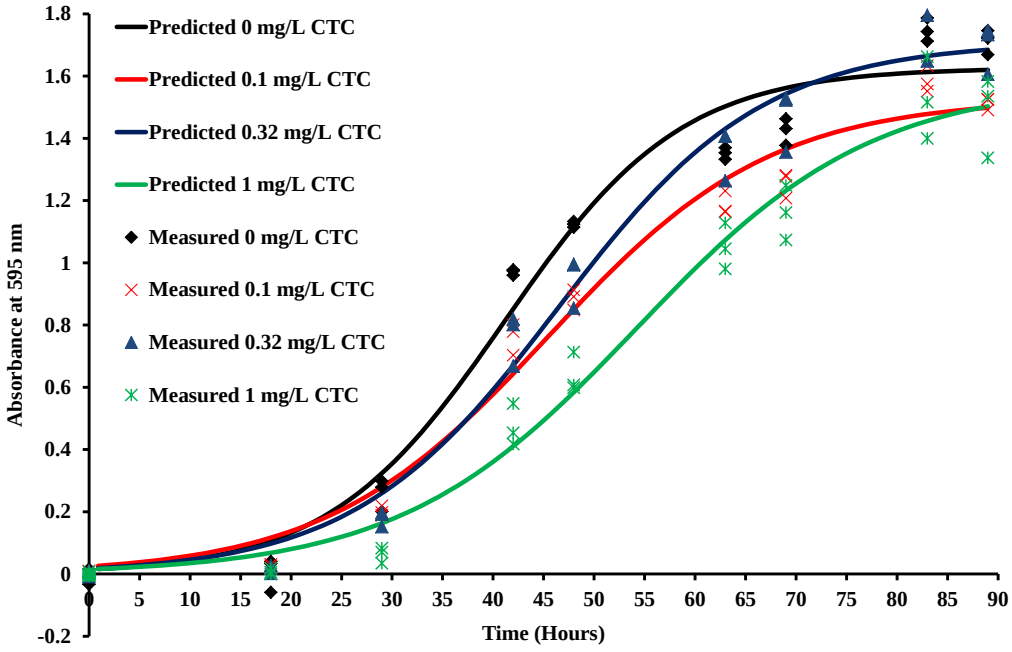


Figure 3.7: Kinetic plots of amino acid utilisation following CTC exposure on a) day 1, b) day 2 and c) day 3.

c)

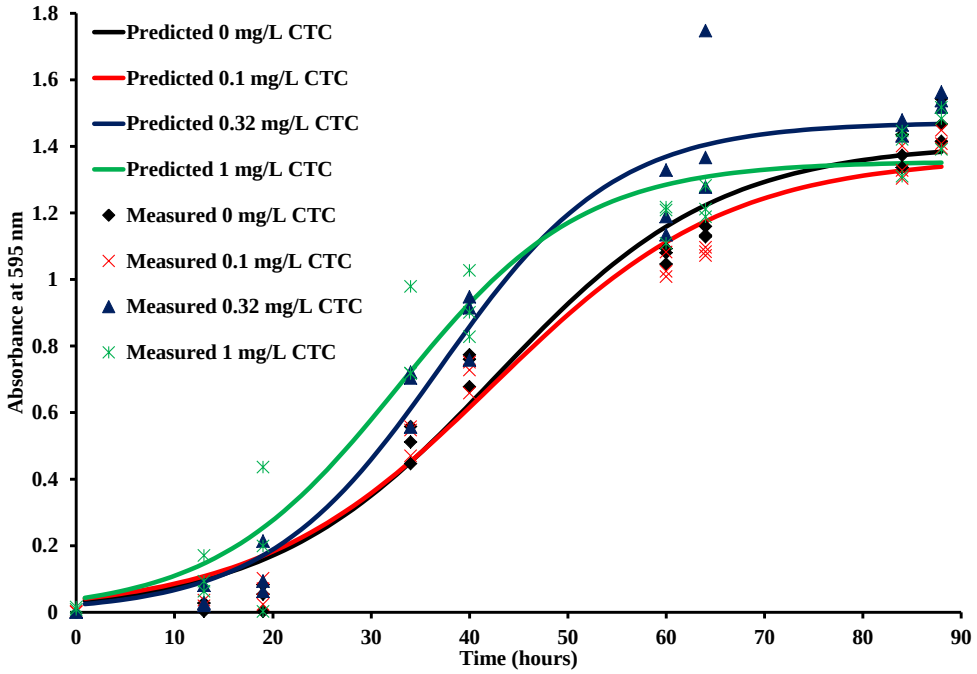
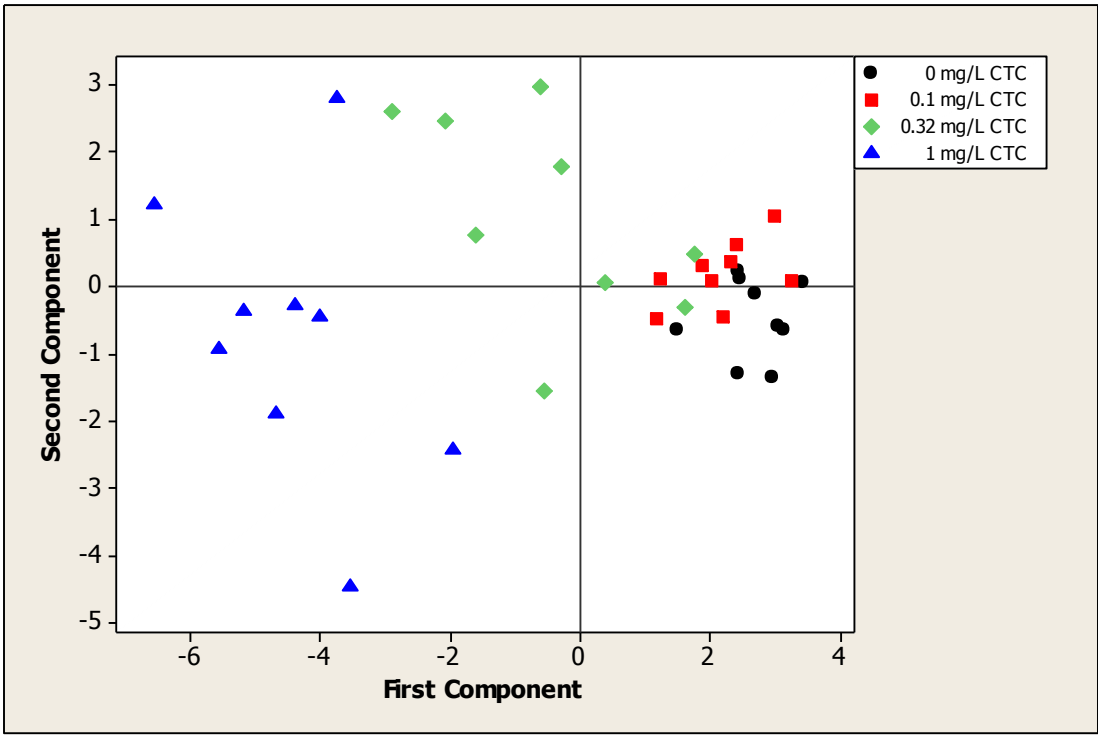


Figure 3.7 (cont).

a)



b)

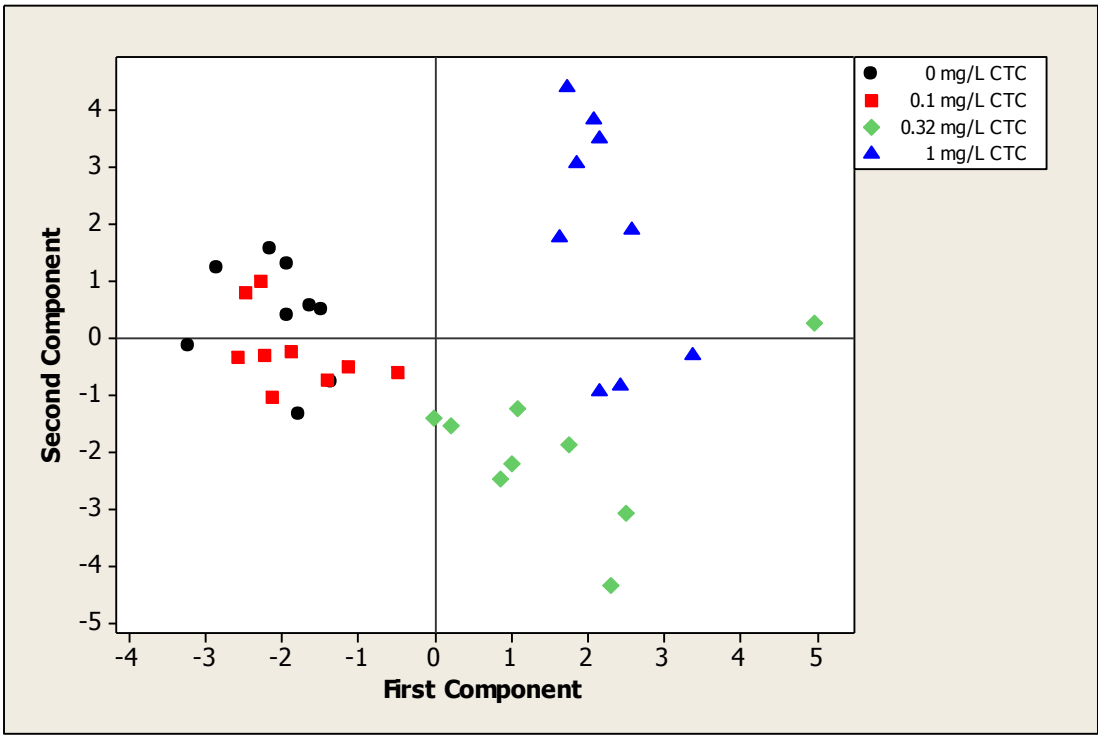
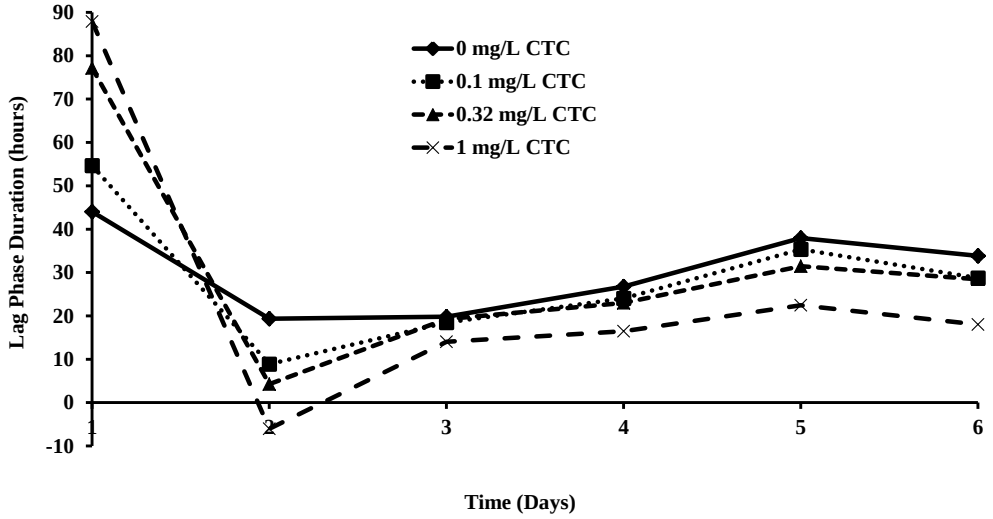


Figure 3.8: Scatter plots of PC scores for the first two principle components for individual replicates from each CTC treatment group representing amino acid utilisation on a) Day 1 and b) Day 4. Day 1 (a) proportion of variation: PC1 = 54.2%, PC2 = 16.2%; Day 4 (b) proportion of variation: PC1 = 23.4%, PC2 = 18%.

a)



b)

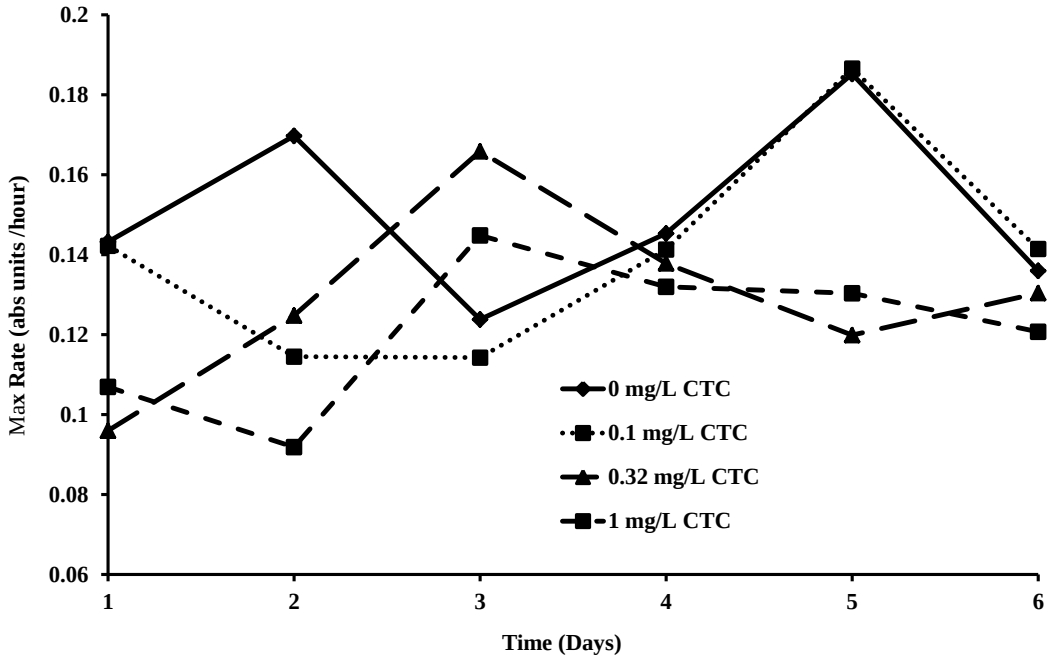


Figure 3.9: a) Lag phase duration versus exposure period time for amino acid utilisation and b) Max rate of amino acid utilisation versus exposure period time following CTC exposure.

Table 3.7: Summary of one-way ANOVA results for lag phase values before amino acid utilization from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	>ns	<*	<ns	<ns	<ns	<ns
0.32	>*	<*	<ns	<ns	<ns	<ns
1	>*	<*	<ns	<*	<*	<*

Table 3.8: Summary of one-way ANOVA results for maximum rate of amino acid utilization values from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	<*	<*	<ns	<ns	>ns	>ns
0.32	<ns	<*	>ns	<ns	<ns	<ns
1	<ns	<*	<ns	<ns	<ns	<*

3.3.4. Effect of CTC on Carbohydrate Utilisation

The data that are shown in figure 3.10 illustrates carbohydrate utilisation on exposure days 1, 2 and 3 that were plotted using a kinetic model. The data in figure 3.11 show PCA plots for the first two principle components scores on days 1 and 4. The data that are shown in figure 3.12 illustrate lag phase duration and the maximum rate of substrate utilisation on exposure days 1-6. The information in tables 3.9 and 3.10 show the statistical significance of lag phase and max rate values compared with control data.

3.3.4.1. Kinetic Model Plots of carbohydrate Utilisation

Carbohydrate utilisation, in terms of slope, onset of colour development and the maximum rate of colour development (and therefore carbohydrate utilisation) was inhibited by all CTC concentrations on day one of the exposure period (figure 3.14a).

On day 2 of the exposure, the plot representing carbohydrate utilisation for 0.32 mg/L CTC exposure resembled the control plot in terms of slope and onset of colour development. Maximum colour development at the end of incubation period was also higher than the control value. Although similar maximum colour development levels (to the control plot) were reached by 0.32 and 1 mg/L curves, a negative effect on carbohydrate utilisation was still visible at these CTC concentrations.

On exposure day 3, plots representing both 0.32 and 1 mg/L showed a quicker onset of colour development and a higher maximum colour development was reached. These data therefore suggested that carbohydrate utilisation was stimulated at these CTC concentrations. Carbohydrate utilisation was still inhibited by CTC in microcosms that had been dosed with 0.1 mg/L CTC however.

3.3.4.2. Lag Phase of Carbohydrate Utilisation versus Exposure Time

On day 1 of the exposure, the lag phase duration was significantly higher as a result of exposure to all CTC doses. In the case of exposure to 1 mg/L CTC, lag phase duration was also significantly higher than the control value on day 2 of the exposure period. On the same day, lag phase duration decreased compared with the control value as a result of exposure to 0.1 and 0.32 mg/L CTC. After day 2, lag phase duration was generally lower than control values at all CTC treatments. At CTC exposures of 0.32 and 1 mg/L, lag phases were significantly lower than control values for the rest of the exposure period.

3.3.4.3. Max Rate of Carbohydrate Utilisation versus Exposure Time

On day 1 of the exposure, the max rate of carbohydrate utilisation was inhibited by all CTC doses. With the exception of microcosms that were exposed to 0.1 mg/L CTC a statistically significant

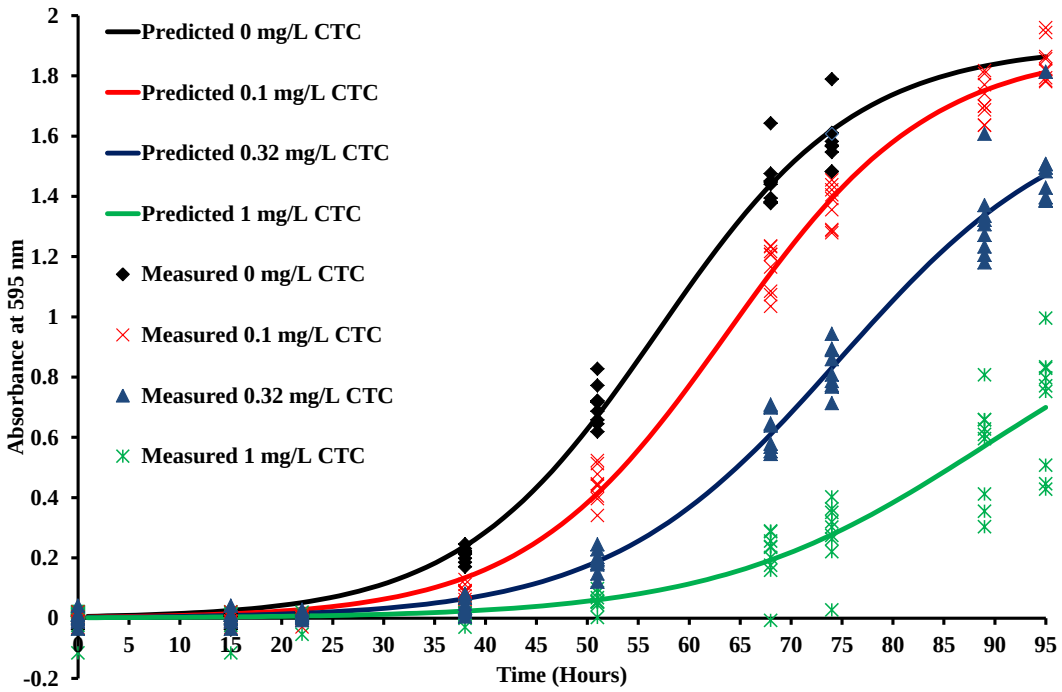
decreases in the max rate of carbohydrate utilisation was observed for all CTC exposures concentrations. This trend continued on exposure day 2 with a significant reduction in max rate of carbohydrate utilisation having been observed in microcosms that had been exposed to 0.1 mg/L CTC. Max rate did not differ significantly at any CTC concentration until day 6 of the exposure period. At this time, significant decreases in the max rate of carbohydrate utilisation were observed as a result of exposure to 0.32 and 1mg/L CTC.

3.3.4.4. PCA Analysis of Carbohydrate Utilisation

The data shown by PCA score plots of the first two principle components on day 1 of the exposure period show three distinct replicate clusters. The first cluster is composed of replicates representing the 1mg/L CTC group. Another distinct cluster depicts replicates exposed to 0.32 mg/L CTC. These data therefore suggest an effect of CTC on carbohydrate utilisation at these two concentrations, in terms of substrate utilisation profile. From these results, it can be suggested that the top two CTC doses caused a change in the community structure of microbes that were utilising carbohydrates. A third cluster is composed of replicates from control and 0.1 mg/L CTC exposure groups suggesting these two treatments result in a similar carbohydrate utilisation profile. It is likely therefore that the portion of the microbial community that was utilising carbohydrates had a similar community structure in microcosms exposed to these CTC concentrations.

A similar trend is apparent on day 4 PCA score plots, suggesting that similar carbon utilisation profiles to day 1 were observed. As such, CTC concentrations of 0.32 and 1 mg/L were still causing changes in the carbohydrate utilisation profile of the microbial community. These data suggest that the portion of the microbial community that could utilise carbohydrates had different community structures from unexposed microbial communities. Day 4 data also suggested that the portion of the microbial community that was able to utilise carbohydrates had a similar community structure in microcosms that had exposed to 0 and 0.1 mg/L CTC.

a)



b)

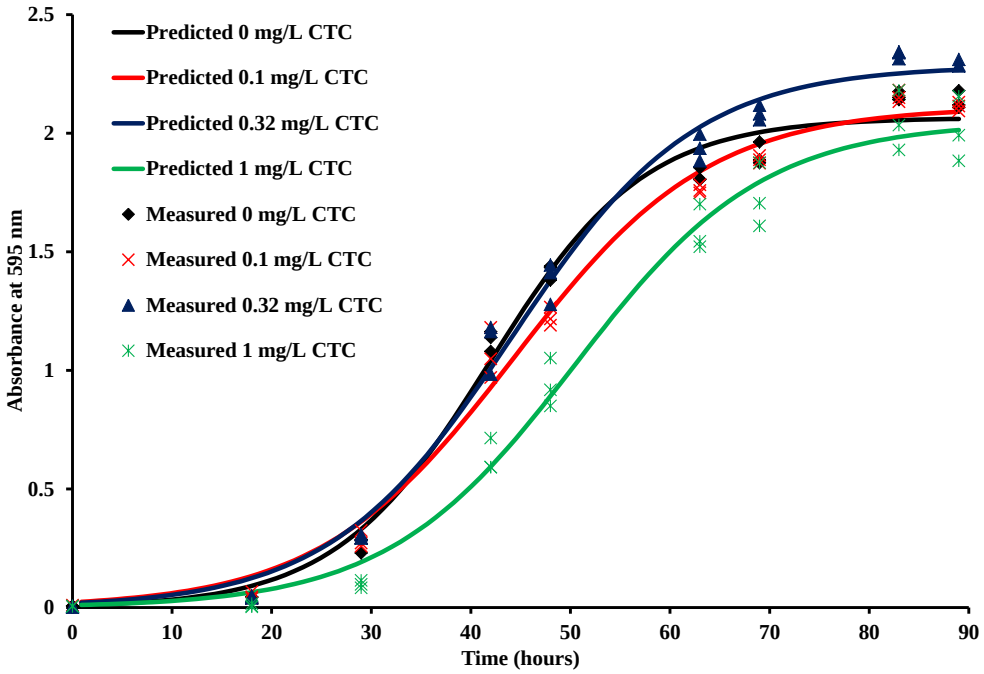


Figure 3.10: Kinetic plots of carbohydrate utilisation following exposure to CTC on a) day 1, b) day 2 and c) day 3.

c)

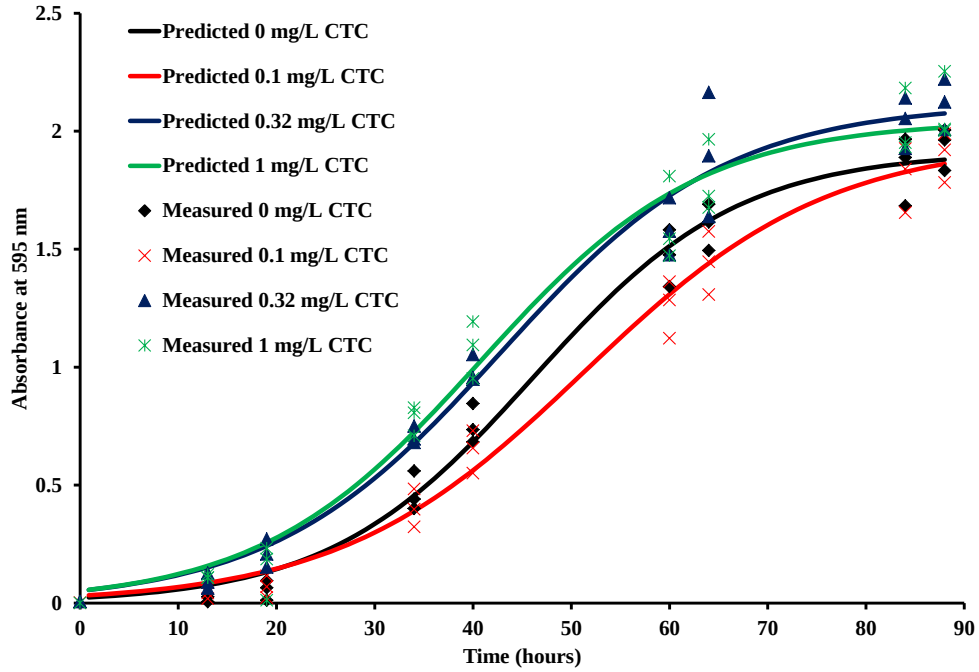
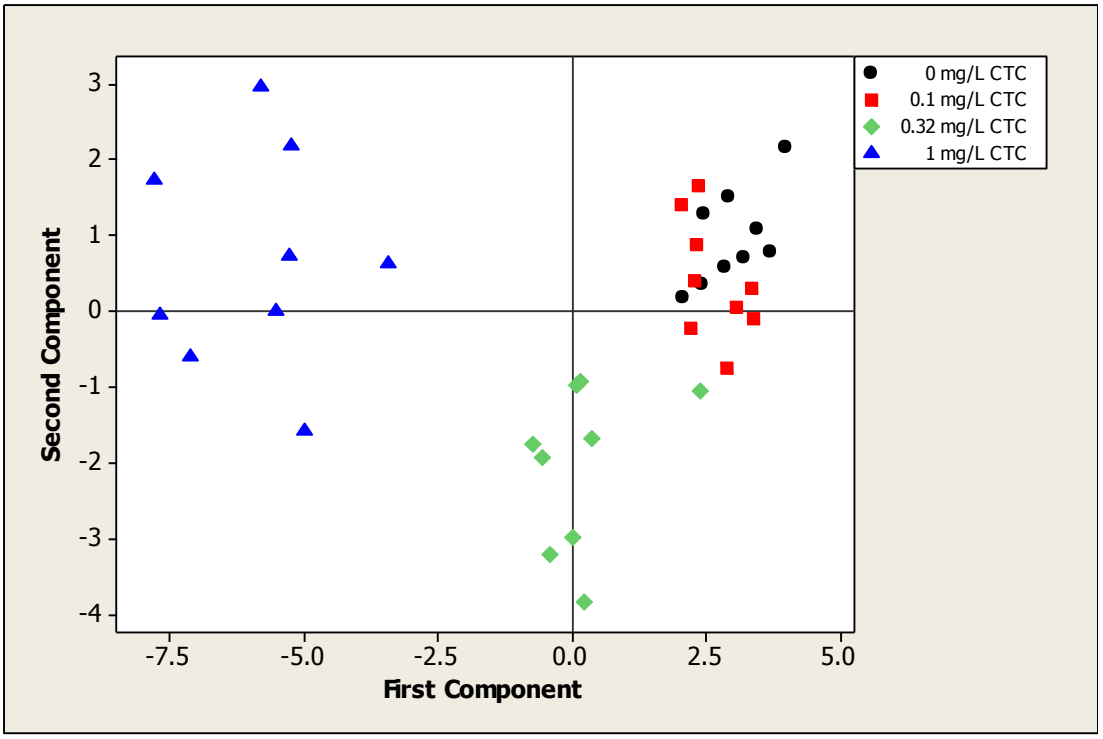


Figure 3.10 (cont).

a)



b)

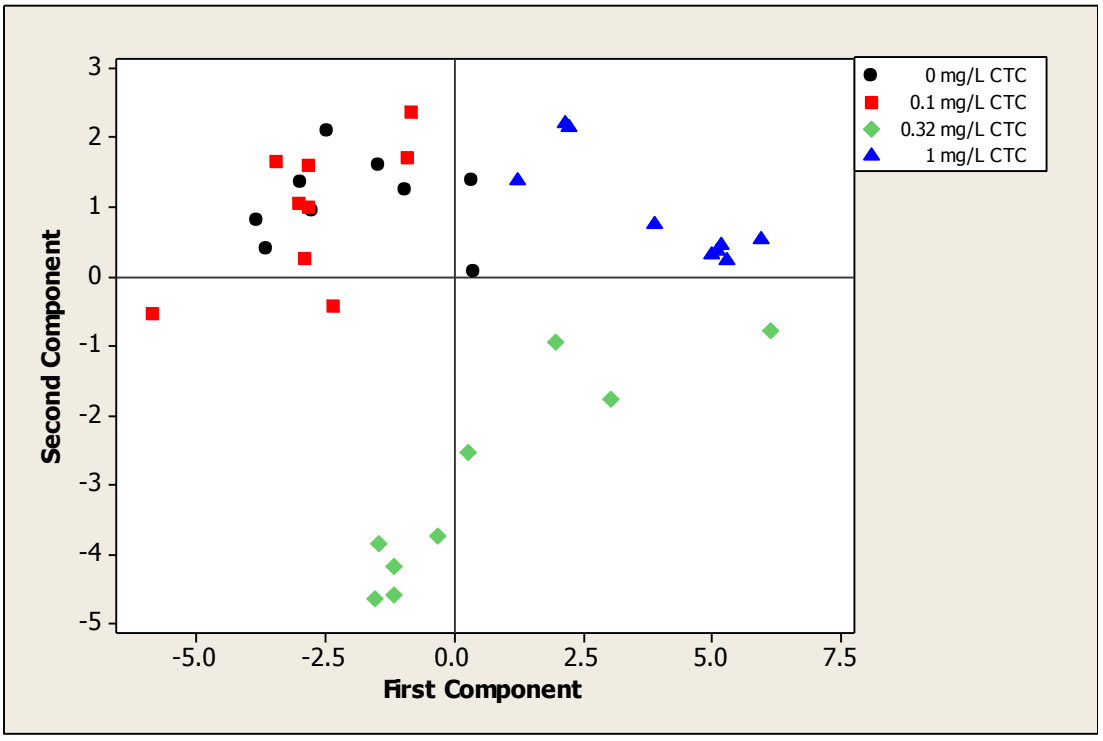
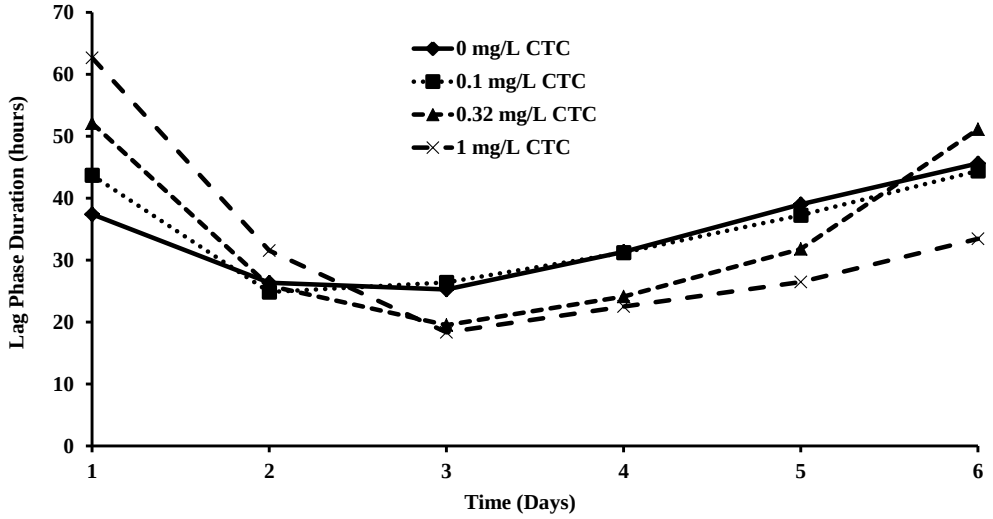


Figure 3.11: Scatter plots of PC scores for the first two principle components for individual replicates from each CTC treatment group representing carbohydrate utilisation on a) day 1 and b) day 4. Day 1 (a) proportion of variation: PC1 = 49.3%, PC2 = 8.6%; Day 4 (b) proportion of variation: PC1 = 37.3%, PC2 = 14.6%.

a)



b)

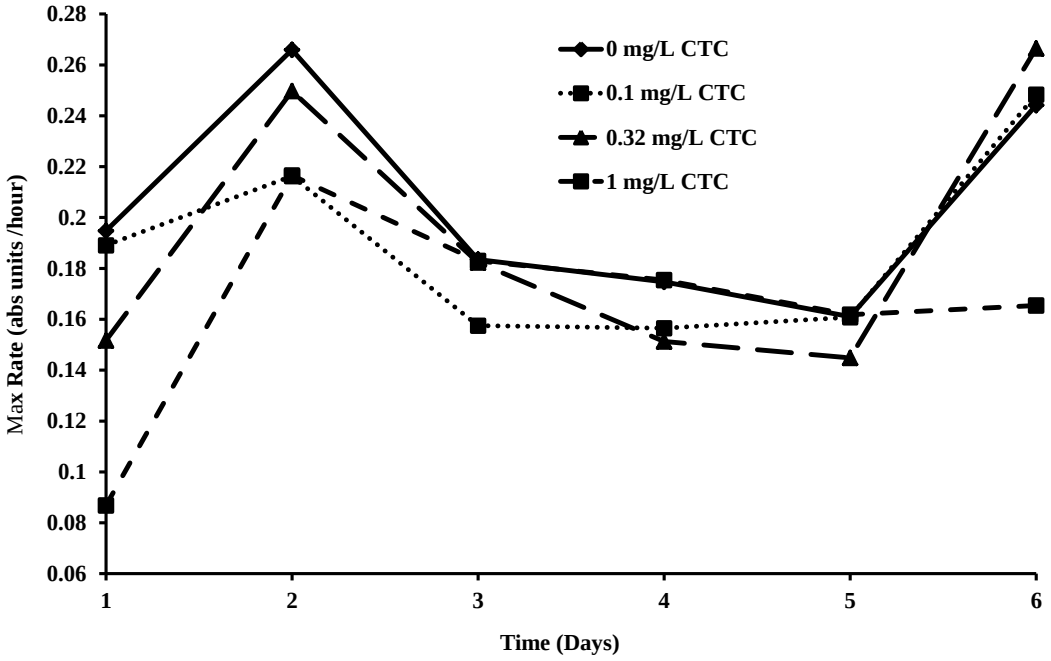


Figure 3.12: a) Lag phase duration versus exposure period time for carbohydrate utilisation and b) Max rate of carbohydrate utilisation versus exposure period time following CTC exposure.

Table 3.9: Summary of one-way ANOVA results for lag phase values before carbohydrate utilization from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	>ns	<*	<ns	<ns	<ns	<ns
0.32	>*	<*	<ns	<ns	<ns	<ns
1	>*	<*	<ns	<*	<*	<*

Table 3.10: Summary of one-way ANOVA results for maximum rate of carbohydrate utilization values from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	<*	<*	<ns	<ns	>ns	>ns
0.32	<ns	<*	>ns	<ns	<ns	<ns
1	<ns	<*	<ns	<ns	<ns	<*

3.3.5. Effect of CTC on Carboxylic Acid Utilisation

The data that are shown shown by figure 3.13 illustrate carboxylic acid utilisation on exposure days 1, 2 and 3 plotted using a kinetic model. The data in figure 3.14 show PCA scatter plots for the first two principle components scores on days 1 and 4. The data in figure 3.15 illustrate lag phase and maximum rate on exposure days 1-6. The information in tables 3.11 and 3.12 show the statistical significance of lag phase and max rate values compared with control data.

3.3.5.1. Kinetic Model Plots of Carboxylic Acid Utilisation

Carboxylic acid utilisation, in terms of colour production onset time and maximum colour development, was inhibited by all CTC concentrations. At CTC concentrations of 0.32 and 1 mg/L, the slopes, when compared with the control slope, also indicated that inhibition of carboxylic acid utilisation had occurred as the result of exposure to CTC (fig 3.13a).

The data shown by day 2 kinetic model plots illustrate a continuing trend from day 1 data. Inhibition of carboxylic acid utilisation was still detected at all CTC concentrations in terms of colour production onset. Slopes of treated plots on day 2 were more similar to control slopes however. Maximum colour development on day 2 was either similar to or higher than control values (fig 3.13b)

The data in day 3 kinetic plots indicate that colour development onset at all CTC concentrations was occurring more rapidly than was shown by control plots. There were also no observable differences in the gradient of plots representing exposed microcosms compared with control data. In addition, the maximum colour development on day 3 was higher at all CTC concentrations compared with control values (fig 3.13c).

3.3.5.2. Lag Phase of Carboxylic Acid versus Exposure Time

On day 1 of the exposure, an increasing lag phase duration was negatively correlated with increasing CTC concentration. The decrease in lag phase duration was shown to be statistically significant. Although lag phase values could still be negatively correlated with increasing CTC concentration on day 2 of the exposure, the increase in lag phase that was statistically significant was caused by exposure to 1 mg/L CTC. Between days 1 and 3 of the exposure period, lag phase values had dropped. The only lag phase value that was significantly higher than the control lag phase duration was at a dose of 1mg/L CTC.

For the remainder of the exposure period (days 3 to 6), lag phase durations remained lower than that of the control. The only lag phase value that was statistically less than the control value was at 1 mg/L CTC (fig 3.15a; table 3.11).

3.3.5.3. Max Rate of Carboxylic Acid Utilisation versus Exposure Time

On day 1 of the exposure, a reduction in the max rate of carboxylic acid utilisation was negatively correlated with increasing CTC concentration. Statistically significant reductions in max rate values were only detected at the two highest concentrations of CTC however. A general rise in the max rate of carboxylic acid utilisation was observed at all exposure concentrations (and in control microcosms) between days 1 and 4, although the max rate of carboxylic acid utilisation was still consistently lower than the control value. It was observed that max rate was significantly lower than the control value in microcosms exposed to 1 mg/L CTC on day 4. On the final day of the exposure period (day 6) significantly lower max rate values (compared with the control max rate) were observed at CTC concentrations of 0.32 and 1 mg/L (figure 3.15b; table 3.12).

3.3.5.4. PCA Analysis of Carboxylic Utilisation

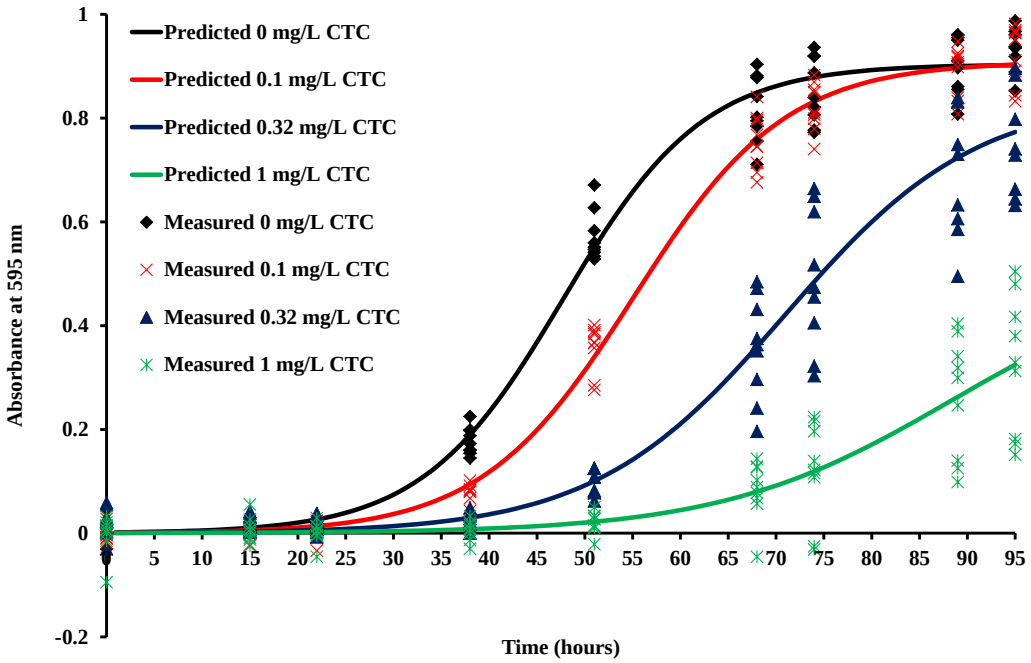
Data illustrated by PCA scatter plots of the first two principle components on day 1 shows a distinct cluster composed of replicates from the 1 mg/L CTC exposure. At this CTC concentration the utilisation profile of carboxylic acids had therefore been altered compared with control profiles. It is likely that the portion of the microbial community that was able to utilise carboxylic acids had a different community structure from the unexposed microbial community. A cluster of five replicates resulted from exposure to 0.32 mg/L. However, four replicates from the 0.32 mg/L grouping were also associated with a cluster that contained replicates from the control and 0.1 mg/L group.

It can therefore be inferred that the carboxylic acid utilisation profile of control and 0.1 mg/L exposed microbial communities had similar carboxylic acid utilisation profiles. Microcosms exposed to 0.32 mg/L had a less distinct carboxylic utilisation profile that could sometimes be associated with control and 0.1 mg/L profiles. These data suggest that the portion of the microbial community that was utilising carboxylic acids on day 1 had similar community structures in microcosms exposed to 0 and 0.1 mg/L CTC. The carboxylic acid utilising microbial community that was exposed to 0.32 mg/L was likely to have a less similar community structure to unexposed communities, although separate clustering would indicate that exposure to this CTC concentration did result in a degree of change in community structure.

Day 4 data indicates that 0.32 mg/L CTC replicates tended to cluster more distinctly from control and 0.1 mg/L replicates but more closely to replicates from the 1 mg/L group. This would suggest that the carboxylic utilisation profile of the microbial community present in microcosms that were exposed to 0.32 mg/L was being altered compared to the microbial community that was present in control microcosms. It is therefore likely that the community structure had changed and had also become more similar to the microbial community that was present in microcosms spiked with 1 mg/L CTC. Replicates from the control and 0.1 mg/L CTC groups continued to cluster on day 4,

suggesting that their carboxylic utilisation profile (and therefore microbial community structure) were similar. Replicates from the 1 mg/L CTC group continued to cluster on day 4. Therefore the carboxylic acid utilisation profile and subsequently the microbial community structure remained distinct from the unexposed group.

a)



b)

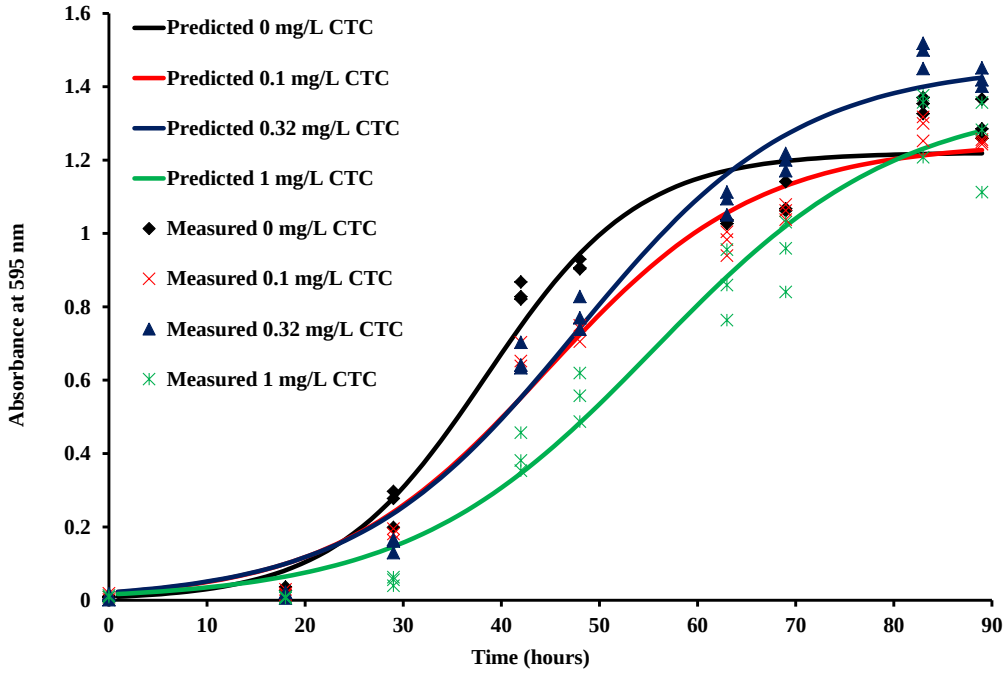


Figure 3.13: Kinetic plots of carboxylic acid utilisation following CTC exposure on a) day 1, b) day 2 and c) day 3.

c)

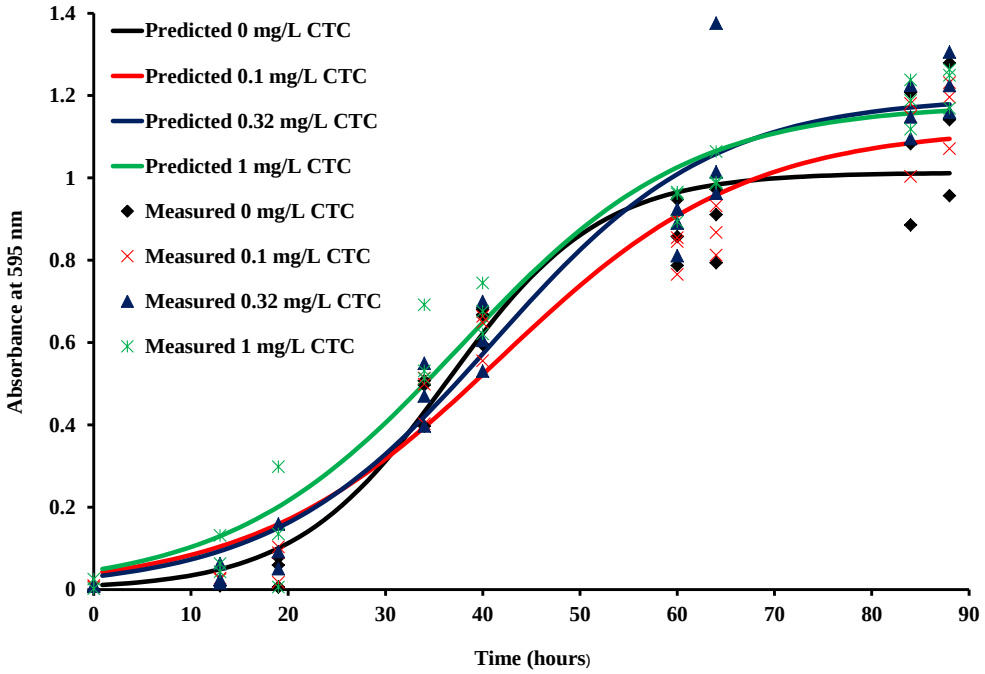
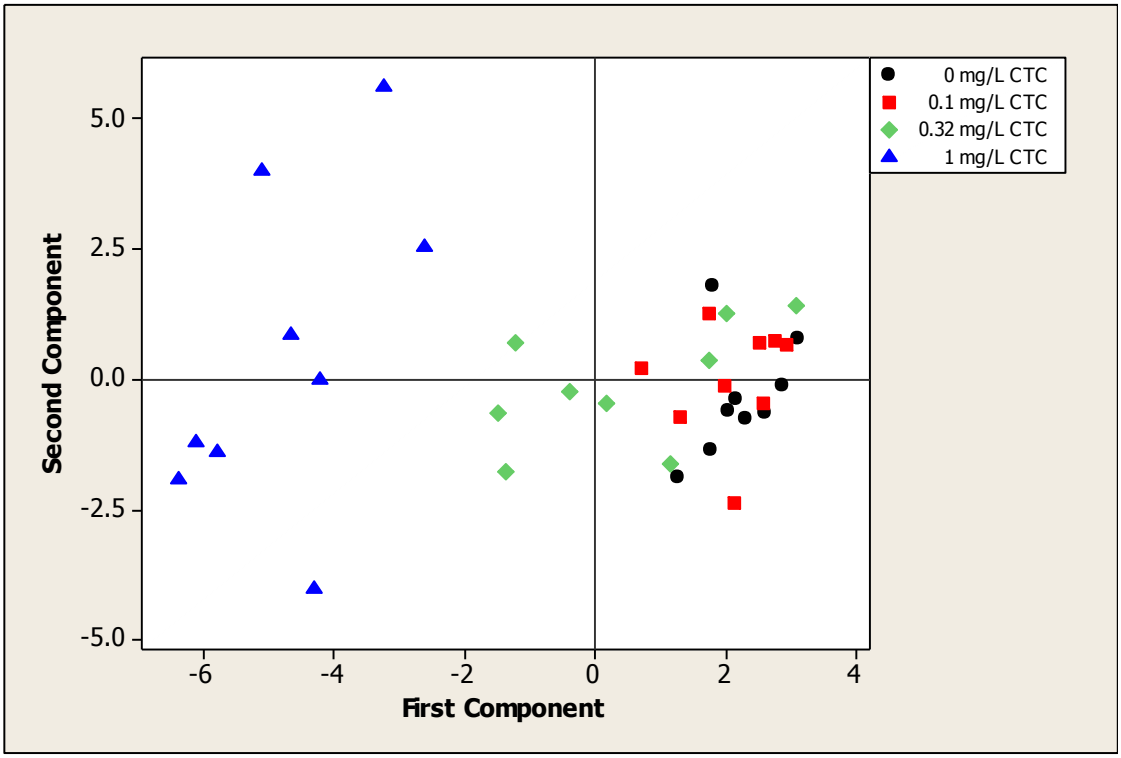


Figure 3.13 (cont).

a)



b)

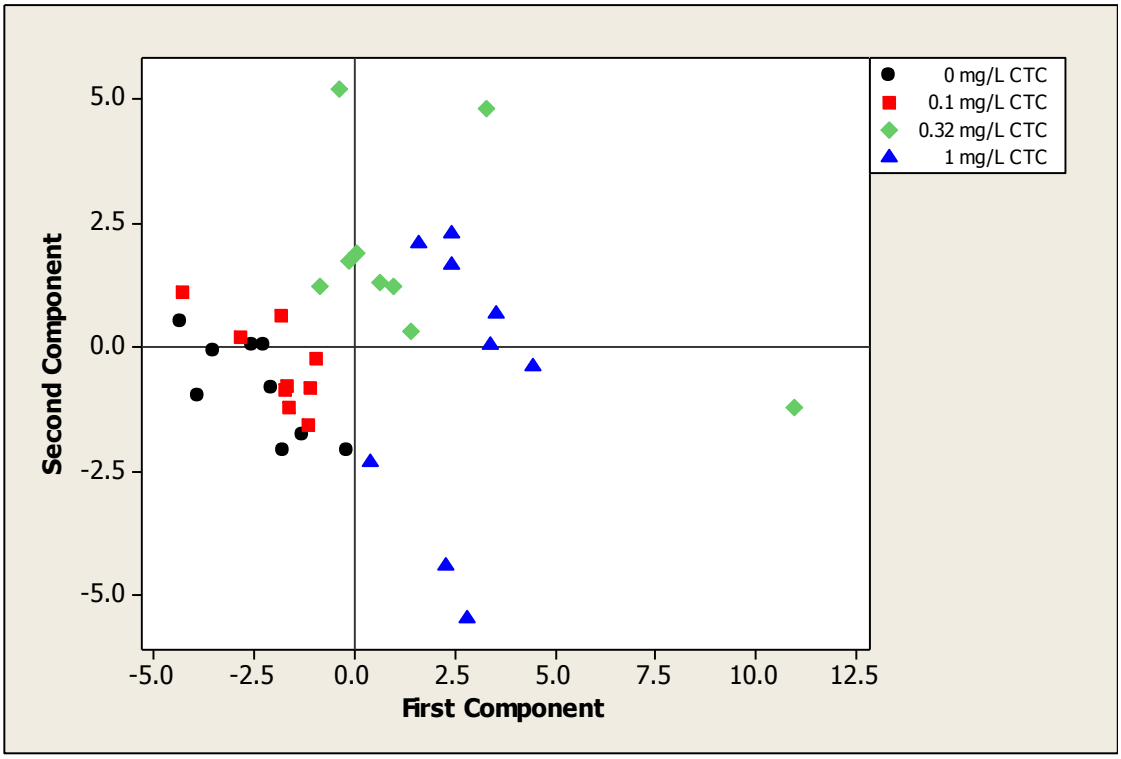
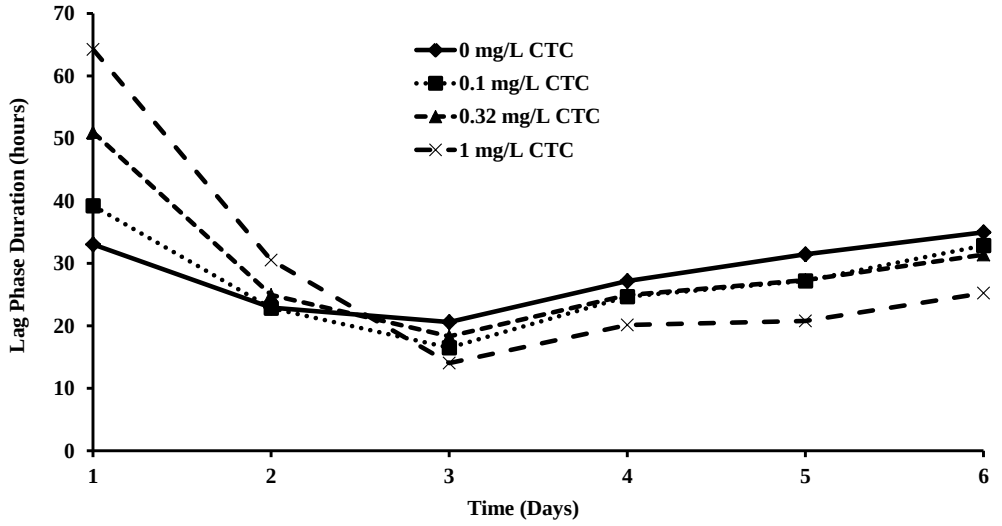


Figure 3.14: Scatter plots of PC scores for the first two principle components for individual replicates from each CTC treatment group representing carboxylic acid utilisation on a) Day 1 and b) Day 4. Day 1 (a) proportion of variation: PC1 = 36%, PC2 = 12%; Day 4 (b) proportion of variation: PC1 = 34.8%, PC2 = 16.8%.

a)



b)

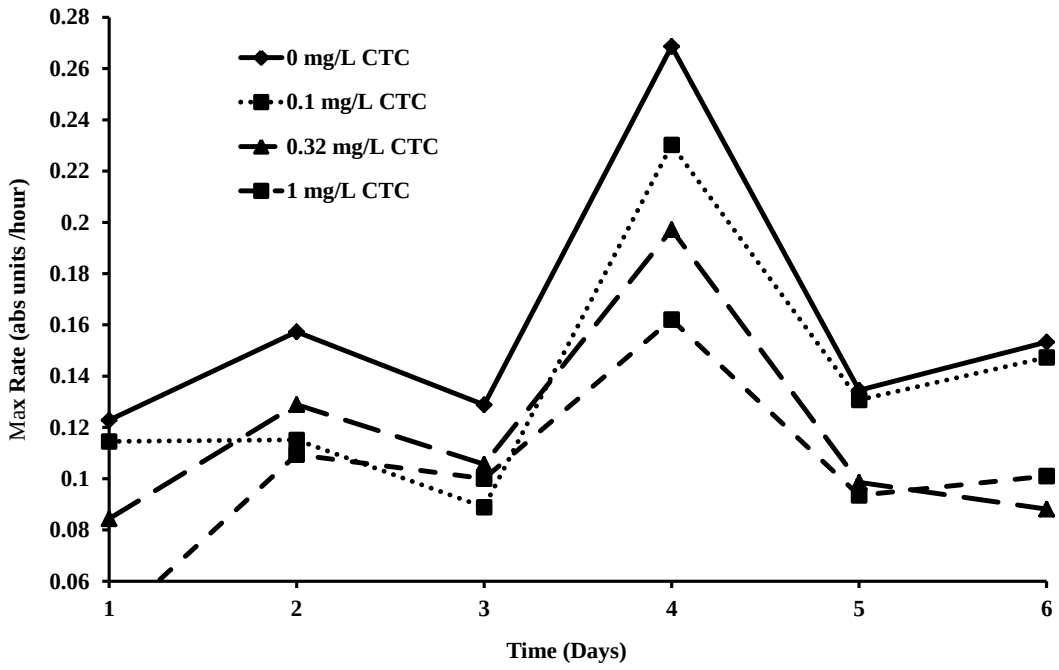


Figure 3.15: a) Lag phase duration versus exposure period time for carboxylic acid utilisation and b) Max rate of carboxylic acid utilisation versus exposure period time following CTC exposure.

Table 3.11: Summary of one-way ANOVA results for lag phase values before carboxylic acid utilization from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	>ns	<ns	<ns	<ns	<ns	<ns
0.32	>*	>ns	<ns	<ns	<ns	<ns
1	>*	>*	<ns	<*	<*	<*

Table 3.12: Summary of one-way ANOVA results for maximum rate ofcarboxylic acid utilization values from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	<ns	<ns	>ns	<ns	<ns	<ns
0.32	<*	<*	<ns	<*	<ns	<ns
1	<*	<*	<ns	<*	<ns	<ns

3.3.6. Effect of CTC on Polymer Utilisation

The data shown by figure 3.16 illustrate carboxylic acid utilisation on exposure days 1, 2 and 3 plotted using a kinetic model. Data in figure 3.17 show PCA plots for the first two principle components scores on days 1 and 4. The data in figure 3.18 illustrate lag phase and maximum rate on exposure days 1-6. The information in tables 3.13 and 3.14 show the statistical significance of lag phase and max rate values compared with control data.

3.3.6.1. Kinetic Plot Models of polymer Utilisation

The data in day 1 kinetic model plots illustrate a trend of polymer utilisation being inhibited by all CTC concentrations. At all concentrations the time of colour production onset, slope gradient and maximum colour development were inhibited compared with data in control kinetic plots (figure 3.16a).

On day 2 of the exposure, data in plots representing polymer utilisation in microcosms exposed to 0.32 mg/L CTC show a steeper gradient and a higher maximum colour development (and therefore polymer utilisation) than illustrated by data in control plots. Plots showing data for l mg/L on the same exposure day point towards an inhibition of colour development onset (figure 3.16b).

On day 3, data from all plots of CTC exposure concentrations show a higher maximum rate of colour development. Inhibition of polymer utilisation was no longer inhibited by 0.32 or 1mg/L CTC exposure. It is unclear as to whether any inhibitory effects were still being observed at 0.1 mg/L CTC exposure on day 3 (figure 3.16c).

3.3.6.2. Lag Phase of Polymer Utilisation versus Exposure Time

On day 1 of the exposure, the lag phase duration increased as a result of exposing microcosms to all CTC concentrations. The longest lag phase duration was observed at 1mg/L CTC exposure, although this reduction was not significantly different from the control lag phase duration. Significant differences in lag phase duration were however observed at 0.1 and 0.32 mg/L CTC exposure.

On the second exposure day, lag phases shortened at all CTC treatments (including control values). Only an exposure concentration of 1mg/L resulted in a statistically shorter lag phase duration however. Lag phases at all CTC doses (not including the control dose) remained similar to the control lag phase duration until the end of the exposure period (day 6). The only exception to this trend was a significant lag phase reduction compared to the control value at 1 mg/L CTC on day 6 being been observed (figure 3.18a).

3.3.6.3. Max Rate of Polymer Utilisation versus Exposure Time

On day 1 of the exposure, a statistically significant decrease in the max rate of polymer utilisation was observed in microcosms that had been spiked with 0.1 and 0.32 mg/L CTC. Conversely, the max rate of polymer utilisation in microcosms that were exposed to 1mg/L increased, although this increase was not statistically significant. On day 2 of the exposure, the max rate of polymer utilisation increased as a result of exposing microbial communities to 0.1 and 0.32 mg/L CTC. The latter exposure concentration resulted in a significantly higher max rate of polymer utilisation compared with the control value. Max rate values for all CTC treatments remained similar to the control max rate until the end of the exposure period (day 6) with the exception of microcosms that had been exposed to 1mg/L CTC on day 4; a significantly higher max rate of polymer utilisation was observed at this time-point and CTC concentration.

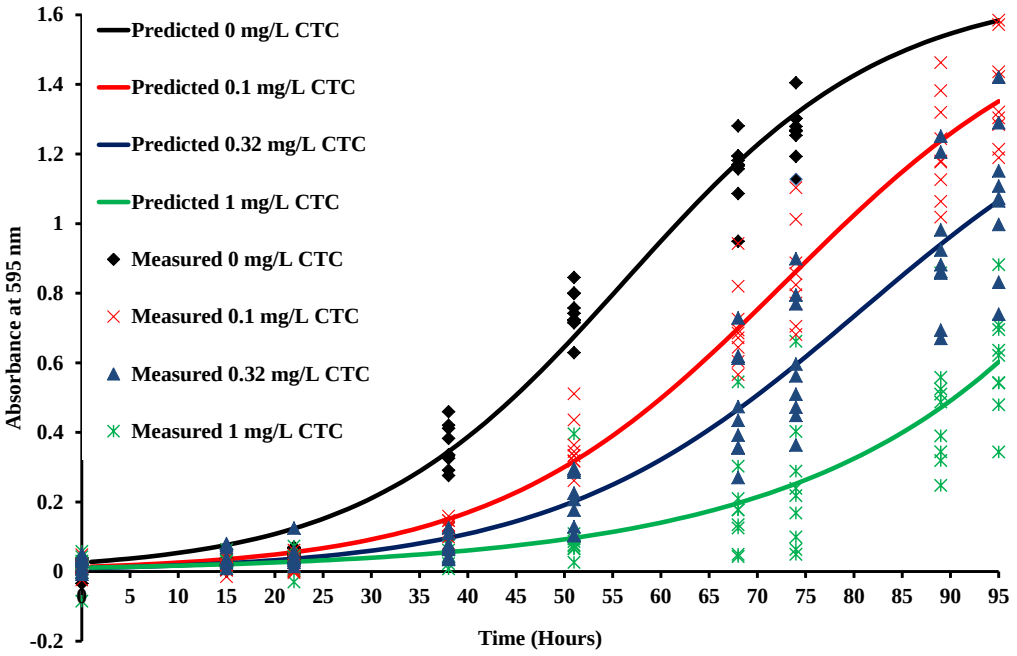
3.3.6.4. PCA Analysis of Polymer Utilisation

Data illustrated by PCA scatter plots of the first two principal component scores on day 1 show two distinct replicate clusters. The first cluster shows distinct grouping of replicates from the control, 0.1 and 0.32 mg/L groups, suggesting that the polymer utilisation profile was similar for these CTC treatments on day 1. In another cluster group, replicates that had been exposed to 1mg/L also cluster closely. This suggests that exposure to the highest CTC concentration results in a distinct polymer utilisation profile also. Some replicates from the 0.32 mg/L CTC group also clustered with this group suggesting that there was some similarity between the polymer utilisation profile of microcosms exposed to 0.32 and 1 mg/L CTC (figure 3.17a).

It can be suggested therefore that the portion of the microbial community that was utilising polymers on day 1 of the exposure had a different community structure than microcosms that were exposed to 1 mg/L CTC. It is also likely that exposure of the polymer-utilising microbial community that was exposed to 0.32 mg/L CTC caused a smaller difference in microbial community structure compared with the unexposed microbial community structure. The results also suggest that this microbial community structure shared some similarity with the microbial community that exposed to 1 mg/L CTC.

The polymer utilisation profile of day 1 is mirrored by the data shown in day 4 PCA scatter plots, although more replicates from the 0.32 mg/L CTC exposure cluster with replicates from the 1mg/L cluster. These data would suggest that the microbial community structure of carboxylic utilising bacteria that were exposed to 0.32 and 1 mg/L CTC were more similar on day 4 (figure 3.17b).

a)



b)

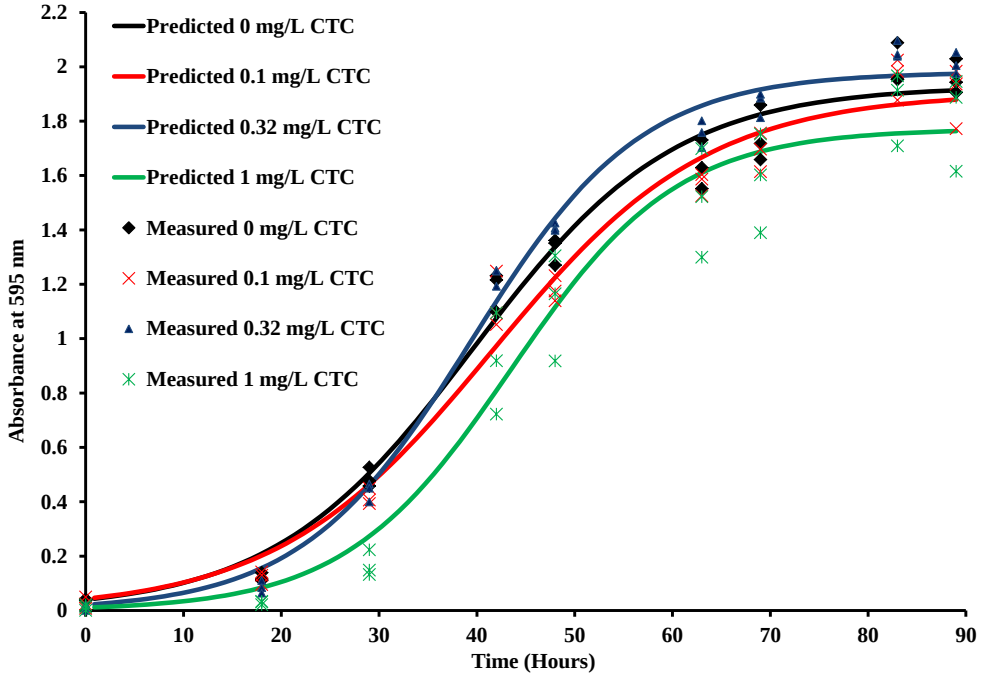


Figure 3.16: Kinetic plots of polymer utilisation following CTC exposure on a) day 1, b) day 2 and c) day 3.

c)

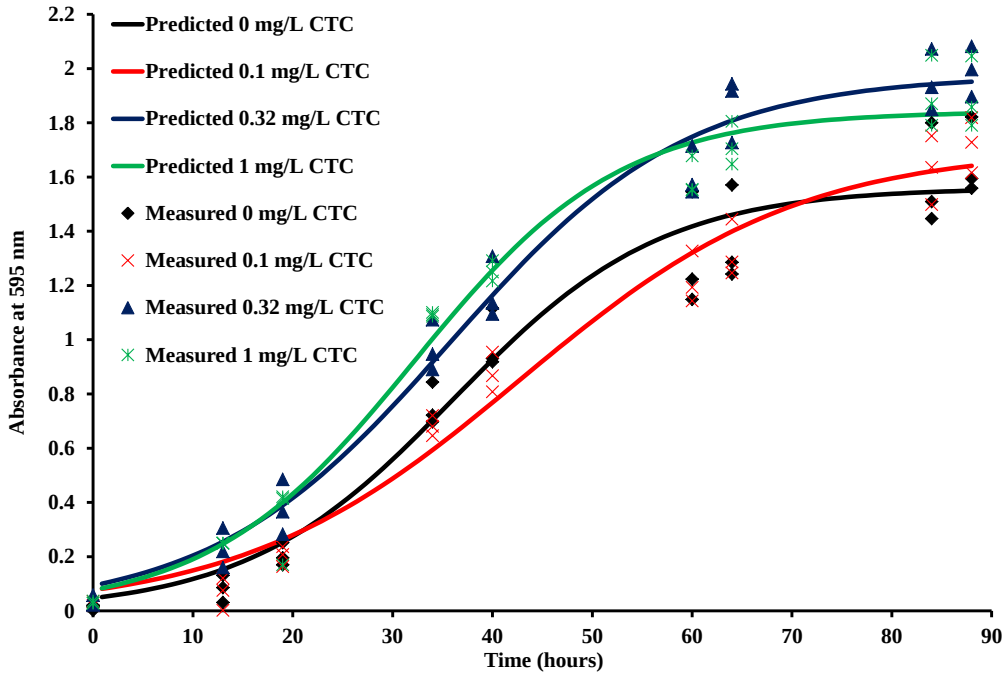
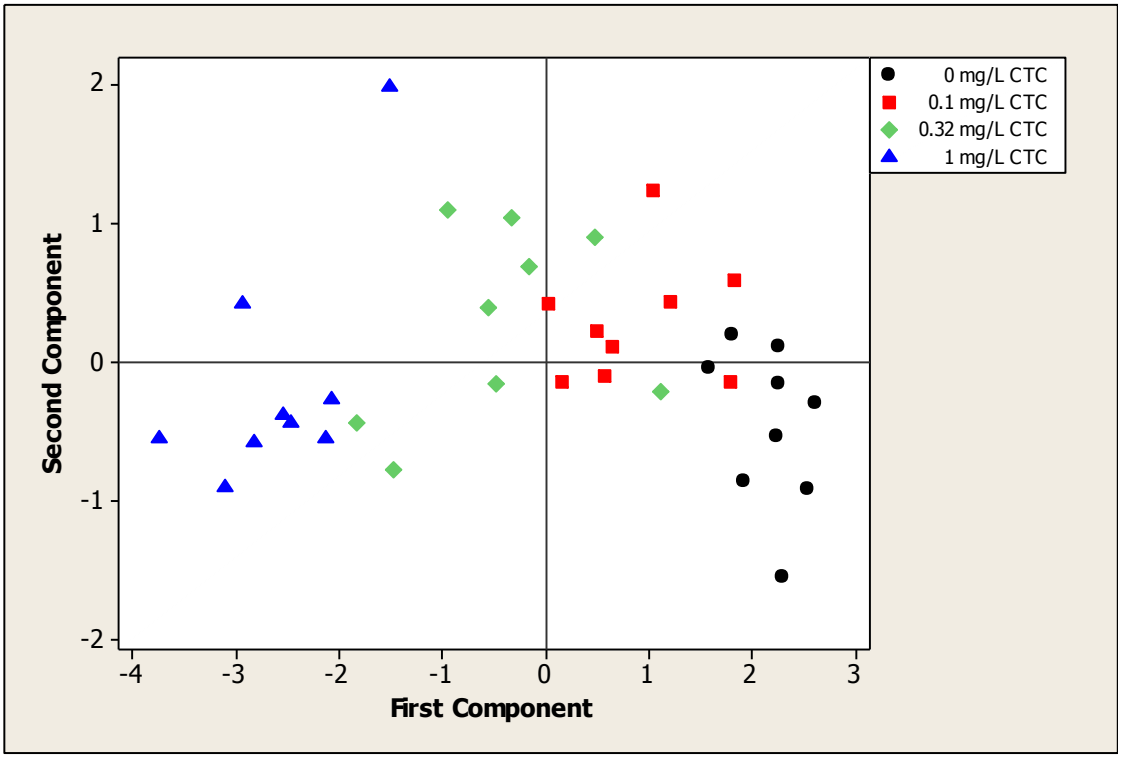


Figure 3.16 (cont).

a)



b)

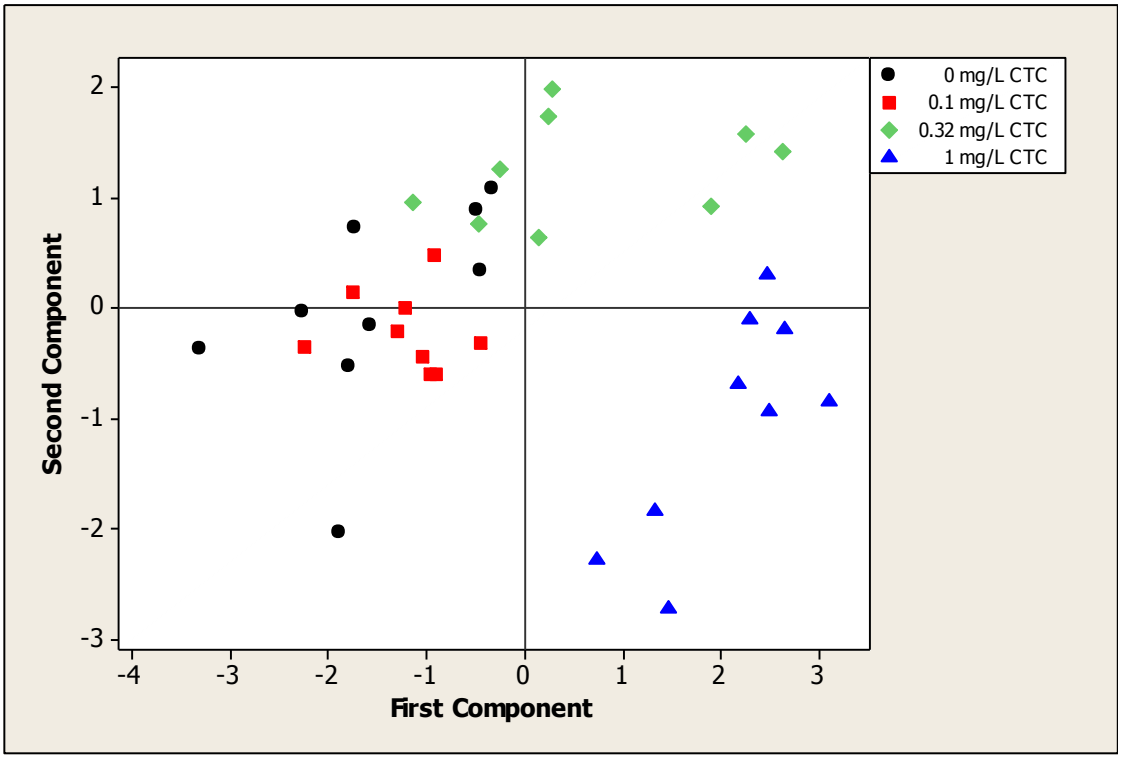
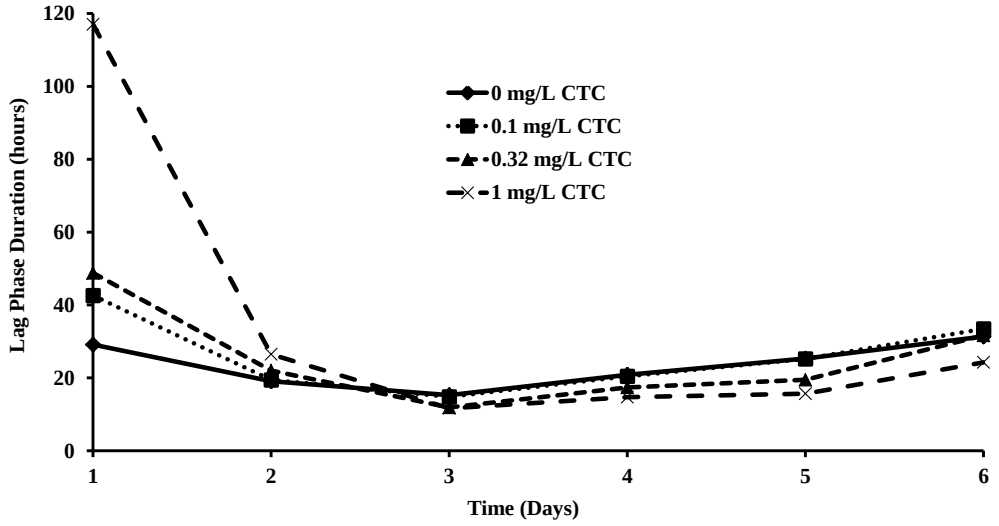


Figure 3.17: Scatter plots of PC scores for the first two principle components for individual replicates from each CTC treatment group representing polymer utilisation on a) Day 1 and b) Day 4. Day 1 (a) proportion of variation: PC1 = 72%, PC2 = 10.1%; Day 4 (b) proportion of variation: PC1 = 59.8%, PC2 = 24.3%.

a)



b)

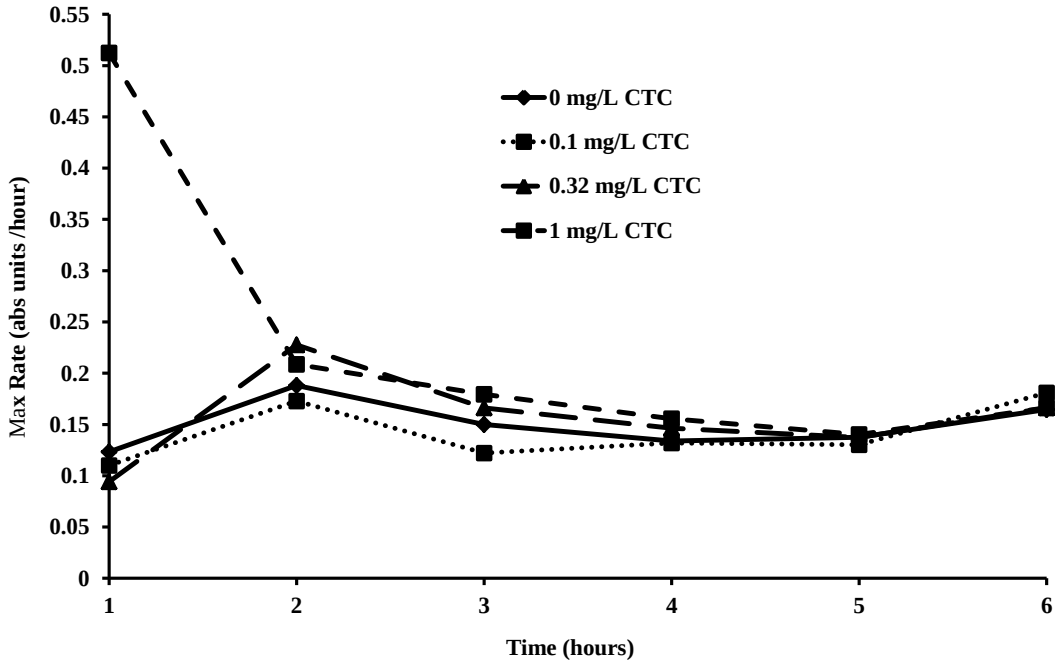


Figure 3.18: a) Lag phase duration versus exposure period time for polymer utilisation and b) Max rate of polymer utilisation versus exposure period time following CTC exposure.

Table 3.13: Summary of one-way ANOVA results for lag phase values before polymer utilization from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	>*	>ns	<ns	<ns	<ns	>ns
0.32	>*	>ns	<ns	<ns	<*	<ns
1	>ns	>*	<ns	<*	<*	<*

Table 3.14: Summary of one-way ANOVA results for maximum rate of polymer utilization values from Biolog data for inocula from microcosms following different exposure times to CTC in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]					
[CTC] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**
0.1	<*	>ns	<ns	<ns	<ns	>ns
0.32	<*	>*	>ns	>ns	<ns	>ns
1	<ns	>ns	>ns	>*	>ns	>ns

3.3.7. Effect of CTC on Total CFU Counts

There were no statistically significant changes in CFU counts compared with control values as a result of exposing microbial communities to any CTC dose on day 1 or day 7 (figure 3.19). These data would suggest that the total number of cells that were being produced by the microbial community was not significantly affected by exposure to any CTC concentration. It can therefore be concluded that microbial communities in general exhibited similar growth regardless of which CTC treatment they were exposed to.

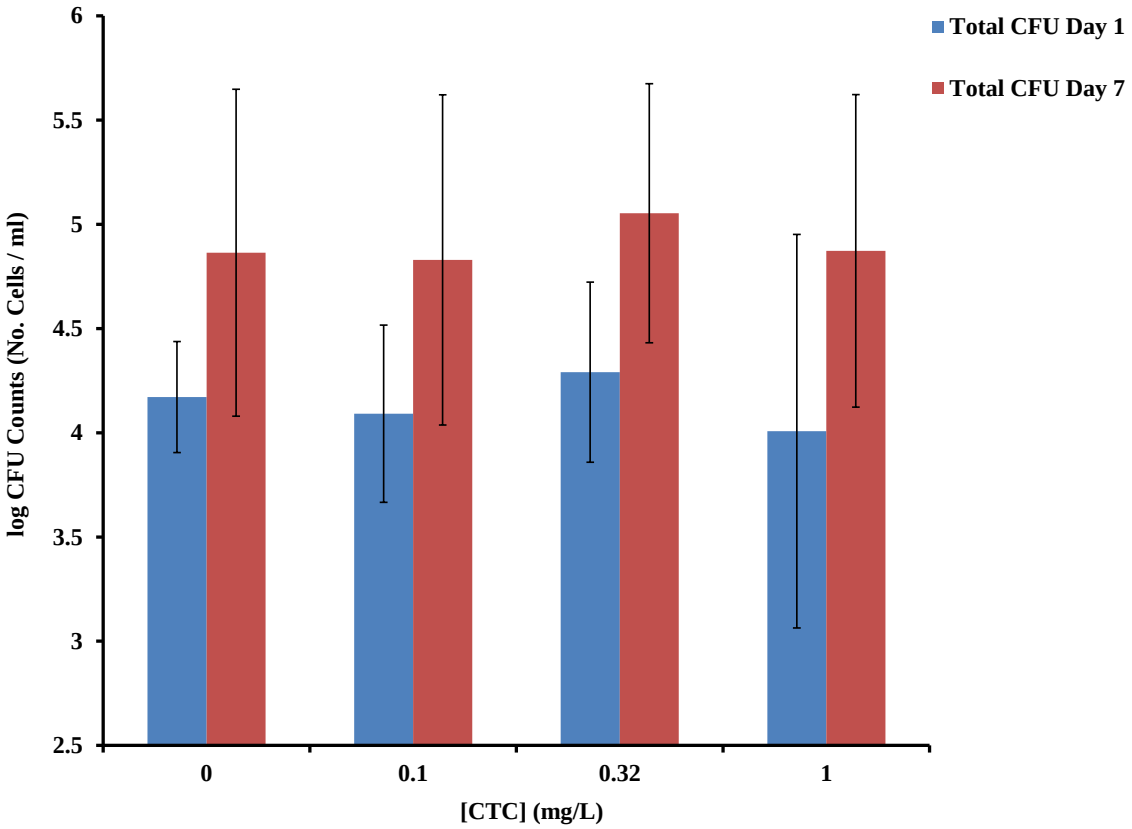


Figure 3.19: Bar graph representing total log CFU counts versus [CTC] on days one and seven of the exposure period. Any statistically significant changes are indicated by asterisk notation (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

3.3.8. Effect of CTC on the Functional Diversity of Multisubstrate Utilisation

The data shown by figure 3.20 indicate changes in the functional diversity of substrates that were being utilised by microbial communities on each day of the exposure. On day 1 of the exposure the functional diversity of substrate utilisation was significantly inhibited ($P < 0.001$) by the two highest CTC concentrations (0.32 and 1 mg/L) as shown by these two treatment groups having a lower diversity index value. These data would suggest that microbial communities that had been exposed to these CTC concentrations were not able to utilise the same range of substrates as communities that were exposed to 0 and 0.1 mg/L CTC were able to utilise.

Data from other days of the exposure show that microbial communities that had been exposed to the two highest CTC concentrations were no longer inhibited on day 2 onwards in terms of the diversity of substrates that they were able to utilise. On days two and three of the exposure, microbial communities at all treatment concentrations showed a similar functional diversity of substrate utilisation. On day 4 however, communities that had been exposed to the two highest CTC doses were able to utilise a significantly greater ($P < 0.001$) diversity of substrates, as shown by a higher diversity index. This trend continued to the end of the exposure in the case of the 1 mg/L CTC treatment group.

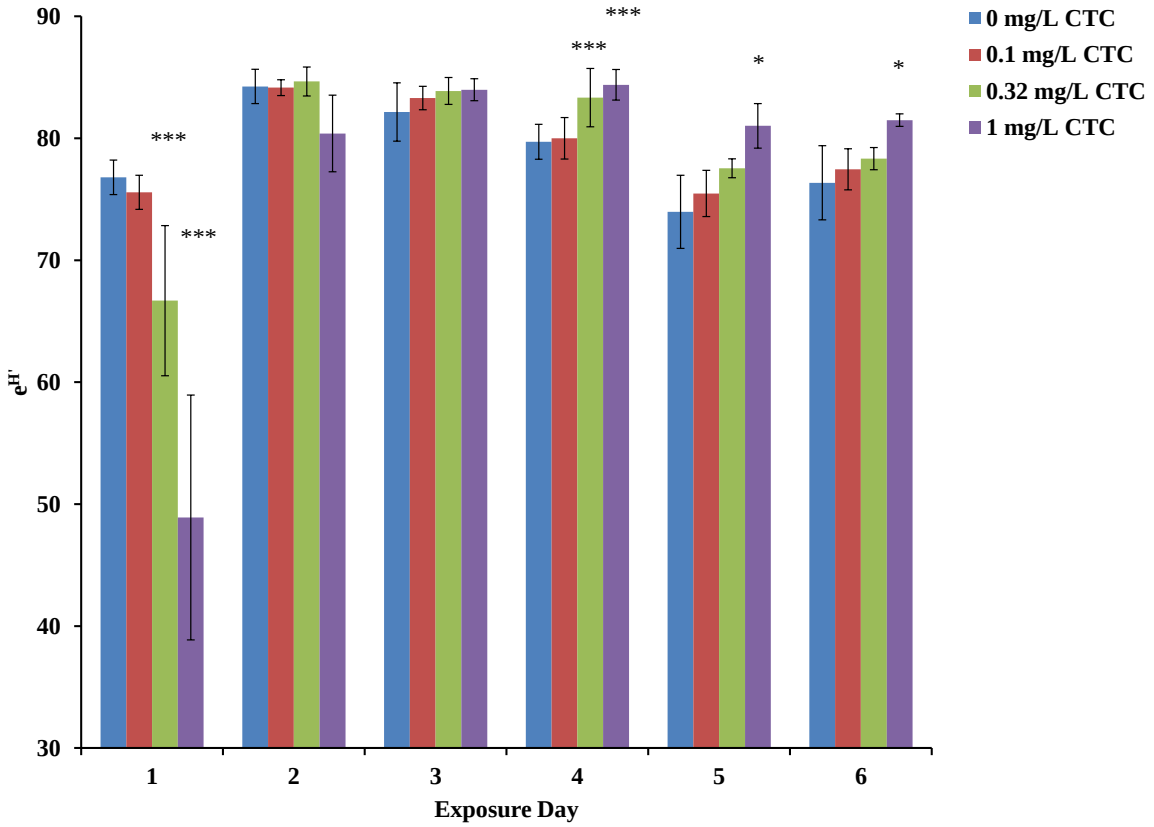


Figure 3.20: Bar graph showing values of the Shannon-Weaver Diversity Index ($e^{H'}$) on each day of the exposure period for each CTC treatment group. Statistical significance is indicated by asterisk notation (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

3.4. Discussion

3.4.1. Effects of CTC on Total Organic Substrate Utilisation

The microcosm/Biolog GN2 phenotype microarray assay-system (which was developed by work described in Chapter 2) was used to explore the potential effects of a candidate tetracycline antibiotic (CTC) on aquatic microbial communities. It was first tested if any inhibition or stimulation of total organic substrate utilisation could be detected in terms of a general trend and by analysis of lag phase duration (following onset of substrate utilisation) and the maximum rate of total substrate utilisation. Data transformed using a 3-parameter kinetic model suggested that an inhibition of total substrate utilisation had occurred on exposure day 1 at all CTC concentrations. Statistical analysis of lag phase duration before onset of total substrate utilisation revealed a significant increase in lag phase duration compared with the control lag phase duration at all CTC doses. A significant decrease in the maximum rate of total substrate utilisation was also observed as a result of exposing microcosms to 0.32 and 1 mg/L CTC.

It is likely that CTC was taken up by bacterial cells rapidly. McMurry *et al*. (1981) for example showed that *E. coli* cells had accumulated 9.5 mg/L tetracycline in just 5 minutes following exposure. It is known that once tetracyclines enter microbial cells they are accumulated (Chopra, 1985). Subsequently, increasing intracellular tetracycline levels exert an antimicrobial effect. The main mode of action of tetracyclines is the inhibition of protein synthesis at the ribosomal level (Tritton, 1977; Gale *et al*., 1981; Chopra, 1985; Rasmussen *et al*., 1991). The binding of prokaryotic tRNA to the 70S ribosome is reduced to around 20% of normal levels (Epe *et al*., 1987). Using cell-free extracts, Holmes and Wild (1967) demonstrated that CTC can exert this effect within 2 hours of exposure to CTC. As a result, it probably took longer for members (or some members) of the microbial community present in microcosms and Biolog GN2 plates to synthesise essential proteins (such as enzymes and transporter proteins) that would have been required to efficiently utilise a range of organic substrates. This most likely explains why an increase in lag phase duration and a reduction in the max rate of substrate utilistion were observed on day 1 at all CTC concentrations. In addition, due to the bacteriostatic effect of CTC, it is possible that the growth of at least a portion of the microbial community present in treated microcosms was inhibited. As a result, potentially fewer bacteria were present in microcosms. CFU data did not show a significant difference in cell numbers (compared with control data) that would have supported this theory however. CFU count data do in fact suggest that no significant changes in cell density occurred as the result of exposing the microbial community to any CTC dose. The possibility that CTC was exerting a growth-independent effect cannot be ruled out therefore. One should bear in mind though that culturing techniques may not be able to detect the majority of the total microbial community; this is due to “the great plate count anomaly.” Accordingly, it has been estimated that less than 1% of the total microbial community can be cultured on (or in) synthetic and semi-synthetic media (Jannasch and Jones, 1959; Staley and Konopka, 1985). As such, there is

a great deal of uncertainty as to whether CFU counts truly represent what is occurring on a whole community level. Also, in 1982 the concept of "viable but non-culturable bacteria" (VBNC) was proposed, whereby microbial cells may be metabolically active but do not reproduce; this portion of the microbial community is not detectable by traditional cell culture techniques (Bogosian and Bourneuf, 2001). A suggestion for future studies therefore would be to use a range of techniques capable detecting distinct portions of the microbial community, such as molecular based techniques (such as detection of total 16S RNA).

It should also be noted that R2A media and the media that is present in Biolog GN2 plate wells (modified LB, or lysogeny broth media) are likely to target different portions of the microbial community. R2A media has a low nutrient formulation which targets slower growing oligotrophic bacteria that are likely to occupy a niche which has a low nutrient level (such as surface waters). Such bacteria generally display relatively slow growth rates. Although Biolog have not published the exact constituents of their growth media, they have revealed that it is a modified LB Medium (Biolog, 2007). LB medium is much richer in nutrients than R2A agar. As a result of this, LB medium is likely to promote the growth of faster growing bacteria which are adapted to exploit more nutrient rich niches. This has also been suggested experimentally by work that has analysed Biolog plates by molecular techniques (Smalla *et al*., 1998). It is therefore unclear whether CFU counts obtained in the current investigation equate to cell densities that may be found in individual Biolog GN2 wells. Future experiments could be designed to address these issues. Firstly, plates from which CFU counts are calculated could contain the same growth medium as is present in Biolog GN2 plates. Both media would therefore be targeting the same (or more similar) portions of the microbial community. A sub-experiment could compare the difference between CFU counts that were obtained by inoculation of both R2A and LB media to see whether R2A media could be used to estimate cell densities in Biolog plates. The type of isolates that were cultured by both methods could also be compared by a molecular technique such as DNA sequencing. Alternatively, Biolog plates could be recreated using R2A broth and commercially available empty (and sterilised) microplates. If this method was tested then all bacteria would be growing on the same substrate. Alternatively, microbial cell densities in both microcosms and Biolog plates could be enumerated using an alternative method, such as measuring total DNA present a given sample (of equal volume).

This also leads one to question of whether R2A plate CFU counts can be used to form the assumption that total cell densities in the inoculum that was used to seed Biolog plates throughout the exposure period were equal. Addressing this issue would be of interest. In particular, if inoculum cell densities are not normalised, then any functional effects that are seen using Biolog data might just be an artefact of there being more or less bacteria present (in individual Biolog GN2 plate wells) that are able to utilise a particular substrate. On the other hand, one could also argue that differences in cell numbers due to exposure (of a microbial community) to a toxicant would

inherently result in differential substrate utilisation between doses, so substrate utlilisation might be expected to be different because of this toxicant-induced reduction in growth. Selecting a method would therefore depend on whether one wished to look at purely growth-independent functional effects or whether one wanted growth-dependent effects to be considered also.

Due to the fact that Biolog plates utilise high nutrient modified LB as a growth media, it is likely that the functional activity of faster growing bacteria was detected during the current study. In addition, such bacteria are also likely to be more abundant at the start of the exposure period as nutrients will be relatively more available compared with a later time point, when a large proportion of nutrients have been consumed by the microbial community (or a portion of the microbial community). It is also worth mentioning that the effect of CTC on slower growing oligotrophic bacteria may not be detected by the current system due to the fact that they may not grow quickly enough to allow their substrate utilisation to be measured by the Biolog GN2 assay. In addition, an exposure period of 7 days may not represent an adequate time frame during which such organisms can be studied. The results presented in the current investigation are likely to better detect relatively acute effects of antibiotics. If future studies wanted to study more relatively chronic effects then a microcosm and assay system that was deigned to be used for a longer time period would have to be employed. When devising future experiments however, one must also bear in mind that previous investigations which have looked at the effects of bacteriostatic antibiotics have concluded that inhibitory effects can only be observed when a relatively high nutrient load is added to a system (Schmitt *et al*., 2005). The design of any future study needs careful consideration of these points.

In addition to kinetic effects, CTC doses of 0.32 and 1 mg/L caused a statistically significant reduction in the functional diversity of substrates that were being utilised by the microbial community (or portion of the microbial community) that were present in corresponding microcosms. It is possible that the growth or physiological function of certain members of the microbial community was being inhibited by the upper two CTC doses. In such a scenario, it is possible that certain members of the inhibited microbial community (present in microcosms spiked with 0.32 and 1`mg/L CTC) had their growth rates reduced to the point that their contribution to the utilisation of certain substrates was not detectable on Biolog GN2 plates. In such a scenario, it is possible that uninhibited members of the microbial community (for example members of the microbial community that were expressing CTC resistance mechanisms) could not exploit the same range of substrates as those that were inhibited (for example bacteria that were present in microcosms spiked with 0 and 0.1 mg/L CTC). It is also possible that certain members of the microbial community in affected microcosms were not able to synthesise the same range of cellular proteins, especially catabolic enzymes, which would have been required to efficiently utilise a more broad range of substrates. This would have occurred due to the inhibition of protein synthesis by CTC. Kong *et al*. (2006) investigated the effect of exposing a soil microbial community to

oxytetracycline at similar concentrations to the present investigation. They observed a general decrease in the diversity of Biolog substrates that could be utilised by the microbial community (or at least a portion of the microbial community). The results are however in general agreement with the current study, suggesting that CTC can have a negative impact on the range of substrates that soil and aquatic microbial communities are able to utilise.

PCA analysis of functional data revealed changes in the pattern of total substrate utilisation at the highest two CTC doses (0.32 and 1 mg/L). This would suggest that the microbial community structure was different in microcosms that had been exposed to these CTC doses. Moreover, these effects could still be observed after effects on kinetic and functional diversity of substrate utilisation could be seen. In fact, changes in the microbial community structure were observed on all days of the exposure period. There are several possibilities why this could be occurring. Firstly, it is likely that previously inhibited bacteria will be able to grow when the system is in recovery. This could be because CTC has been degraded or such bacteria have developed a degree of resistance to CTC. It is also known that the bacteriostatic effect of CTC is transient, meaning that the effect of CTC will eventually stop (Chopra, 1985). There are no prior studies indicating the time-frame this may occur within in the context of aquatic microbial communities however. As time progresses, bacteria with different growth rates could be thriving at different time points compared to when they would have done in an unexposed system. Or indeed resistant members of the microbial community may be exploiting nutrients within the system. As members of the dosed (with 0.32 and 1 mg/L CTC) community appear at different times, their pattern of substrate utilisation is likely to be different. Alternatively, if resistant bacteria are present in dosed but not unexposed microcosms, they could also exhibit a different pattern of substrate utilisation. It could also be the case that the same resistant and susceptible bacteria are present in both exposed and unexposed systems, but in exposed microcosms the resistant community members are more able to compete for the available resources. In such a scenario, substrate utilisation patterns (and subsequent microbial community structures) could also be different in affected and unaffected systems.

Although patterns of substrate utilisation suggest that differences in community structure have occurred between dose groups, future work should validate these findings using molecular approaches such as PCR-DGGE. Other investigations have used this approach to good effect previously. Nelson *et al*. (2011) for example analysed V3 regions of bacterial 16S rRNA (ribosomal RNA) which was extracted from soil microbial communities that had been exposed to CTC.

Day 1 results are in agreement with other studies that have tracked the effects of tetracyclines on total substrate utilisation. Kong *et al*. (2006) observed a significant reduction in total substrate utilisation by soil microbial communities as a result of exposure to oxytetracycline. A lowest oxytetracycline concentration of 0.46 mg/L was found to significantly reduce the maximum colour

development that was formed in Biolog GN2 plates over time. The authors also reported an increased onset of colour production. Although this was not numerically or statistically quantified the observation is in agreement with the data that were obtained during this study. Stone *et al*. (2011) observed that aged manure application which contained 11.6 mg/L CTC reduced overall substrate utilisation by soil microbial communities at certain depths in soil columns (30 cm).

Kong *et al*. (2006) used PCA score plots of the first two principal components to look at the clustering of different oxytetracycline exposure groups compared with an unexposed control group. All oxytetracycline exposure groups clustered independently from the control group, with the top three oxytetracycline concentrations forming a separate cluster. In the present study separation of the two highest CTC concentrations was observed. The data in both studies therefore illustrate changes in total substrate utilisation profiles by environmental microbes a result of exposure to tetracycline antibiotics. In another investigation, Stone *et al*. (2011) found that manure containing aged antibiotic residues (tylosin and CTC) caused changes in substrate utilisation patterns at different soil depths (10 and 30 cm). The authors speculated that exposure of the soil microbial community to antibiotics had caused a shift in the structure of the microbial community. Other studies have also investigated changes in microbial community as indicated by substrate utilisation profiles. Maul *et al*. (2006) exposed leaf-bound microbial communities to the antibiotic ciprofloxacin. A significant variation in PC1 (principal component 1) and PC2 (principal component 2) scores were observed at a dose of 0.1 mg/L ciprofloxacin, suggesting that a change in total carbon utilisation profiles occurred compared with controls. Schmitt *et al*. (2005) reported a shift in PCA scores on the second axis, suggesting a change in the total substrate utilisation profile as result of exposing soil microbial extracts to the antibiotic sulfachloropyridazine. The results of these and the present study suggest that a range of antibiotics can affect the metabolic physiology of microbial communities in a range of environments.

The inhibitory effects of CTC that were observed the on the max rate of total substrate utilisation and lag phase duration were also observed on day 2 of the exposure period at the highest CTC concentration (1 mg/L). It is likely the same reasons that were mentioned previously were responsible for these prolonged effects. There was no effect on either lag phase duration or the max rate of substrate utilisation as result of exposure to 0.1 and 0.32 mg/L CTC on day 2. These data suggest that the microbial community in microcosms exposed to CTC concentrations below 1 mg/L were recovering from the inhibitory effects of intoxication. By day 3 of the exposure period no inhibitory effects were being observed at the top CTC exposure concentration in terms of either increased lag phase durations or a reduction in the max rate of total substrate utilisation. These data suggest that microcosms exposed to 1 mg/L were also recovering by day 3. By day 4 lag phases for all CTC treatments were lower than control values, suggesting microbial function had actually been stimulated. This could potentially be explained by less toxic waste products being present (such as ammonia) and more nutrients (essential for microbial growth and cellular function) being available

due to the previously reduced uptake and metabolism of macromolecules, salts etc. due to CTC intoxication. This could have potentially occurred due to the fact that inhibition of the microbial community (or a portion of the microbial community) may have resulted in less metabolism of nutrients within the microcosm and therefore less potentially toxic by-products (such as ammonia) of metabolism being formed. Alternatively, previously inhibited bacteria may have been growing and exploiting nutrients now that they had potentially recovered from the toxic effects of CTC. As a result, it may have taken longer for these community members to become abundant enough to have a measurable kinetic impact on substrate utilisation. Also, some members of the microbial community could have formed resistance (by horizontal gene transfer, for example) earlier in the exposure due and were now present in sufficient numbers to utilise a measurable level of substrates.

The recovery of the system was also mirrored by diversity index data, with no inhibition of the functional diversity of substrate utilisation being witnessed after day 1. On days 2 and 3 of the exposure period the functional diversity of substrate utilisation was similar at all CTC doses before an increase could be observed later on in the exposure period (on day 4 at 0.32 and 1 mg/L CTC). These data provide evidence that the portion of the microbial community that was being investigated was able to utilise a greater number of substrates during the recovery period. This could have been caused by previously inhibited members of the microbial community being able to exploit a greater number of substrates as they recovered from the effects of CTC intoxication. Such bacteria may have already thrived then died in exposure scenarios during which their growth or physiological functioning was not inhibited, in control microcosms for example. It is possible that no other member (or members) of the microbial community could utilise the same range of substrates once such members of the community had died. As such, the utilisation of a reduced range of substrates would have lowered the value of the diversity index.

The recovery of the microbial community observed from day 2 onwards can possibly be attributed to one or more mechanisms of antibiotic resistance being expressed by a portion of the microbial community. A number of intrinsic, mutational and acquired mechanisms of resistance exist which result in aquatic microbes being protected from the toxic effects of tetracyclines (and other antibiotics). This is usually dependent on bacteria possessing one or more resistance genotypes. Tetracycline mechanisms of resistance include drug efflux, ribosomal protection and biochemical modification of tetracycline molecules (Speer *et al*., 1992; Davies, 2007). Members of the microbial community could potentially be able to thrive in an environment containing CTC if they possessed one or more of these resistant phenotypes. This leads to the possibility that a resistant member (or resistant members) of the microbial community that would normally be out-competed (during a non-exposed scenario) by a susceptible member could start to compete for the resources that the inhibited member cannot now utilise. In such a scenario, it is possible that the cell density

within a microcosm system would not necessarily change. In such a scenario, CFU data from the current investigation would support such a theory.

In addition, the possibility for microbes to pass resistance genes to other members of the microbial community that did not previously possess copies of that specific gene has been reported (e.g.: Droge *et al*., 1999). If members of the microbial community are becoming resistant at the start of the exposure period, it is likely that the faster growing members of the microbial community (which are able to utilise the relatively large abundance and diversity of nutrients that are present nearer the beginning of the exposure period) are forming resistance. If tetracycline resistance was spreading throughout the microbial community (or at least a portion of it) then it is highly likely that at least one member of the community possessed a resistant genotype at the start of the exposure. Chapter 5 will address the issue of antibiotic resistance in more depth.

It is also possible that the parent compound (CTC) is being degraded. It is difficult to evaluate this due to the conflicting nature of the results that have been generated by previous studies. For example, Verma *et al*. (2007) have reported a degradation half-life of CTC of between 13-18 days in the aquatic environment. Sanderson *et al*. (2005) on the other hand reported a shorter degradation half-life of 1-4 days. In the scenario that the shorter half-life is correct, then the recovery of the microbial community may have been at least in part facilitated by the dissipation of CTC from the microcosm system. The likelihood of this occurring is reduced somewhat in the scenario that a longer degradation half-life was required to remove CTC from microcosm. Tetracycline degradation would likely have been more rapid in the presence of light (Verma *et al*., 2007), but the current investigation was conducted under dark conditions. During the course of the current study, time constraints did not allow for CTC analysis. As the merits of such an analysis are clear, future investigation should allow for this. In any case, it is noteworthy that at least two of the reported tetracycline mechanisms of resistance correspond to the biochemical alteration of the CTC molecule, which would result in biodegradation or biotransformation of the tetracycline molecule. *Tet* X for example codes for a flavoprotein that catalyses the monohydroxylation of the Tetracycline-mg^{2+} binding domain. After several hours the tetracycline molecule degrades into several degradation products (Wright, 2005). The enzymatic activity of *tet* (37) is very similar to *tet* (X) in that a monohydroxylation reaction is catalysed which is NADPH-dependent (Diaz-Torres *et al*., 2003).

Despite the fact that total substrate utilisation had recovered by day 2 in terms of functional diversity and day 3 in terms of the lag phase duration and max rate of substrate utilisation, a reduction in the max rate of substrate utilisation was observed at 1 mg/L CTC on day 6 of the exposure. A possible explanation for this could be that a toxic metabolite of CTC was exerting a toxic effect upon (at the very least) a portion of the microbial community. A number of CTC metabolites (5a, 6-anhydrochlortetracycline, isochlortetracycline, *N*-desmethylchlortetracycline,

apochlortetracycline, apo-epichlortetracycline, or possible a 64 combinations of keto and enol tautomers) could have been formed by the biochemical action of certain members of the microbial community Halling-Sorensen (2002) investigated the toxicity of tetracycline degradation products on single species (*Pseudomonas*, *Agrobacterium sp.*, *Moxella sp.* and *Bacillus sp.*) growth tests. Although *Pseudomonas* was not very sensitive to tetracycline degradation products (MIC_{50} values ranged from 0.25-32 mg/L), it was found that soil isolates were more sensitive, with MIC_{50} values ranging from 0.25 mg/L). Halling-Sorensen *et al.* (2002) also observed inhibition of CFU counts of activated sludge microorganisms as a result of exposure to tetracycline degradation products. At the lower end of the results scale an EC_{50} value of 0.03 mg/L were observed as a result of exposure to 5a, 6-anhydrotetracycline hydrochloride, an identical value to that obtained as a result of exposure to CTC. Given the low toxicity values of tetracycline degradation products (which are in the same order of magnitude of effects that have been observed in the present investigation) it is possible that these could be exerting a toxic effect on day 6 of the exposure.

Tetracyclines are the most used veterinary antibiotics in the EU (Kools *et al.*, 2008). They can enter the aquatic environment via a number of routes, such as surface run-off and aquaculture chemotherapy effluent (Halling-Sorensen *et al*., 1998; Yang and Carlson, 2003). Consequently, the occurrence of tetracyclines in the aquatic environment has been reported in several publications (eg: Koplin *et al*., 2002). The present study has shown the inhibitory effects of CTC on the ability of aquatic microbes to utilise an average of 95 distinct substrates. Jankhe and Craven (1995) reported the ability of aquatic microorganisms to utilise a broad range of molecules contained within DOC (dissolved organic carbon). DOC has been reported to contain a wide range of organic molecules. Among the most prevalent of these are carbohydrates, carboxylic acids, amines and amino acids (McDowell and Likens, 1988). DOC has been shown to contain up to 50% humic substances, highlighting the importance of carboxylated DOC constituents. Humic DOC components have been shown to be important for bacterial production in the aquatic environment (Moran and Hodson, 1990). Biolog GN2 plates also contain carbohydrates, carboxylic acids, amino acids, amines and amides, all of which have been shown to be important constituents of DOC in the aquatic environment.

The results of the present study therefore suggest that the presence of CTC may inhibit the ability of aquatic microbes to utilise and degrade this broad range of molecules contained within DOC. Tranvik and Hofle (1987) commented on the high efficiency with which microbes normally achieve this. If CTC inhibits the ability of aquatic microbes to utilise DOC then this could result in a reduced microbial biomass in aquatic environments. Although this may potentially increase oxygen levels in the aquatic environment (potentially making more oxygen available to aerobic organisms), the absence of certain bacteria may also result in key processes within nutrient cycles not being performed as efficiently. For example, a reduction in key nitrifying, denitrifying and xenobiotic-degrading bacteria may have adverse effects on nitrogen cycling and on the removal of

pollutants from the aquatic environment. In addition to this, bacteria provide a key food source for aquatic organisms such as heterotrophic flagellates and protozoa. In turn, these organisms are preyed upon and so on until energy is ultimately transferred to an apex predator (an organism that has no predator of its own). A reduction in bacterial numbers may therefore affect the normal transfer of energy through aquatic food chains. Blomqvist *et al*. (2001) demonstrated the importance of DOC in terms of its conversion into microbial biomass. This also means that, via the incorporation of DOC into bacterial biomass, aquatic bacteria act as important carbon sink. Carbon sinks play an important role in carbon sequestration from the atmosphere (removal or reduction in carbon dioxide levels). As such, bacterial biomass plays an important biogeochemical role in regulating the earth's climate.

The degradation of a wide array of autochthonous and allochtonous organic matter (such as humus, undigested food and dead animal material) in the aquatic environment requires microbial communities to be efficient organic multi substrate utilisers. Within the carbon cycle, bacterial breakdown of DOC can be liberated back into the environment via the release of carbon dioxide, which is commonly regarded as being the most critical greenhouse gas on the planet (Siegenthaler and Sarmiento, 1993). DOC degradation therefore plays an important role in releasing gasses which regulate the planet's temperature over time (Berner, 1990). Other inorganic nutrients (such as nitrates and phosphates) are also liberated during microbial degradation of DOC. These may be taken up as nutrients by, for example, aquatic plants, or they may enter another nutrient cycle (such as the nitrogen cycle) for further processing. Any effect on DOC degradation in the aquatic environment therefore has the potential to interfere with climate and nutrient cycling.

Previous studies have shown that oxytetracycline inhibits the ability of bacteria to break down the broad range of volatile organic substances in manure. Biogass production was therefore reduced. Inhibition of biogass production (both aerobic and anaerobic) as a result of exposing microbial communities to oxytetracycline ranged from 27-50% across three studies (Sankvist, 1984; Gamal-El-Din, 1986; Arikan Rital *et al*., 2006). In one study, methane production was inhibited for six consecutive days, although oxytetracycline was doesd at a high rate (100 mg/L) (Sankvist *et al*., 1984). Landi *et al*. (1993) also reported a significant decrease in soil respiration rates as a result of exposure to streptomycin. As the substrate being respired was SOM (soil organic matter), this pointed to the inhibition of multisubstrate utilisation by soil microbial communities. The inhibition of organic substrate utilisation by different antibiotics has therefore been reported in both the aquatic and terrestrial environment. It should be pointed out however that the exposure concentrations were higher during these studies (the minium exposure was 9.8 mg/kg).

The current investigation has taken the approach of looking at how aquatic environmental bacteria utilise component parts of DOC (substrates from each Biolog guild) in the presence of a toxicant, rather than looking at how environmental bacteria may metabolise a mixture of often more

complex molecules. As such, the current investigation has concentrated on looking at how the component parts of organic matter may be metabolised by the microbial community in the aquatic environment in the presence of an antimicrobial toxicant. A possible line of future investigation therefore may be to investigate how CTC affects the degradation/utilisation of a range of more complex molecules that may be found in the environment.

Microbial consortia are also responsible for the degradation (and therefore utilisation) of a wide range of organic substrates in the aquatic environment (Pelz, 1999; Mishra, 2001). The tendency for CTC to inhibit multi substrate utilisation could also have implications for the degradation of anthropogenic organic compounds. The potential for this to occur has been previously observed in soil studies. Chun *et al*. (2005) observed that CTC (as well as sulfamethazine and tylosin) reduced the ability of a soil microbial community to degrade the synthetic hormone 17 β-estradiol into its transformation product estrone. In another study conducted in soil, Allen and Walker (1987) reported that the degradation of three pesticides was inhibited by the antibiotic novobiocin. Montiero *et al*. (2009) have also reported the inhibition of naproxen biodegradation as the result of exposing microbial communities to the veterinary antibiotic tylosin. Based on the results of these studies, it is possible that CTC could inhibit the degradation of xenobiotic compounds by microbial communities (or portions of microbial communities).

3.4.2. Effect of CTC on Amine and Amide Utilisation

Although day 1 kinetic plots (figure 3.8a) would suggest an inhibition of amine and amide utilisation across all CTC exposure concentrations, the only statistically significant inhibitory effect was observed as result of microcosms being exposed to 0.1 mg/L (tables 3.5 and 3.6). It is likely that statistical significance was not observed in the case of max rate and lag phase duration due to the high standard deviation (and therefore variation) among replicate Biolog plates. Future studies of this type that incorporated a larger number of samples (to increase *n*) would reduce inter sample variability and increase the likelihood of gaining a higher degree of statistical significance.

A significant increase in lag phase duration was however observed on day 2 of the exposure period in microcosms exposed to 1 mg/L CTC (table 3.5). A significant decrease in the max rate of amine and amide utilisation was also observed in microcosms exposed to 0.32 mg/L CTC (table 3.6). As with total substrate utilisation, the reasons behind these inhibitory effects of CTC are likely to result from the compounds mode of action (as discussed in the previous section).

PCA analysis (figures 3.5a and 3.5b) of amine and amide utilisation revealed that a similar profile was shared by control/0.1 mg/L and 0.32/1 mg/L microcosm replicates on day 1. A similar trend was observed on day 4 suggesting amine and amide utilisation followed a similar profile. These results suggest that exposure to CTC alters amine and amide utilisation profiles even after a recovery had been detected by data from other end-points. It is likely that the action of CTC (or its metabolites and transformation products) is having an effect on the structure of the microbial

community which are responsible for the degradation of amines and amides in the aquatic environment.

Amines and amides enter the nitrogen cycle where bacteria convert them into nitrite and nitrate via the processes of ammonification and nitrification (Paul and Clark, 1996). CTC concentrations of 0.32 and 1 mg/L are therefore causing changes in the microbial community structure of aquatic bacteria that are responsible for these processes. This would explain a change in the metabolic fingerprint that was expressed by exposed (to CTC) microbial communities. This would have occurred because different microbial communities are likely to possess differential metabolic systems. Indeed, the Biolog GN2 system was originally designed to differentiate microbial species and strains based on differential substrate metabolism (Preston-Mafham *et al*., 2002). To confirm these changes in the community structure of amine and amide utilisation bacteria, future studies should apply a molecular technique such as PCR-DGGE. This would confirm any changes in the community structure of amine and amide utilisers within the nitrogen cycle. Furthermore, sequencing on DGGE bands would reveal more about which particular bacteria were present in dosed and control (unexposed) communities. One could therefore make some conclusions on which biogeochemical mechanisms were present or missing from each community.

After day 2 of the exposure period there were no statistically significant inhibitory effects on amine and amide utilisation observed with the exception of a significant reduction in max rate of amine utilisation on day 6 as the result of exposure of microcosm to 1mg/L CTC. The general recovery associated with amine and amide utilisation could be explained by the emergence of members of the microbial community that possess resistant phenotypes. A possible reason for day 6 inhibition of max rate of amine and amide utilisation could be the toxicity of one or more degradation product of CTC. Halling-Sorensen (2002) investigated the toxicity of CTC degradation products on single species (*Pseudomonas*, *Agrobacterium sp.*, *Moxella sp.* and *Bacillus sp.*) growth tests. Although *Pseudomonas* was not very sensitive to CTC degradation products (MIC_{50} values ranged from 0.25-32 mg/L), it was found that soil isolates were more sensitive, with MIC_{50} values ranging from 0.25 mg/L). Halling-Sorensen *et al.* (2002) also observed inhibition of CFU counts of activated sludge microorganisms as a result of exposure to tetracycline degradation products. At the lower end of the results scale EC_{50} values of 0.03 mg/L were observed as a result of exposure to 5a, 6-anhydrotetracycline hydrochloride, an identical value to that obtained as a result of exposure to CTC. It is possible that the significant reduction of the max rate of amine and amide utilisation was caused by a toxic degradation or transformation product of CTC considering their potential to exert toxicity upon environmental microorganisms at similar (and lower) concentrations to those that were tested in the current study.

Kong *et al.* (2006) also observed a decrease in amine and amide utilisation (quantified by maximum colour development) as a result of exposing Biolog GN2 plates (inoculated with soil extract) to different oxytetracycline concentrations. At the highest concentration amine and amide

utilisation was almost completely inhibited, although significant effects were observed between 1 and 5μM. CTC concentrations of between 2.8 and 28 μM were used in the present study. Effects of tetracycline were therefore observed at similar exposure concentrations in both studies.

Amines and amides can be utilised in a number of ways by environmental bacteria. They may enter the nitrogen cycle where they are first subject to the process of ammonification. When ammonia has been produced via this reaction it is then subject to further biogeochemical processing in the process of nitrification, whereby nitrite and nitrate are formed. Nitrogen in these forms can be utilised by bacteria (Paul and Clark, 1996). Amines and amides can therefore act as an important nitrogen source for environmental microbes.

Inhibition of amine and amide utilisation as a result of CTC exposure could indicate a potential inhibitory effect on the ammonification and nitrification in the aquatic environment. Previous work has detected inhibitory effects of specific aspects of the nitrogen cycle as a result of exposure to tetracyclines. Halling Sorensen *et al.* (2000) detected inhibition of the growth of the aquatic nitrifying cyanobacterial species *Microcystis aerugenosa* as a result of exposure to CTC and tetracycline. In the case of both compounds an EC_{50} value of < 0.1 mg/L was observed. Halling Sorensen (2000) also detected significant inhibition of nitrification as a result of exposing activated sludge communities to CTC, oxytetracycline and tetracycline. In the same study, tetracyclines were found to inhibit the growth of cyanobacteria, with EC_{50} values of between 0.002 and 0.5 mg/L being observed. The data in these studies provide a link between the inhibition of nitrogen utilisation and exposure to tetracyclines. The findings of this study therefore provide support for some of the results of the current investigation.

Another study (Klaver and Mathews, 1994) detected almost complete inhibition of nitrification in aquaria experiments as a result of exposure to oxytetracycline, observing EC_{50} values of between 8.6 and 29 mg/L. Although the concentration of tetracycline used was much higher than in the current study potential effects of tetracyclines on nitrification were still observed.

3.4.3. Effects of CTC on Amino Acid Utilisation

Amino utilisation lag phase was found to be significantly inhibited by exposure to 0.32 and 1 mg/L CTC on exposure day 1. The max rate of amino acid utilisation was significantly reduced as the result of exposure of microbial communities to 0.1 mg/L CTC on day 1 and by all CTC concentrations on day 2 (figure 3.4; tables 3.7 and 3.8). The high variation between samples may again explain why a statistical significance was not observed in microcosms exposed to higher CTC concentrations. There are various reasons that can explain the reduced rate and increased lag phase duration observed on days 1 and 2. Firstly, amino acids can be utilised as a nitrogen-based energy source by heterotrophic environmental microbes in the aquatic environment (Flores and Herrroro, 1994). For them to achieve this they must process amino acids via ammonification and nitrification reactions. Due to the inhibition of protein synthesis (as a result of CTC exposure)

production of enzymes (such as nitrogenases and amino acid dehydrogenases) that are required for these processes may be inhibited and the utilisation of nitrogenous compounds such as amino acids may be inhibited. The inhibition of an important stage in amino acid utilisation in the nitrogen cycle, deamination, as the result of exposure of chick ruminal microbes to CTC has been previously observed (Broderick and Balthrop, 1979). It is therefore a possibility that the same process is occurring in aquatic microbial communities that are likely to share some enzymatic homology with chick ruminal bacteria. In terms of effects on the nitrogen cycle, similar effects could be potentially observed as described for amine and amide utilisation.

Secondly, amino acid uptake may be reduced due to the fact that CTC has inhibited amino acid (in the form of aminoacyl tRNA) binding to the 70S ribosomal subunit (Epe *et al.*, 1987) and amino acids will therefore not have an intracellular binding target. Wheeler and Kirchman (1986) had previously reported a > 58% decrease in amino acid uptake as a result of exposing marine bacteria to the protein synthesis inhibitor chloramphenicol. Verma *et al.* (2007) observed a significant reduction in leucine incorporation rates by river water microbial communities as result of exposure to 5 μg/L tetracycline (compared with a lower CTC exposure concentration of 100 μg/L during the present study). In another study, Kong *et al.* (2006) observed a significant reduction in amino acid utilisation as the result of exposing soil microbial communities to oxytetracycline. The concentrations range they used was in the same order of magnitude as was tested in the present study. These results are in agreement with the current investigation, with results from other studies suggesting that antibiotics that inhibit protein synthesis may also inhibit amino acid uptake. Although a significant reduction in the max rate of amino acid utilisation was observed on days 1 and 2, the lag phase duration on day 2 of the exposure period was significantly lower than control values at all levels of CTC exposure (table 3.7). The reduction was greatest at a CTC concentration of 1mg/L, followed by 0.32 mg/L then 0.1 mg/L. In the case of 1 mg/L CTC exposure, no lag phase was recorded at all. In terms of lag phase duration, the recovery observed in the case of amino acids was extremely fast compared with the recovery that was observed for other substrate guilds. It would benefit the microbial community if they were able to recover amino acid utilisation as quickly as possible, as they are required to synthesise essential proteins such as enzymes that are necessary to utilise other substrate guilds.

The formation of resistant members of the microbial community being able to utilise amino acids may explain the reduction in lag phase duration. The short onset of amino acid utilisation could also be explained by a rapid uptake of amino acids that were required to bind with intracellular tRNA. Holmes and Wild (1967) observed an initial increase in RNA synthesis during *E. coli* exposure to CTC. In addition, this RNA was stable and stored intracellularly. During the recovery period following CTC-mediated inhibition of protein synthesis, RNA remained stable and ribosomes were synthesised. It is this increase in RNA and ribosomal synthesis that may explain the reduced lag phase during the recovery period following the inhibition of protein synthesis.

A significant reduction in the max rate of amino acid utilisation however would suggest that a certain amount of inhibition was still occurring. This may be reflect the fact that only a certain portion of the microbial community can tolerate the inhibitory effects of CTC and some of the community was still inhibited. As was seen in the case of amine and amide synthesis, there was also a significant reduction in the max rate of amino acid utilisation at the highest CTC concentration (1 mg/L) on day 6 of the exposure period (table 3.8). Again, this could possibly be explained by the potential toxicity of CTC metabolites or transformation products (Halling-Sorensen *et al*., 2002).

PCA analysis of amino acid utilisation profiles on day 1 (figure 3.5a) revealed that control/0.1 mg/L and 0.32/1 mg/L microcosm replicates shared a similar profile as suggested by the presence of two distinct clusters on PCA score plots. A similar trend was observed on day 4 (figure 3.5b), suggesting that amino acid utilisation followed a similar profile. The results suggest that exposure of microbial communities to 0.32 and 1 mg/L CTC alters amino acid utilisation profiles even after a recovery has been detected by data from other end-points. It is likely however that PCA data in the present study is representative of the action of CTC (or its metabolites and transformation products) having an effect on the structure of the microbial community due to the effect it is having on the metabolic profiles of the microbial community. In particular, the portion of the microbial community that is likely being affected is certain bacterial species that are involved in nitrogen cycling. Broderick and Balthrop (1979) have previously shown that CTC can interfere with deamination of amino acids in chick ruminal bacteria. Due to the presence of conserved biochemical mechanisms between different bacteria, it is possible that certain portions of the microbial which perform deamination reactions are also being affected in aquatic microcosms and therefore the aquatic environment in general. From the data provided in this study, it is not clear which particular bacteria are present in microbial communities from each dose group. The extent to which discrete biogeocheical processes were being performed by distinct microbial communites (within different microcosms) is therefore not known. Future studies should therefore utilise molecular techniques to confirm community shifts (eg: PCR-DGGE) and whole genome sequencing to establish the identity and biogeochemical functioning of community members.

3.4.4. Effects of CTC on Carbohydrate Utilisation

Carbohydrate utilisation was significantly inhibited on day 1 in terms of an increase in lag phase duration at all CTC exposure concentrations (Table 3.9). There was also a significant decrease in the max rate of carbohydrate utilisation on day 1 in microcosms exposed to 0.32 and 1 mg/L CTC. A significant reduction in max rate of carbohydrates was also observed on day 2 in microcosms exposed to 0.1 mg/L CTC (Table 3.10). These inhibitory effects can be explained by the mode of action of CTC in terms of the inhibition of protein synthesis. Many carbohydrates require transporter proteins to enter the cytoplasm of microbial cells. Examples of such proteins are the ABC carbohydrate uptake transporters family 1 (MalF and MalG) and carbohydrate uptake

transporters family 2 (RbsC and RbsD). It is therefore possible that inhibition of the synthesis of these proteins is caused by exposure of the microbial community to CTC. Certain carbohydrates could therefore not enter the cytoplasm of microbial cells. It is also a possibility that the synthesis of oxidative enzymes that are responsible for the transfer of electrons from carbohydrates to ADP (to from ATP) as an energy liberating system are being inhibited by CTC. Examples of these enzymes include α-galactosidases, glucoamylase and lactase.

There has been a body of work that has been conducted into the effects of tetracyclines on carbohydrate metabolism. Thiele-Bruhn and Beck (2005) examined the effect of oxytetracycline on glucose-induced respiration. They observed 24-hour ED_{50} values of between 19.1 and 31.2 µg/L. Values gained were statistically significant. In contrast, Zielezny *et al.* (2006) observed no significant in glucose-induced respiration rates as a result of exposing soil microbial communities to CTC. Vaclavik *et al.* (2004) observed an increase in glucose-induced respiration rates in the order of 1.3-1.7 times as a result of exposure to tetracycline and CTC in soil studies. In a study that quantified enzyme activity associated with glucose utilisation it was observed that oxytetracycline significantly reduced dehydrogenase activity in soil mesocosms after 7 days exposure (Boleas *et al*., 2005).

Kong *et al.* (2006) tracked carbohydrate utilisation in Biolog plates that had been inoculated with soil extracts and oxytetracycline. They observed an inhibition in the maximum level of carbohydrates utilised as a result of increasing CTC concentration. The results from the literature therefore portray a mixed picture in terms of the effects of CTC on carbohydrate metabolism and utilisation. Maul *et al.* (2006) observed a 2.7-3.5-fold reduction in carbohydrate utilisation as a result of exposing leaf-bound microbial communities to the antibiotic ciprofloxacin (100 µg/L). This work demonstrated the inhibitory effects of antibiotics to microbial communities in the environment and provides data in support of some of the findings from the present study.

The main consequence of a reduction in carbohydrate utilisation is the inability of the microbial community to gain energy from preferred organic sources. In the absence of a suitable energy source the growth of certain portions of the microbial community will likely be inhibited. This has been demonstrated by whole-lake DOC addition experiments, in which a significant increase in aquatic microbial biomass was observed as a result of adding increased DOC (Blomqvist *et al*., 2001). The high degree with which aquatic microbes are able to convert glucose into biomass (20% of total DOC) highlights the importance of carbohydrate utilisation to aquatic microbial communities (Tranvik and Hofle, 1987). It has previously been discussed within the current chapter the importance of microbial biomass in terms of food chains and acting as a carbon sink. In addition, a wide range of biochemical processes are reliant upon the energy that microbial communities gain from carbohydrates, such as active transport of molecules in and out of cells and maintenance of buoyancy systems in cyanobacterial cells (Chu *et al.*, 2007).

After day 2 of the exposure period carbohydrate utilisation data exhibited a recovery in terms of a decrease in the lag phase duration (table 3.9). As has been see with the utilisation of other substrates during this study, this recovery may be the result of several factors, such as formation of tetracycline resistance or degradation of CTC for example. In the case of max rate data however significant reductions compared with control data were still seen on day 4 (at 0.1 mg/L CTC) and day 6 (at 0.32 and 1mg/L CTC). It is a possibility therefore that carbohydrate metabolism was being inhibited by toxic metabolites and transformation products of CTC (Halling-Sorensen *et al*., 2002). Due to the relatively low CTC concentration that was inhibiting the max rate of carbohydrate utilization at periods throughout the exposure period, carbohydrate metabolism was one of the sensitive end-points that was used in the present study (in terms of looking at the effects of CTC).

PCA analysis of carbohydrate utilisation profiles on day 1 and (figure 3.11a) of carbohydrate utilisation revealed that control/0.1 mg/L and 0.32/1 mg/L day 1 replicates shared a similar profile as represented by their distinct clustering. A similar trend was also observed on day 4 (figure 3.11b), suggesting that carbohydrate utilisation shared a similar profile at this time-point. These data suggest that exposure to CTC alters carbohydrate utilisation profiles even after a recovery has been detected by data from other end-points. It is possible that the action of CTC (or its metabolites and transformation products) is having an effect on the structure of the microbial community as shown by the effect that it is having on the pattern of metabolic profiles of the microbial community. In such a scenario, a specific portion of the microbial that utilises carbohydrates as an energy source (which is likely to be performing their metabolic action within the carbon cycle) may be inhibited at a particular CTC concentration but not at lower doses. In the absence of this particular carbohydrate utiliser (or utilisers in the case that more than one member of the microbial community is inhibited at a certain CTC dose) other members of the microbial (which may have been outcompeted previously) may be able now utilise carbohydrates. If different member of the microbial community are present at different CTC doses, then it probable that at least a portion of the respective microbial communities will have display a different carbohydrate utilisation profile.

3.4.5. Effect of CTC on Carboxylic Acid Utilisation

Carboxylic acid utilisation was shown to exhibit an inhibitory effect on lag phase duration on day 1 of the exposure period in microcosms that had been exposed to 0.32 and 1 mg/L CTC (table 3.11). An inhibitory effect on lag phase duration was also observed on day 2 as a result of exposure to 1 mg/L CTC. An identical pattern of inhibition was also observed on the max rate of carboxylic acid utilisation (table 3.12).

Kong *et al.* (2006) also observed an inhibition of carboxylic acid utilisation as a result of exposing soil extracts to oxytetracycline (similar concentrations to the present study). Inhibitory effects were

also observed at a similar oxytetracyline concentration as used in the current study. This indicates that carboxylic acid utilisation can be inhibited in both the terrestrial and aquatic environment at similar concentrations.

Carboxylic acids represent an important energy source for environmental microbes. Molecules such as citric acid, pyruvic acid, succinic acid and lactic acid can enter energy liberating biochemical pathways such as the tricarboxylic acid cycle, directly (Reactome, 2010). Carboxylic acids are also formed from the cleavage of fatty materials in the environment in the form of fatty acids. Naumann (1918) first showed the utilisation of humic materials by aquatic microorganisms. Humic acids contain high carboxylic acid content (Kawahigashi and Sumida, 2006). These form an important part of the carbon cycle in terms of the recycling of dead animal and plant material. Inhibition of carboxylic acid utilisation as result of CTC exposure in the environment could therefore inhibit the ability of environmental microbes to produce energy and degrade certain molecules within the carbon cycle.

Guven *et al*. (2005) demonstrated the ability of certain Archaea species (such as *Candidatus* Brocadia and *Canditus* Kuenenia) to utilise organic acids (such as propionate) using nitrite/nitrate instead of oxygen as terminal electron acceptor. The important role of these organisms in the nitrogen cycle was therefore shown. Inhibition of carboxylic acid utilisation could possibly affect this part of the nitrogen cycle, although it is unclear if these types of reactions are taking place on Biolog GN2 plates. Analysis of microbial by molecular techniques (such as DNA sequencing) may be able to give an insight into the portion of the microbial community that is present within CTC-dosed microcosms however.

Carboxylic acid utilisation showed a recovery (in terms of lag phase duration and max rate of utilisation) at all CTC concentrations on day 3 of the exposure period. As with the recovery observed with the utilisation of other substrates, the increased appearance of resistant phenotypes and the subsequent synthesis of enzymes capable of utilising carboxylic acids is a possible explanation for this. In such a scenario, members of the microbial community which are not affected by CTC may be now be able to exploit resources that they not previously able to due to them being outcompeted. Alternatively, populations of slower growing or resistant bacteria which were able to significantly utilise carboxylic acids (which were present from the start of the exposure period) could now be sufficiently abundant to utilise the carboxylic acids.

PCA analysis was conducted with regard to carboxylic acid utilisation. Day 1 replicates exposed to 1 mg/L and some replicates from the 0.32 mg/L group clustered independently. Replicates from the control group clustered with replicates from the 0.1 mg/L group. These data indicate that on day 1of the exposure, the carboxylic acid utilisation profile of microbial communities was altered mainly as the result of exposure to 1 mg/L CTC and to a lesser extent by exposure to 0.32 mg/L CTC. The carboxylic utilisation profile was not altered by exposure of microcosms to 0.1 mg/L

CTC. Day 4 data illustrated a similar picture with regard to the 0.1 mg/L / control cluster, although 1 mg/L replicates clustered closer to these groups. The carboxylic acid utilisation profile was therefore changed as a result of exposure to 1 mg/L CTC, but had become more similar to 0.1 mg/L/control treatments by day 4. The majority of 0.32 mg/L replicates clustered closer to 1 mg/L replicates on day 4, although some were separately clustered from all other treatments. These data indicate that microbial communities exposed to 0.32 mg/L and 1 mg/L shared similar carboxylic utilisation profiles, with some replicates from the 0.32 mg/L group having a unique carboxylic utilisation profile. It can be therefore be inferred that the microbial community structure was different as result of exposure 1 mg/L CTC and (to a lesser extent) 0.32 mg/L CTC. At these time points, it is likely therefore that the community structure of carboxylic utilising bacteria was different at these doses.

3.4.6. Effect of CTC on Polymer Utilisation

Polymer utilisation was significantly inhibited in terms of lag phase duration as a result of exposing microcosms to CTC concentrations of 0.1 and 0.32 mg/L CTC. A significant reduction in the max rate of polymer utilisation was also detected due to exposure of microcosms to the same CTC concentrations. Although exposure of microcosms to 1 mg/L CTC did not result in significant effects to lag phase, a high increase was still recorded. A high increase in the max rate of polymer utilisation was also observed. It is likely that these values were not found to be significant due to the high standard deviation of regression values calculated by the 3-parameter kinetic model. Future studies should therefore repeat similar exposure studies with a higher degree of sample replication in to reduce any possible variation. Significant inhibitory effects were also seen on day 2 in terms of lag phase (in microcosms exposed to 1 mg/L CTC) and max rate of polymer utilisation (in microcosms exposed to 0.32 mg/L CTC).

Kong *et al.* (2006) exposed soil extract inoculated Biolog plates with oxytetracycline at similar concentrations to the present study. They observed a significant reduction in maximum colour production (and therefore polymer utilisation) as a result of exposure to oxytetracycline. The results of this study are therefore in agreement with the current study.

Environmental microorganisms are responsible for the degradation of polymers within ecosystems as the result of biosynthesis of lipases, ureases, esterases and proteases (Fleming, 1998; Lugauskas, 2003). Microorganisms also secrete enzymes into their environment to progressively reduce their molecular weight, producing oligomers, dimmers and monomers (Lucas *et al*., 2008). They have also been shown to be capable of degrading polymers that are widely regarded as being recalcitrant to degradation (Shimano, 2001; Howard, 2002; Szostac-Kotowa, 2004; Shah *et al*., 2008). Microrganisms ultimately assimilate polymers as cellular biomass and energy (Lucas *et al*., 2008). Any inhibition of the microbial community in terms of polymer utilisation could therefore result in the reduced functioning of such processes within the aquatic environment.

A recovery in lag phase duration was observed in microcosms exposed to 0.1 and 0.32 mg/L CTC. A recovery in lag phase duration was not observed until one day later, indicating that aquatic systems take longer to recover from higher CTC exposures. In terms of the max rate of polymer utilisation a recovery was observed on day 2 at the lowest and highest CTC concentration. Effects were still being seen in microcosms exposed to 0.32 mg/L CTC. It could be that significant effects were again not detected due to the high degree of variation between samples however. By day 3 all microcosms had recovered, suggesting polymer utilisation is back to normality at this time point. At this point a large proportion of the microbial community is likely to have become resistant to the effects if CTC via one or more resistance mechanisms (Speer *et al.*, 1992).

The data in PCA score plots on day one revealed that a change in the polymer utilisation profile was only caused by exposure of microbial communities to 1 mg/L CTC. All other groups clustered with control replicates, with the exception of two replicates from the 0.32 mg/L group (that clustered with 1 mg/L replicates). On day 4 a similar pattern emerged except more 0.32 mg/L replicates clustered with 1 mg/L replicates. This data indicates that the polymer utilisation profile was only altered by exposure of microbial communities to 1 mg/L CTC, with some changes caused by exposure to 0.32 mg/L CTC. Although there is no president to these results in the available literature, it is likely that aquatic microbes in 1 mg/L microcosms form a different community structure to other microcosms. These data may suggest therefore that exposure to relatively high CTC concentrations may alter the manner in which aquatic environmental microbes to degrade polymers of natural and anthropogenic origin. For example, CTC exposure may reduce the diversity of microbes which are able to degrade a range of polymers. This could be investigated further with the use of molecular tools such DNA sequencing with BLAST analysis.

3.4.7. Effect of CTC on CFU Counts

In the present study no significant effects were seen on CFU counts as a result of exposing Aquatic microbial communities to three concentrations of chortetracycline. In other studies, Halling Sorensen *et al.* (2002) observed an EC_{50} values of as 0.03 mg/L for CTC and 5a, 6-CTC when studying the effect of these compounds on CFU counts. During this study activated sludge microbial communities were exposed to tetracyclines, indicating that the growth of activated sludge communities may be more sensitive to tetracyclines than river water communities. Colinas *et al.* (1993) also showed a significant decrease in CFU counts in soils exposed to oxytetracycline, with a 20% reduction in the CFU count having been observed. It is possible that the growth of soil microbial communities is more sensitive to tetracyclines. Alternatively, it may be the case that the portion of the aquatic microbial community that is affected by tetracyclines is not culturable using traditional culturing techniques. A final possibility for not seeing any inhibition of microbial growth in the present study could be the presence of more resistant members of the aquatic microbial community than in other studies. One piece of evidence that supports this view is the

relatively high increase in CFU between days 1 and 7 as a result exposure to 1 mg/L CTC. Although this change was not statistically significantly different compared with CFU changes in the control, the variation between agar plates was extremely high. This reduces the chances of gaining a significantly different value.

Chapter 4: Effect of Sulfamethoxazole on Microbial Functioning in Aquatic Systems

4. Effects of Sulfamethoxazole on Microbial Function

4.1. Sulfonamides

In the early 1930s it was observed that certain azo group containing dyes had antibacterial properties. Further research into these properties by the German pathologist and bacteriologist Gerhard Johannes Paul Domagk led to the synthesis of the first commercially available antibiotic, prontosil. This 1939 Nobel Prize winning discovery was the beginning of the modern antimicrobial therapy revolution and heralded the first in a series of commercially available sulfonamide compounds (table 4.1). The importance of Domagk's initial research was highlighted by the treatment of Tuberculosis post World War II by the sulphonamides thiosemicarbazone and isoniazid. The success of sulfa drugs led to the unregulated manufacture of hundreds of tons of sulfa-containing drugs worldwide until the "Elixir Sulfonamide Disaster" (during which hundreds of people were poisoned by ethylene glycol impurities that were present in sulfonamide drugs) led to the first regulation of antibiotic production (Carpenter, 2010).

Table 4.1: Chronology of sulfonamide drug discovery.

Chemical name	Generic Name	Year of Discovery
4-[[2, 4-diaminophenyl) azo] benzenesulfonamide	prontosil	1935
4-aminobenzenesulfonamide	sulfanilamide	1936
N-[(4-aminophenyl) sulfonyl] acetamide	sulfacetamide	1939
2-(p-aminobenzenesulphonamide)-4, 6-dimethylpyridine	sulfadimidine/sulfamethazine	1942/1943
N1-2-pyrimidinylsulfanilamide	sulfadiazine	1947
4-amino-*N*-(5-methyl-1, 2-oxazol-3-yl) benzenesulfonamide	sulfamethoxazole	1961

4.1.2. Chemistry of Sulfonamides

Sulfonamide antibiotics are composed of a sulfonyl group covalently bonded to an amine group with the general formula RSO_2NH_2, where R represents an organic group. Sulfonamides are synthesised from a sulfonic acid precursor by replacement of a hydroxyl group with an amine group. Sulfonamide chemistry is often affected by pH. Sulfonamides are ionogenic compounds. As such they are subject to pH-dependent ionisation. The two ionisable groups on sulfonamide molecules are the anilinic amine and amide moieties. A cationic species (SA^+) is mainly formed at low pHs and an anionic species (SA^-) being mainly formed at higher pHs. Between the two pk_a values (pk_a1 and pk_a2), a neutral sulfonamide species can exist (SA^0). The neutral species is in tautomeric equilibrium with another zwiterionic sulfonamide species ($SA^{\pm}$) although this form is only reported to account for < 0.2% of speciation in favourable conditions (Gao and Pederson, 2005).

4.1.3. Sulfonamide Mode of Action

The enzyme 6-hydroxymethyl-7, 8-dihydropteroate synthase (DHPS) catalyzes the condensation of para-aminobenzoic acid (pABA) with 6-hydroxymethyl-7, 8-dihydropterin-pyrophosphate to form 6-hydroxymethyl-7, 8-dihydropteroate and pyrophosphate. DHPS is essential in the synthesis of folate in prokaryotes and plants. Sulfonamide antibiotics act as a structural analogue of pABA and the folate pool is therefore reduced in inhibited organisms. As a result, cells are not able to grow and occasionally cell death is recorded also (Baca *et al.*, 2000).

The action of sulfonamides has been reported to be pH-dependent. Mengelers *et al*. (1997) for example showed that the toxicity of several sulfonamides was dependent on the pk_a2 values and the extracellular pH of which the bacteria inhabit. Based on the fact that the intracellular bacterial pH does not normally exceed 8 there is a pk_a2 limit of 6.5 on the sulfonamide species that can enter the bacterial cell. Therefore sulfonamides with a pk_a2 of greater than 6.5 will not enter the bacterial cell.

Changes in the intracellular pH of the bacterial cell will also affect the uptake of sulfonamides. Even a small shift in intracellular pH can cause an increase in sulfonamide uptake (assuming an optimum pk_a2 for uptake). However, if intracellular and extracellular pHs are similar, no significant changes in sulfonamide uptake will be recorded and the greater the pH difference between intra- and extracellular space the greater the uptake of sulfonamides will be. The extracellular pH therefore has an effect on sulfonamide uptake also. In general, the uptake of sulfonamides with low pk_a2 values is highly dependent on extracellular pH, assuming that intracellular bacterial pH is within the normal range of between 5 and 9. Considering an environmental pH optimum of 7, the sulfonamides with the potential for the most effectiveness are sulfisoxasole and sulfamethoxazole (Zarfl *et al.*, 2008; Madigan *et al.*, 2003; Roland *et al.* 1979).

Tappe *et al.* (2008) tested these concepts further and investigated two bacteria with a good and poor ability to regulate pH. They observed that bacteria with a strong ability to regulate pH would be strongly inhibited by sulfonamides in low pH environments. Conversely, they observed that bacteria that are poor regulators of pH are likely to be mildly inhibited by sulfonamides at low pH and affected more at a pH of 7 or 8. This study showed the importance of internal bacterial pH (pHi) in evaluating the antibiotic activity of sulfonamides.

4.1.4. Sulfonamide Usage

Sulfonamide antibiotics are used in both human and veterinary antimicrobial chemotherapy. In human medicine, sulfonamides are mainly used to treat urinary and upper respiratory tract infections. They can also be used to treat bacterial pneumonia, shigellosis and *Nocardia* infection (Connor, 1998). Sulfonamide antibiotics are mainly used to treat coccoidal infections in both commercial farm animals and pets. Veterinary sulfonamide antibiotics are controlled under the EC directive 1831/2003 (European Union Register of Feed Additivies).

4.1.5. Occurrence of Sulfonamide Antibiotic in the Environment

Sulfonamides have been detected in several environmental matrices including soil (e.g.: Shelver *et al*., 2010) surface water (e.g.: Young and Carlson, 2004) and groundwater (e.g.: Holm *et al.*, 1995). Reported occurrences of sulfonamides are summarized in table 4.2. The data in table 4.2 show that sulfonamides have detected mainly in aquatic environments. All reported detection of sulfonamides in the soil environment has been in areas that have been amended with manure from sulfonamide – treated animals. As with tetracycline detection in the soil environment this makes sense as manure is a major exposure route of veterinary antibiotics.

It can also be seen from table 4.2 that sulfonamides have been detected at levels that are very close to LOD or LOQ levels. This would suggest that detectable levels of sulfonalides in the environment are on the edge of what can be detected. As with other antibiotcs that have been detected in the environment, sulfonamide levels are extremely low.

Table 4.2: Reported occurrence of sulfonamide antibiotics in various environmental compartments. Data in italics indicates limit of quantification.

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentrations	Reference (s)
sulfamerazine	Surface water	USA	0.05 μg/L	nd and 0.05 μg/L	Yang and Carlson, 2004b
	Lake water	USA	0.05 μg/L	0.19 μg/L	Yang and Carlson, 2004b
sulfamethazine	Stream water	USA	0.02 μg/L - 0.12 μg/L	*0.05 μg/L*	Kolpin *et al*., 2002
	Stream water	USA	0.22 μg/L	*0.05 μg/L*	Kolpin *et al.,* 2002
	Lake water	USA	0.22 μg/L	0.05 μg/L	Yang and Carlson, 2004b
	Surface water	USA	0.0010 μg/L	< 0.0010 μg/L	Skadsen *et al.,* 2004
	Groundwater (landfill site)	Denmark	*20 μg/L*	< 20 μg/L – 900 μg/L	Holm *et al.,* 1995
	Groundwater	Germany	*0.02 μg/L*	0.16 μg/L	Hirsch *et al.*, 1999
sulfamethoxazole	Surface water	Germany	*0.02 μg/L*	0.03 - 0.48 μg/L	Hirsch *et al.*, 1999
	Surface water (tributaries)	Germany	*0.030 μg/L*	< 0.030 μg/L & 0.040 μg/L	Wiegel *et al*., 2004
	Surface water (in April 1998)	Germany, Czech Rep.	*0.030 μg/L*	0.030 - 0.070 μg/L	Wiegel *et al.,* 2004
	Surface water (upstream STP) discharge)	Sweden	NS	< 0.001	Bendz *et al*., 2005
	Surface water (downstream STP discharge)	Sweden	NS	0 - 0.01 μg/L	Bendz *et al*., 2005
	Surface water (upstream STP)	UK	<0.050 μg/L	0.050 μg/L	Ashton *et al.,* 2004

Table 4.2 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentrations	Reference (s)
sulfamethoxazole (cont)	Surface water (downstream STP)	UK	<0.050 µg/L	0.050 µg/L	Ashton *et al.*, 2004
	Surface water	UK	<0.050 µg/L	0.050 µg/L	Hilton and Thomas, 2003
	Stream water	USA	*0.05 µg/L*	0.15 - 1.9 µg/L	Kolpin *et al*., 2002
	Stream water	USA	*0.023 µg/L*	0.066 µg/L - 0.52 µg/L	Kolpin *et al*., 2002
	Surface water	USA	0.0010 µg/L	0.010 ± 0.007 µg/L	Skadsen *et al.*, 2004
	Lake water	USA	0.05 µg/L	0.06 µg/L	Yang and Carlson, 2004b
	Surface water	USA	0.007 µg/L	0.023 µg/L	Heberer *et al.*, 2001
	Surface water	USA	0.05 µg/L	0.05 µg/L & 0.12 µg/L	Yang and Carlson, 2004b
	Groundwater	Germany	0.0018 µg/L	0.410 µg/L	Sacher *et al.*, 2001
	Groundwater	USA	0.023 µg/L	0.002 µg/L	Heberer *et al.*, 2001
	Groundwater	Germany	0.025 µg/L	< LOQ – 0.11 µg/L ± 0.07 µg/L	Ternes *et al*., 2007
	Groundwater	USA	*0.05 µg/L*	0.01 µg/L & 0.08 µg/L	Karthikeyan & Bleam, 2003
	Groundwater	Germany	0.02 µg/L	0.47 µg/L	Hirsch *et al.*, 1999
sulfathiazole	Surface water	USA	0.0010 µg/L	< 0.0010 µg/L	Skadsen *et al.*, 2004
sulfathiazole	Surface water	USA	0.0010 µg/L	< 0.0010 µg/L	Skadsen *et al.*, 2004

Table 4.2 (cont).

Compound	Matrix	Location	LOD/*LOQ*	Reported Concentrations	Reference (s)
sulfamethizole	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 330 µg/L	Holm *et al*., 1995
sulfadiazine	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 1160 µg/L	Holm *et al.*, 1995
sulfadiazine	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 1160 µg/L	Holm *et al.*, 1995
sulfanilamide	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 300 µg/L	Holm *et al*., 1995
sulfaguanidine	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 1600 µg/L	Holm *et al*., 1995
sulfanilic acid	Groundwater (landfill site)	Denmark	20 µg/L	< 20 µg/L – 10440 µg/L	Holm *et al.*, 1995
acetyl-sulfamethoxazole	Surface water (upstream STP)	UK	0.050 µg/L	< 0.050 µg/L	Ashton *et al.*, 2004
	Surface water (downstream STP)	UK	0.050 µg/L	< 0.050 µg/L – 0.239 µg/L	Ashton *et al*., 2004
	Surface water	UK	0.050 µg/L	< 0.050 µg/L - 0.240 µg/L	Hilton and Thomas, 2003

LOD = Limit of Detection
LOQ = Limit of Quatification

4.1.6. Fate and Behaviour of Sulfonamides in the Environment

4.1.6.1. Sorption

Several studies have investigated the fate and behaviour of sulfonamides in several environmental matrices, although the vast majority of these studies have concentrated on the terrestrial environment.

The environmental behaviour of sulfonamides has been shown to be affected by sorption to soil mineral constituents such as montmorillomite, illite, and ferrihydrite and metal oxides; studies have also shown these processes to be pH-dependent (Khale and Stamm, 2007; Thiele-Bruhn *et al*., 2004). The sorption of sulfonamides to micelle–clay systems has further highlighted the importance of sulfonamide sorption to inorganic particles (Polubesova *et al.*, 2006).

The tendency for sulfonamides to interact with phenolic, carboxylic and *N*-heterocyclic compounds has also been investigated, highlighting the importance of compounds found in SOM to the sorption of sulfonamides (Thiele-Bruhn *et al.*, 2004). Bialk *et al.* (2005) demonstrated the sorption of sulfonamides to natural organic matter (NOM), showing that the compounds sorbed to the model NOM constituents that weer investigatd. Kahle and Stamm (2007) showed that this process was also highly pH-dependent as well as being affected by higher ionic strength (due to multiple ionisation sites of sulfonamides). In general, sulfonamides tend to form "unextractable fractions" with environmental matrices which has made studying the fate and behaviour of sulfonamides (and their chemical species) difficult (Huschek *et al.*, 2008). Indeed, data from sorption and transport studies have provided data which suggests that permanently bound sulfonamide-soil complexes have been formed (Kruezig *et al*., 2005; Hamscher *et al.*, 2005).

The sorption of sulfonamides to a variety of different soil types has been investigated. Heise *et al.* (2006) observed that a high proportion of sulfadiazine and sulfamethoxazole represented unextractable bound residues (93%) when added to whole soils. Forster *et al*. (2009) showed that residual fractions of both sulfadiazine and its *N*-acetylated metabolite were trapped in whole soils where they were likely to persist for several years. Thiele-Bruhn *et al*. (2004) observed a greater degree of sorption of sulfonamides to soils with finer particles compared to soils with a relatively more coarse structure. The authors linked higher SOM with an increased adsorption of sulfonamides. Another study however found no significant differences between sulfonamide sorption behaviour in a silt and sandy loam (Acclinelli *et al.*, 2007).

4.1.6.2. Transportation of Sulfonamides

Several studies have also looked at the movement of sulfonamides through different soils. Burkhardt and Stahm (2007) investigated the movement of sulfonamides (sulfadimidine, sulfadiazine and sulfathiazole) through an undisturbed loamy grassland soil. They found that all

three compounds studied were highly mobile through the soil column via preferential flow in pore water with as much test compound being detected 50 cm down as was detected 5 cm from the top.

Blackwell *et al.* (2007) investigated the movement of sulfonamides through a sandy loam soil. The authors reported that sulfachloropyridazine was highly motile through the soil column, with a relatively high concentration (0.78 µg/L) being detected at a depth of 40 cm after 20 days. The same authors conducted another study (in 2009) to investigate the movement of sulfachloropyridazine through a clay soil into drainage water. They were able to detect a considerable sulfachloropyridazine concentration in drainage water (613 µg/L in year 1 and 6.1 µg/L in year 2), indicating that the compound had moved through the soil column.

Lapen *et al.* (2008) also investigated the movement of two sulfonamides (sulfamethoxazole and sulfapyridine) from sewage sludge through a soil column following a series of simulated precipitation events. The two compounds could only be detected at levels above the LOQ in tile drainage water following the first tile drain event but not after subsequent simulated rainfalls. The results from this study would therefore suggest that sulfonamides had moved rapidly through the soil column into drainage water.

Weiss *et al.* (2008) also studied the movement of a sulfonamide (sulfamethazine) through soil following simulated precipitation events. In this study the test compound was contained within seepage water rather than sewage sludge. Two different soil environments were tested (grassland versus arable cropping sites). The authors observed losses (of sulfamethizine) to drainage water of between 2.8 and 5.4% in arable cropping soils compared with 10% losses in grassland areas. The results were hypothesised to have been caused by an increase in preferential macropore transport in grassland soils due to the increased presence of undisturbed macropores in grassland soils. Werhan *et al.* (2007) also observed significant leaching of a sulfonamide compound (sulfadiazine) through a soil column. This study additionally demonstrated that leaching of sulfonamide residues is increased if the compound is frequently applied at higher concentrations. There would appear to be a general agreement therefore that sulfonamides show a degree of motility through a variety of soil columns despite the sorptative properties that are displayed by the compounds. As result, sulfonamides can be transported to drainage waters with the possibility that further transport to adjacent water bodies will also take place. Another potential exposure route of sulfonamides into the aquatic environment is surface runoff. Blackwell *et al.* (2009) demonstrated this under field conditions. During the experiment they observed a sulfachloropyridazine concentration of 25.9 µg/L in surface runoff water. These data suggest that these dissolved sulfonamide residues could enter the aquatic environment.

Although the movement of sulfonamides through soil has been well reported in the literature, transport within the aquatic environment has been less well documented. One report that investigated sulfonamide (sulfachloropyridazine, sulfadimethoxine and sulfamethoxazole)

movement from sewage effluent downstream through a receiving river however found that the compounds travelled kilometre scale distances with relatively low uptake velocities. This one study therefore suggested that sulfonamides are very mobile in the aquatic environment.

4.1.6.3. Dissipation of Sulfonamide Antibiotics - The Terrestrial Environment

The degradation of several sulfonamides has been investigated in the terrestrial environment. Heuer *et al.* (2008) applied ^{14}C sulfadiazine-spiked manure to a soil system. Although no mineralisation was shown to have taken place, rapid deacetylation of the major metabolite (*N*-acetyl sulfadiazine) occurred in soil. Forster *et al*. (2009) also observed significant deactylation of *N*-acetyl sulfadiazine in soils spiked with sulfadiazine and fresh manure. They also observed that the subsequent product of this (sulfadiazine) was further transformed to 4-OH-sulfadiazine. In terms of total dissipation, a DT_{25} of 16.8 days was observed in cambisol compared with a DT_{50} of 250 days in luvisol.

The degradation of sulfachloropyridazine in soil has also been studied. Blackwell *et al.* (2007) tracked the dissipation of sulfachloropyridazine in spiked sandy loam soils. DT_{50} and DT_{90} values of 3.5 and 18.9 days were observed respectively, indicating that the compound had dissipated relatively quickly. Accinelli *et al.* (2007) also measured dissipation rates of sulfachloropyridazine in a sandy loam soil. The authors of this study reported a DT_{50} of 21.3 days. A slightly lower DT_{50} value (18.6 days) was observed for sulfachloropyridazine dissipation in a silt loam soil although dissipation was further increased by the addition of fresh manure to the soil system.

Another study has looked at the degradation kinetics of sulfadimethoxine in soils spiked with manure-containing sulfonamides. Using a kinetic model it was observed that the degradation rate constant increased with decreasing initial concentration. The authors attributed this to the antimicrobial action of sulfadimethoxine at higher concentrations. Higher degradation rates were also observed with increasing manure and moisture contents; the former is therefore in agreement with other results (Accinelli *et al.*, 2007; Wang *et al.*, 2006).

4.1.6.4. Dissipation of Sulfonamide Antibiotics - The Aquatic Environment

Although the transport of sulfonamide antibiotics in aquatic systems has not been extensively studied, there is a body of literature that describes their dissipation in the aquatic environment. Many of these investigations have measured sulfonamide dissipation in rivers. Bendz *et al.* (2005) studied the dissipation of sulfamethoxazole in a Swedish river. Sulfamethoxazole was shown to be relatively persistent, with a concentration of just below 0.03 µg/L being measured at the furthest point downstream of an STP compared with an STP effluent concentration of 0.05 µg/L. Tamtam *et al.* (2008) also investigated sulfamethoxazole dissipation downstream of an STP in a French river. They observed 50% removal of sulfamethoxazole during the study. This was attributed to a

40% river dilution due to a high tributary flow rate. It was concluded therefore that sulfamethoxazole was likely to be persistent. Haggard and Bartsch (2009) observed similarly low removal rates of seven sulfonamide antibiotics (sulfachloropyridazine, sulfadiazine, sulfadimethoxine, sulfamerazine, sulfamethazine, sulfamethoxazole and sulfathiazine) down an aquatic gradient of several kilometres. In addition, low uptake levels from the water column were observed, suggesting that minimal degradation had occurred. Conkle *et al.* (2008) also tracked the dissipation of sulfamethoxazole (and sulfapyridine) from STP effluent through a wetland and ultimately a lake. The study detected no changes in the concentration of either sulfonamide antibiotic in STP effluent compared with wetland and lake water. These data would suggest that the sulfonamides tested are persistent in the aquatic environment.

Benotti and Brownawell (2008) have conducted the sole study that has investigated sulfonamide degradation in the marine environment. Of the broad range of pharmaceuticals tested, sulfamethoxazole was one of the most persistent tested, with a lower half-life of 85 days having been measured.

Other studies have attempted to decipher the possible mechanisms which may be responsible for sulfonamide degradation / transformation. Radke *et al.* (2009) for example investigated sulfamethoxazole and sulfamethoxazole metabolite dissipation in water and water sediment systems. They found that the compounds tested were only significantly removed by systems that included sediment, apart from the metabolite, sulfamethoxazole-*N1*-glucuronide, which was significantly removed in water alone. Due to the lack of significant sorption taking place, the authors concluded that the sulfonamides tested were removed by the degradative action of sediment microorganisms.

Lai and Hou (2008) investigated the influence of light and microbial activity on the degradation rates of four sulfonamides (sulfamethoxazole, sulfadiazine, sulfadimethoxine and sulfamethazine) in eel pond water and sediment. All compounds were shown to be transformed by light treatment alone. Light and non-sterile conditions resulted in a reduction in half-lives in both water and sediment suggesting a possible role of microbes in the biotransformation of sulfonamides. However, only sulfamethoxazole was shown to undergo biotransformation in non-sterile dark controls in both water and sediment. This result was particularly interesting given that a number of other studies have concluded that sulfamethoxazole is not biodegraded in conventional closed bottle tests (Alexy *et al.*, 2004; Al-Amad *et al.*, 1999).

4.1.7. Reported Effects of Sulfonamide Antibiotics on Microbial Function in the Environment

The effects of sulfonamide antibiotics on various aspects of microbial function in the environment have been investigated. Single species testing has accounted for many of these studies. Isidori *et al.* (2005) observed an EC_{50} value of 23.3 mg/L when exposing the Microtox system to

sulfamethoxazole. Kim *et al.* (2007) also used the Microtox test to study the effects of sulfonamides. They reported EC_{50} values (5 and 15 minutes) of 26.4 mg/L 53.7 mg/L for sulfachlorpyridazine and 74.2mg/L and 78.1mg/L for sulfamethoxazole.

Ando *et al*. (2007) investigated the effect of sulfadimethoxine on the growth of eight cyanobacterial species. Interestingly, the sulfonamide antibiotic was shown to be much less toxic than the other compounds tested. It exerted an EC_{50} value of between 2.3 and >2000 mg/L. All other antibiotics tested exerted EC_{50} values of < 1 mg/L.

Other studies have looked at the effect of sulfonamides on microbial respiration. Thiele-Bruhn and Beck (2005) for example tracked the effect of sulfapyridine on substrate induced respiration (SIR). They observed EC_{50} values of 6.2 and 11.5 μg/L for two soils. In another experiment, Zielezny *et al.* (2006) tracked SIR in soils spiked with sulfadiazine. They observed an inhibition of respiration rates in the presence of glucose only, highlighting the importance of nutrient addition when studying bacteriostatic compounds. Vaclavik *et al.* (2004) recorded a 0.8 fold decrease in respiration rate as a result of exposing soil mesocosms to sulfachloropyridazine.

Halling-Sorenson *et al.* (2002) studied the effect of sulfadiazine activity over time in activated sludge and soil pore water. They showed this by demonstrating a reduction in CFU counts. This study was the only investigation that utilized CFU counts as an end point in the detection of sulfonamide toxicity in the environment. Brandt *et al.* (2009) also showed a reduction in bacterial growth using ^{3}H leucine incorporation. This was significantly reduced by exposure of the microbial community to 0.1 μg/g sulfadiazine.

Other teams have investigated the effect of sulfonamides on specific cellular function. Thiele-Bruhn (2005) for instance tested the effect of sulfonamides on iron metabolism. All of the sulfonamides that were tested (sulfadimethoxine, sulfadiazine, sulfadimidine and sufadimidine) tested exerted ED_{10} values of < 100 mg/kg. Schmitt *et al.* (2005) tested the effect of sulfachloropyridazine on multisubstrate utilisation. The authors reported that sulfachloropyridazine shifted physiological fingerprints on the second PCA axis compared with controls, suggesting that a change in community structure had occurred. Loftin *et al.* (2005) investigated the effect of three sulfonamide antibiotics (sulfathiazole, sulfamethazine and sulfadimethoxine) on anaerobic metabolism. They found that two of the sulfonamide antibiotics significantly inhibited methane production after 72 hours and all three compounds inhibited methane production after 336 hours.

Two studies have also investigated the impact of sulfonamide on the biodegradation of xenobiotics. Chun *et al*. (2005) found that sulfamethazine significantly inhibited the dissipation of the synthetic hormone 17 β-Estradiol in soil. More recently, Monteiro and Boxall (2009) observed a reduction in naproxen dissipation as the result of exposing microbial communities to sulfamethazine.

4.1.8. Aims and Objectives of Chapter

The overall aim of the work described in this Chapter was to assess the potential effects of SMX on aquatic microbial communities. This was achieved using the following specific objectives;

1) To utilize the methods developed in Chapter 2 as well as measures of colony forming units to assess the effects of SMX on microbial population numbers and the ecological functioning of microbial communities in aquatic systems.

2) To explore the potential for recovery of microbial communities following SMX exposure in terms of ecological functioning.

3) To link functional data to effects of SMX in terms of the likely effect SMX may be exerting and to link these data to how these effects may be affecting wider ecological processes in the aquatic environment.

4.2. Materials and Methods

4.2.1. Study outline

The literature explored in previous chapters has revealed that sulfonamide antibiotics have been detected in the aquatic environment. Moreover, despite a range of reported effects having been detected in several environmental matrices there is still a lack of knowledge regarding the effects of sulfonamide antibiotics in the aquatic environment. In this chapter methods developed in Chapter 2 will be utilised to investigate the effects of sulfonamide antibiotics on microbial function in aquatic environment. The effects of sulphonamide exposure on CFU (colony forming units will also be assessed.

4.2.2. Chemicals

All materials and chemicals were purchased and prepared according to section 2.2.1. R2A agar was purchased from Oxoid (Cambridge, UK). Sulfamethoxazole was purchased from Sigma Aldrich (Poole, UK).

4.2.3 Surface water

Surface water was collected and prepared according to section 2.2.2.

4.2.4. Activated Sludge and Preparation of Treated Sewage

Activated sludge was collected and prepared according to section 2.2.3.

4.2.5. Preparation of OECD Synthetic Sewage

OECD synthetic sewage was prepared according to section 2.2.4.

4.2.6. Preparation of R2A Plates

R2A plates were prepared according to section 3.8.5.

4.2.7. Sulfamethoxazole Exposure Experiments

Twelve microcosms were set-up as described in section 2.2.6.1. Nine microcosms were spiked with an aqueous sulfamethoxazole solution. Three microcosms were spiked to a nominal sulfamethoxazole concentration of 1 mg/L, three to a nominal sulfamethoxazole concentration of 0.32 mg/L and three to a nominal sulfamethoxazole concentration of 0.1 mg/L. Three control microcosms received no sulfamethoxazole solution. A time series experiment was then conducted as described in sections 2.2.7.2.

4.2.8. CFU (Colony Forming Unit) Counts

CFU counts were performed according to section 3.9.1.

4.2.9. Data Analysis

Data were analysed according to section 3.9.2.

4.3. Results

4.3.1.1. Effect of Sulfamethoxazole on Total Substrate Utilisation

The data in figure 4.1 (a, b and c) show kinetic model plots of total substrate utilisation on days 1, 4 and 7 of the exposure. These graphs were included as they illustrated the main events during the exposure period at regular intervals. Figure 4.2 (a and b) shows PCA score plots representing multivariate data on days 1 and 7. The data in figure 4.3 (a and b) show lag phase and maximum rate graphs on all days of the exposure period. Tables 4.3 and 4.4 show statistical analyses of modelled max rate and lag phase duration values on days 1-7.

4.3.1.2. Kinetic Model Plots

Kinetic plots showing day 1 exposure data for total substrate utilisation shows that maximum absorbances reached their highest levels on 0 mg/L SMX plots and their lowest levels on 1 mg/L SMX plots. Plots depicting day 4 kinetic data show that microcosms that had been treated with 1 mg/L SMX had recovered slightly as far as a visual trend can tell. However, the two middle treatments had now showed more of an inhibition compared with the highest treatment in terms of an increased visual lag phase (0.1 and 0.32 mg/L SMX) and the maximum absorbance that plots had reached (0.32 mg/L SMX). All treatments still showed an inhibitory trend compared with control plots. Plots showing day 7 data reveal that all treatments had shown a further recovery in terms of the absorbance maxima, slope steepness and apparent lag phase. Inhibition was still observable with plots depicting data for 0.1 and 0.32 mg/L SMX showing the greatest inhibition.

4.3.1.3. Lag Phase Duration versus Exposure Time

The data shown by figure 4.4a and table 4.3 shows that the lag phase duration following total substrate utilisation was significantly increased as a result of exposing aquatic microcosms to 0.32 and 1 mg/L SMX. On day 2 of the exposure, microcosms that had been exposed to 1 mg/L SMX still displayed a significantly higher lag phase duration. However these microcosms had showed a recovery by day 3 and even a significantly reduced lag phase duration by day 5. The lag phase following total substrate utilisation as a result of exposure to 0.32 mg/L SMX showed a complete recovery by day 2. A significant increase in lag phase duration was observed on day 4 of the exposure in microcosms that had been exposed to 0.1 mg/L SMX. No further effects were seen at this concentration for the remainder of the exposure period however.

4.3.1.4. Max Rate versus Exposure Time

The data shown by figure 4.4b and table 4.4 show significant widespread effects of SMX on the max rate total substrate utilisation. On day 1 of the exposure a significant reduction in the max rate of total substrate utilisation was observed as a result of exposure to 0.32 and 1 mg/L SMX, although no significant effect was observed in microcosms exposed to the lower SMX

concentration (0.1 mg/L). On days 2 and 3 of the study significant reductions in the max rate of total substrate utilisation were seen at all SMX concentrations. After this, a recovery was observed in 1 mg/L SMX-treated microcosms. Significant reductions in the max rate of substrate utilisation were seen until the end of the investigation in microcosms that had been treated with 0.32 mg/L SMX. This was also the case with microcosms that were exposed to 0.1 mg/L SMX, except no effects were observed on days 5 or 6.

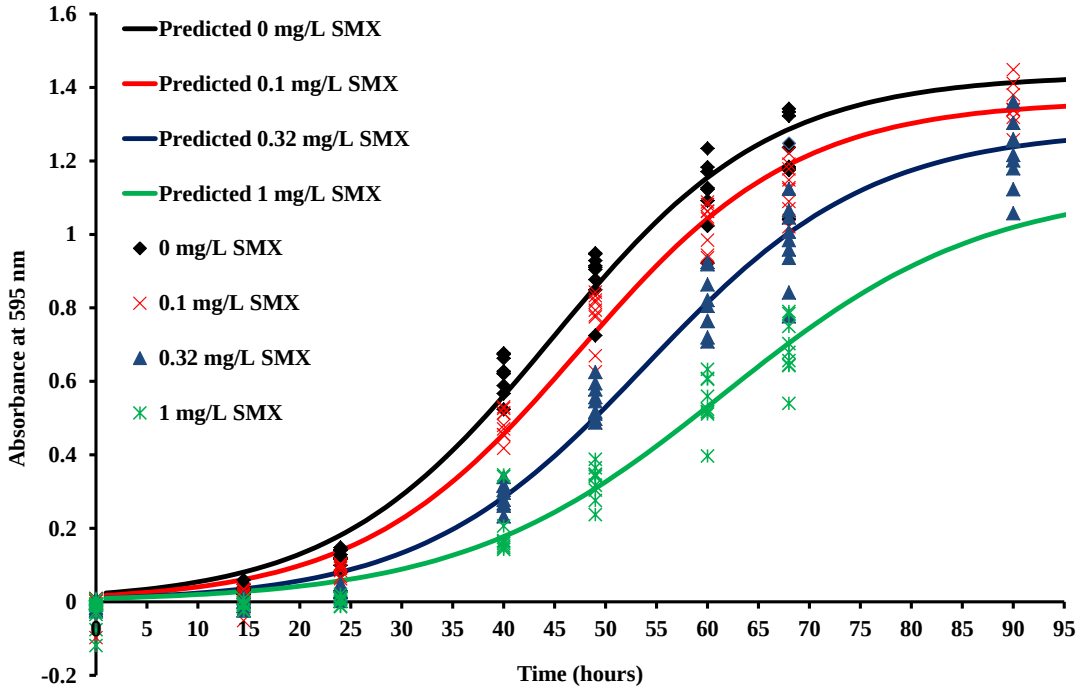
4.3.1.5. PCA Analysis of Total Substrate Utilisation

PCA plots depicting day 1 exposure data show that replicates from each treatment clustered almost exactly by SMX dose, with minimal mixing of replicates between treatments. This would indicate that total substrate utilisation had resulted in 4 distinct microbial community structures, based on their individual "metabolic fingerprint."

By day 4 of the exposure, a different picture had been formed according to PCA score plots. These plots show a higher degree of mixing between replicates from all SMX treatments, including between control and 1 mg/L doses. A distinct replicate cluster containing only 0.1 mg/L replicates could also be observed, suggesting that this treatment had caused the most change in the total substrate utilisation pattern of the microbial community.

By day 7 of the exposure, replicates from the two highest SMX treatments were relatively well separated, with some slight mixing between replicates representing 1 and 0.32 mg/L and 0.1 and 0.32 mg/L. There was significant replicate mixing between day 7 clusters representing control and 0.1 mg/L SMX treatments, suggesting that the total substrate utilisation profile was similar at these doses. It can be inferred from this that the microbial communities within microcosms treated with these treatments utilised total substrates in a similar manner.

a)



b)

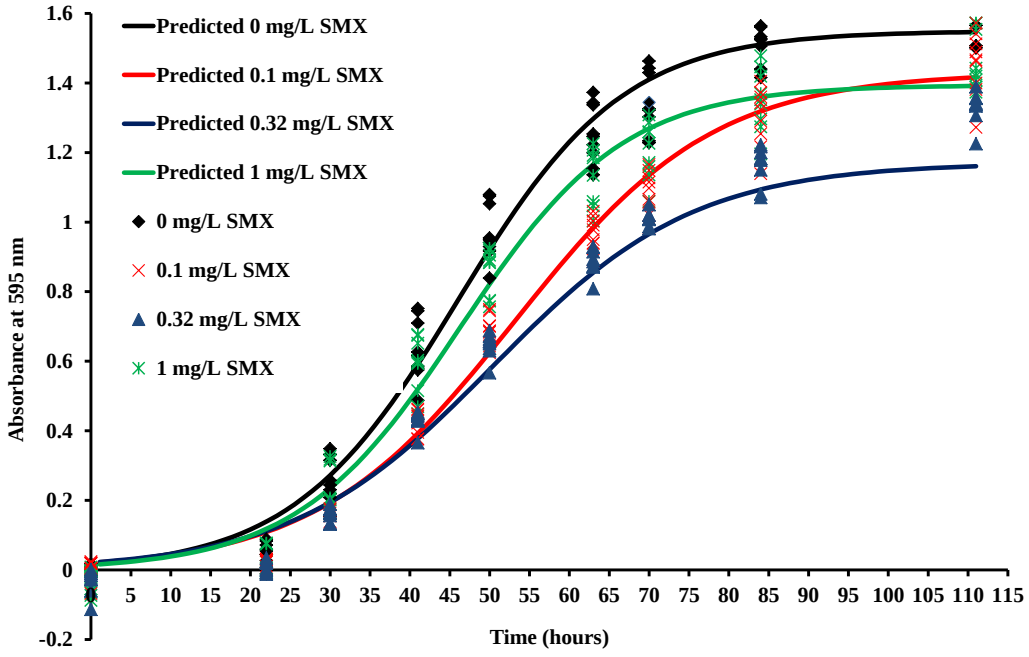


Figure 4.1: Kinetic plots of total substrate utilisation following SMX exposure on a) day 1, b) day 4 and c) day 7.

c)

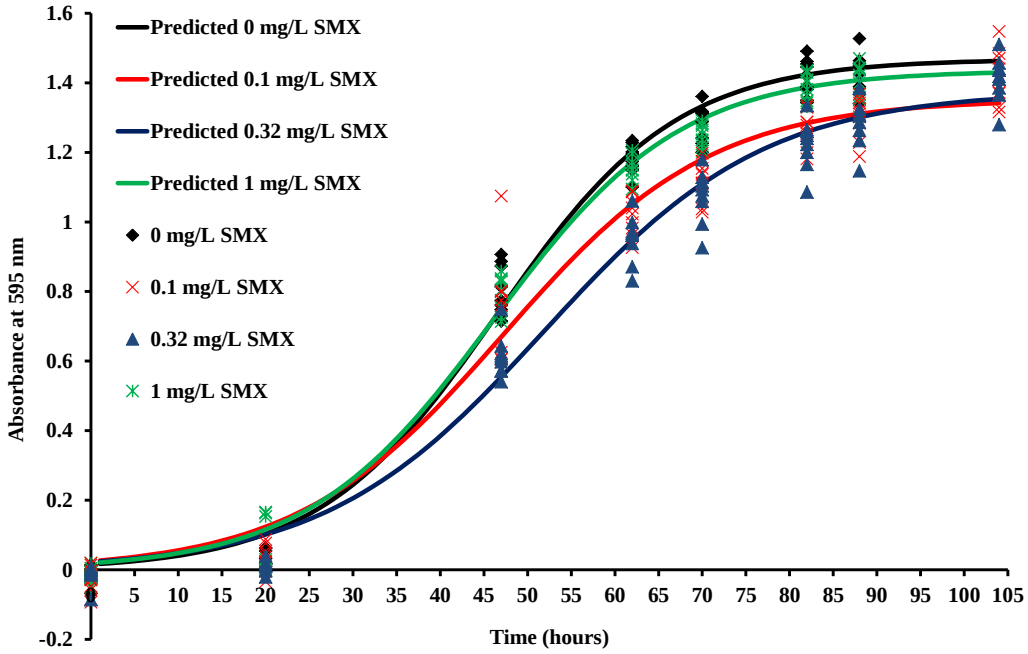
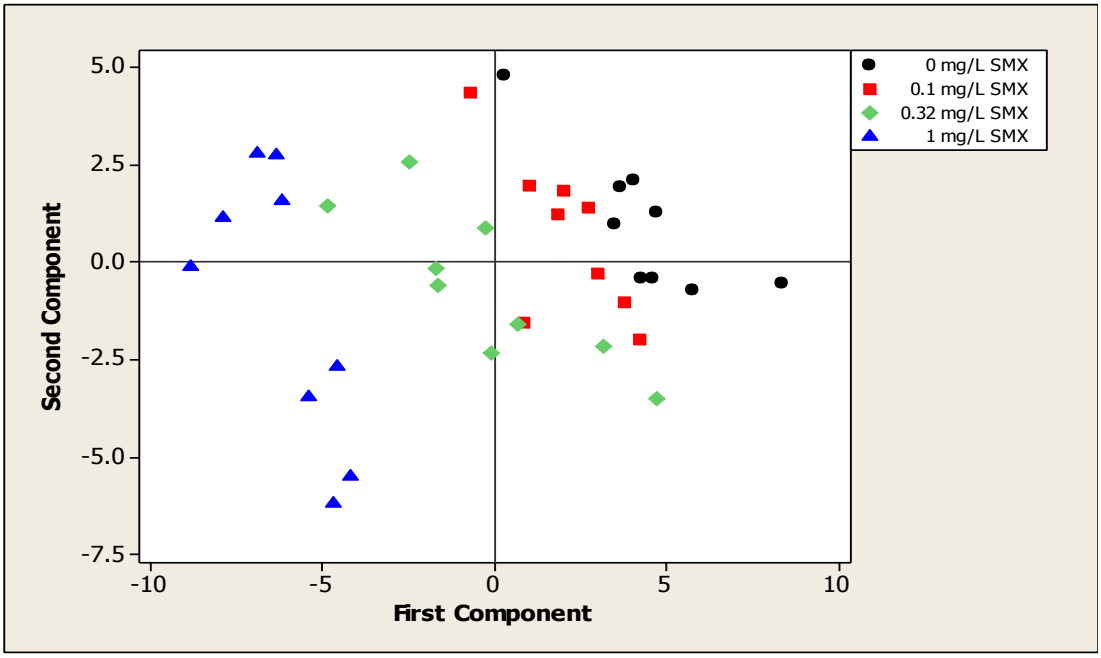


Figure 4.1 (cont).

a)



b)

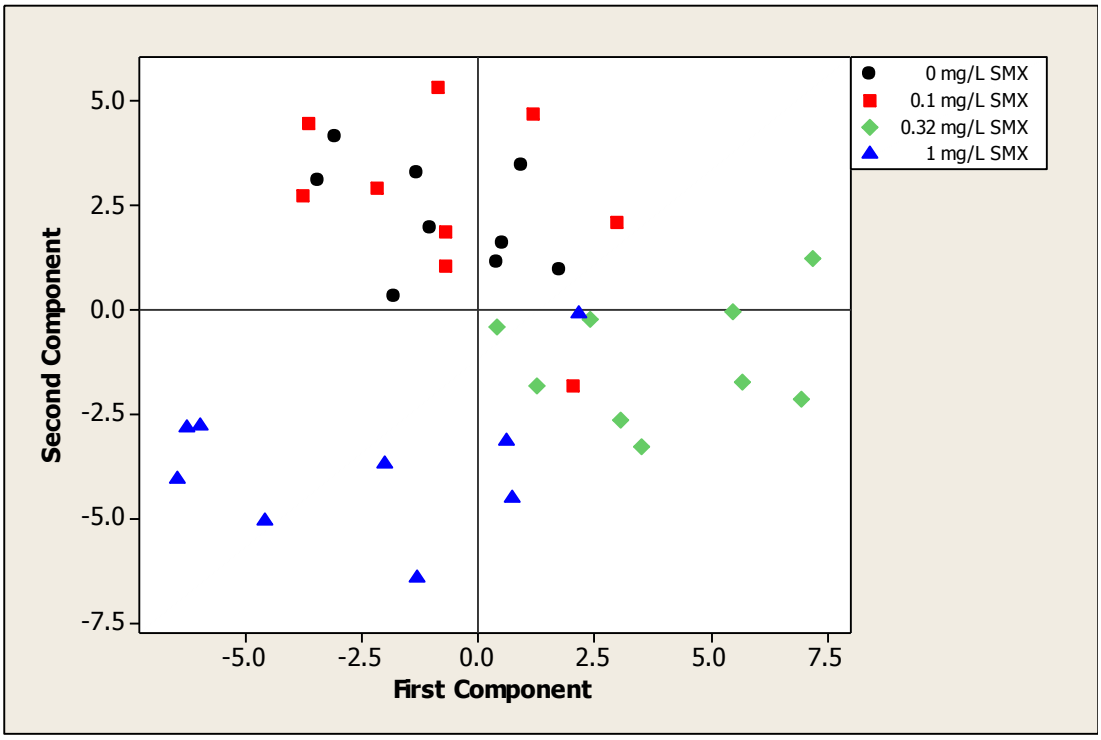
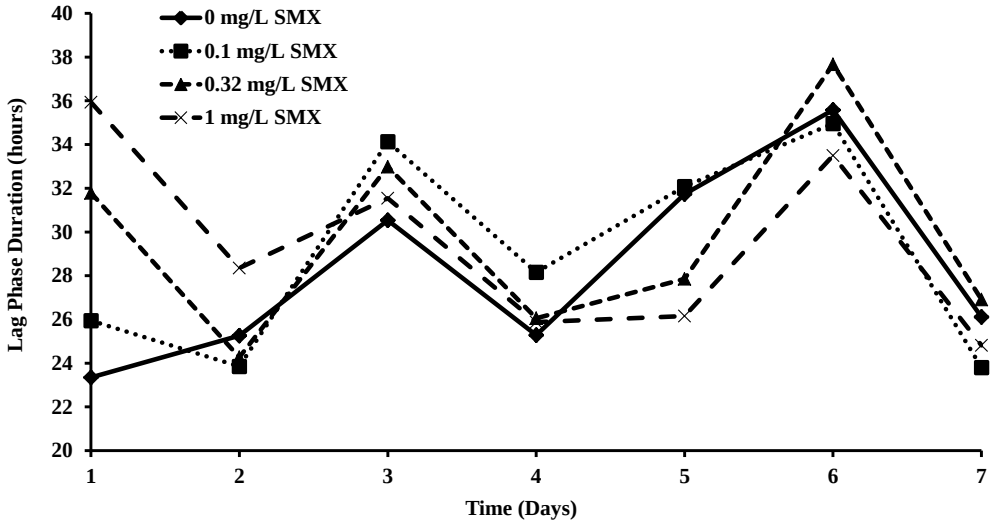


Figure 4.2: Scatter plots of PC scores for the first two principle components for individual replicates from each SMX treatment groups representing total substrate utilisation on a) day 1 and b) day 7. Day 1 (a) proportion of variation: PC1 = 69.3%, PC2 = 14.8%; Day 4 (b) proportion of variation: PC1 = 42.9%, PC2 = 23.4%.

a)



b)

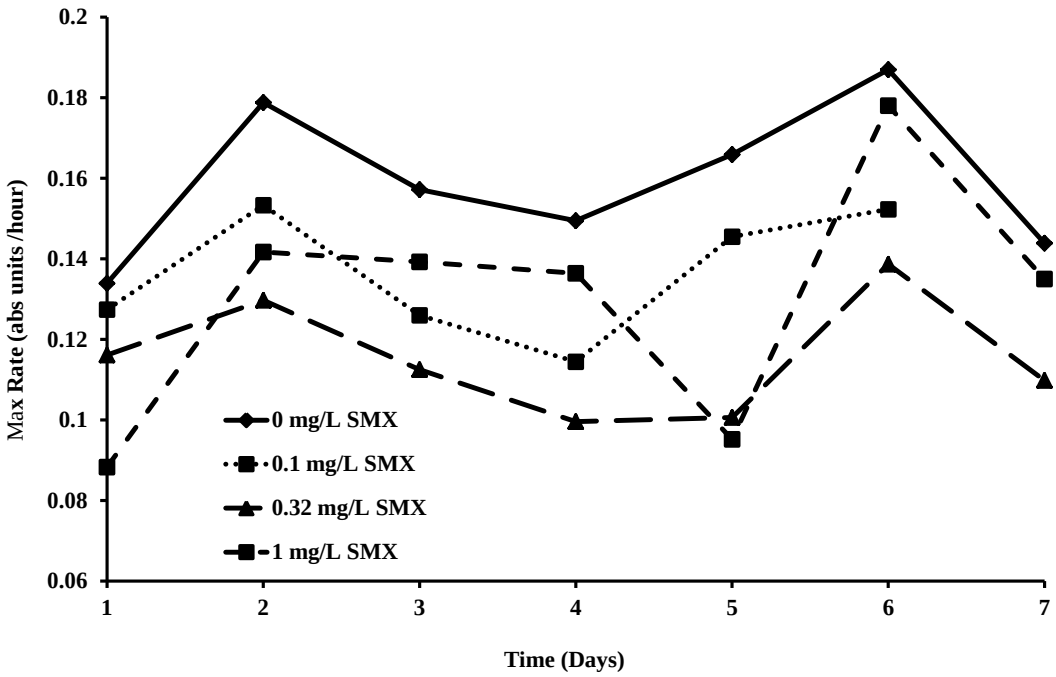


Figure 4.3: a) Lag phase duration versus exposure period time for total substrate utilisation and b) Max rate of total substrate utilisation versus exposure period time following exposure to SMX.

Table 4.3: Summary of one-way ANOVA results for lag phase values before total substrate utilization from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Lag Phase Duration[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	>ns	<ns	>ns	*>	>ns	<ns	<ns
0.32	>*	<ns	>ns	>ns	<ns	>ns	>ns
1	>*	>*	>ns	>ns	<*	<ns	<ns

Table 4.4: Summary of one-way ANOVA results for maximum rate of total substrate utilization values from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Max Rate[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	=	<*	<*	<*	<ns	<ns	<*
0.32	<*	<*	<*	<*	<*	<*	<*
1	<*	<*	<*	<ns	<ns	<ns	<ns

4.3.2. Effect of SMX on Amine and Amide Utilisation

The data in figure 4.4 show kinetic plots for amine and amine utilisation on days 1 (a), 4 (b) and 7 (c). Data shown in figure 4.5 show PCA score plots of amine and amide utilisation for the first two principle components on days 1 (a), 4 (b) and 7 (c). Figure 4.6 shows data representing lag phase duration and max rate values on days 1-7 of the exposure period. Tables 4.5 and 4.6 show statistical analysis of lag phase duration and max rate values of amine and amide utilisation in aquatic microcosms.

4.3.2.1. Kinetic Model Plots of Amine and Amide Utilisation

Kinetic model plots showing amine and amide utilisation show that SMX exposure resulted in an apparent inhibition of amine and amide utilisation on day 1 of the exposure in terms of lag phase, rate and the maximum absorbance that was recorded. The only exception was the maximum absorbance that was reached by 1 mg/L SMX plots; this exceeded the maximum absorbance reached by plots depecicting microcosms that had been exposed to 0.32 mg/L SMX. An apparent recovery by microcosms exposed to 1 mg/L SMX can be seen in plots depicting day 4 kinetic data, with slopes closely resembling those of control plots. On day 4, kinetic plots show that inhibitory effects can be seen at 0.1 and 0.32 mg/L SMX, with the most inhibition being observed at 0.32 mg/L SMX. On day 7, kinetic plots show that microcosms that had been treated with 0.1 and 0.32 mg/L SMX show no inhibition, with both curves showing a steeper slope and a higher maximum absorbance compared with the control slope. A recovery was therefore suggested at these two SMX concentrations. However, an inhibitory effect could still be observed in microcosms exposed to 0.32 mg/L SMX in terms of lag phase duration, maximum absorbance and slope steepness.

4.3.2.2. Lag Phase Duration: Amines and Amides

In terms of lag phase duration no significant increases could be observed as a result of exposing aquatic microcosms to 0.1 or 0.32 mg/L SMX on any day of the exposure period. However, a significantly higher lag phase duration was observed in microcosms that had been exposed to 1 mg/L SMX on days 1 and 2 of the study. A recovery started on day 3 onwards in these microcosms however, as shown by reuced lags times. In general, there was a trend for lag phase duration to decrease over time at all SMX concentrations. No apparent trend could be detected in control treatments.

4.3.2.3. Max Rate of Amine and Amide Utilisation

Although the graph showing max rate data on days 1-7 of the study shows the max rate of amine and amide utilisation was generally lower in treated microcosms, a statistically significant reduction in the max rate of amine and amide utilisation was only observed on day 4 of the exposure. These were observed in microcosms that had been exposed to 0.1 and 0.32 mg/L SMX.

No significant effects were observed as a result of treating microcosms to 1 mg/L SMX on any day of the study. In general, there was a trend of max rate of amine and amide utilisation increasing with time as result of all treatments.

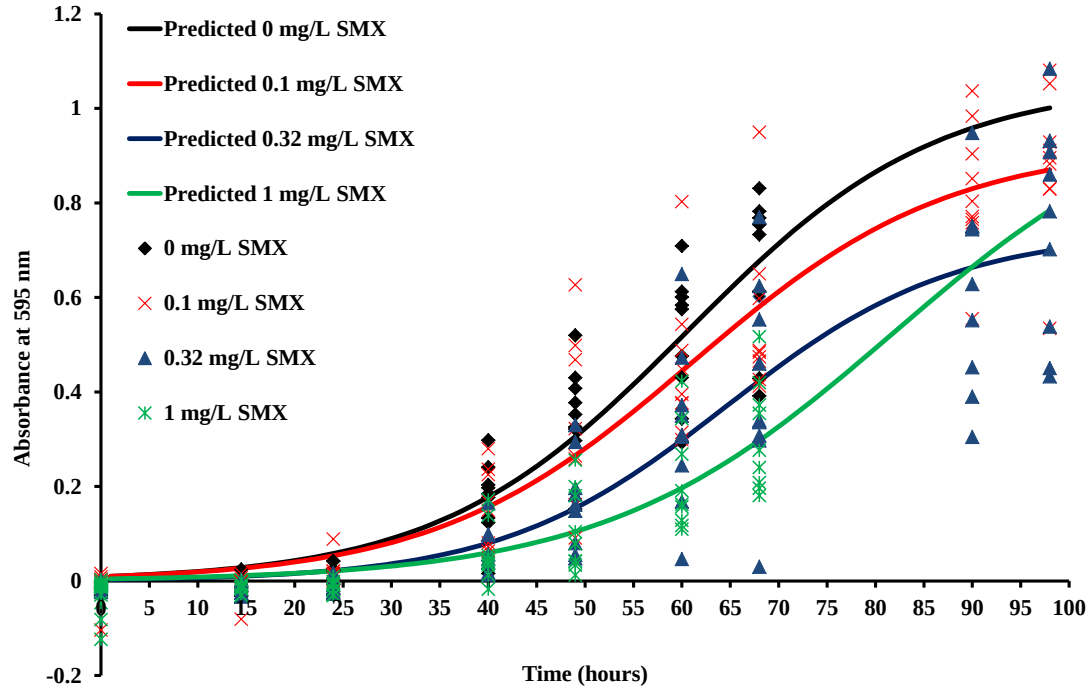
4.3.2.4. PCA Analysis of Amine and Amide Utilisation

PCA score plots of amine and amide utilisation illustrating day 1 substrate utilisation shows that there was a large degree of replicate mixing from different SMX treatments. Mixing of replicates could also be observed at all SMX treatments and with control replicates also. However, there was another large replicate cluster that grouped treated replicates from all SMX treatments but no control replicates. This would suggest that a portion of the microbial community was utilising amines and amides slightly differently at all SMX doses but a portion of the microbial community was also utilising amines and amides in a similar manner. These data suggest that the microbial community structure for microbes that were utilising amines and amides was slightly different in microcosms exposed to all SMX concentrations.

On day 4 of the exposure, replicates from treated microcosms tended to cluster together with only minimum mixing with control replicates. There was however a large degree of mixing between replicates from microcosms that had been exposed to all SMX concentrations. These results would suggest that the microbial community in all treated microcosms were utilising amines and amides differently form the microbial community that was present in control microcosms. It is therefore possible that the community structure of amine and amide utilising microbes was different as the result of exposing microcosms to SMX.

A similar picture was observed on day 7 of the study; again, replicates from treated microcosms tended to cluster. There was however a greater degree of mixing between control and treated replicates on day 7, suggesting that the amine and amide utilising microbial community became slightly more similar to control replicates in terms of amine and amide utilisation.

a)



b)

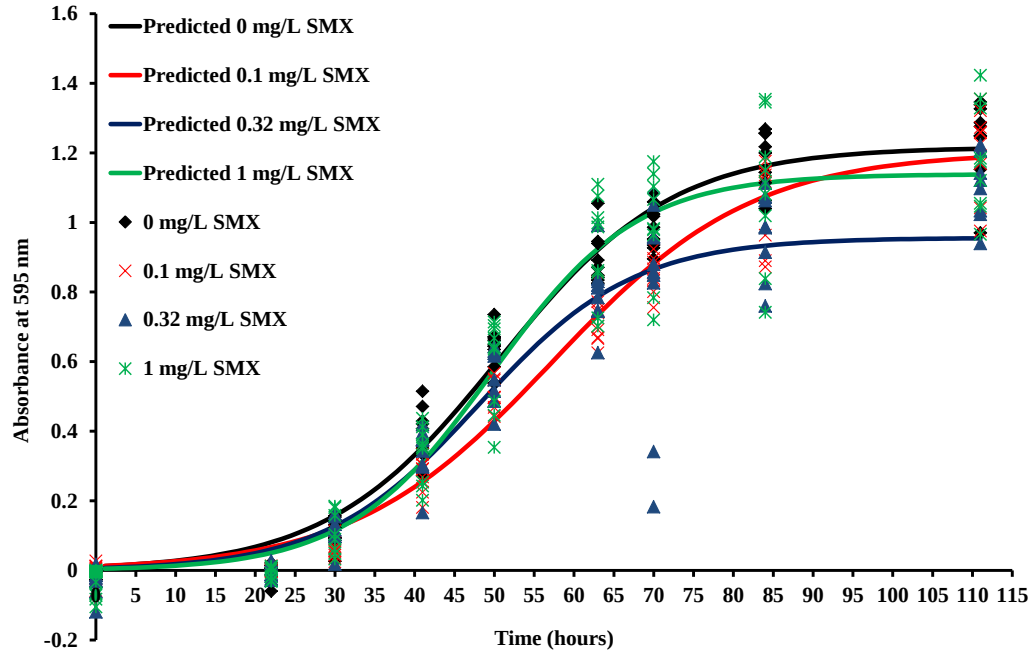


Figure 4.4: Kinetic plots of amine and amide utilisation following SMX exposure on a) day 1, b) day 4 and c) day 7.

c)

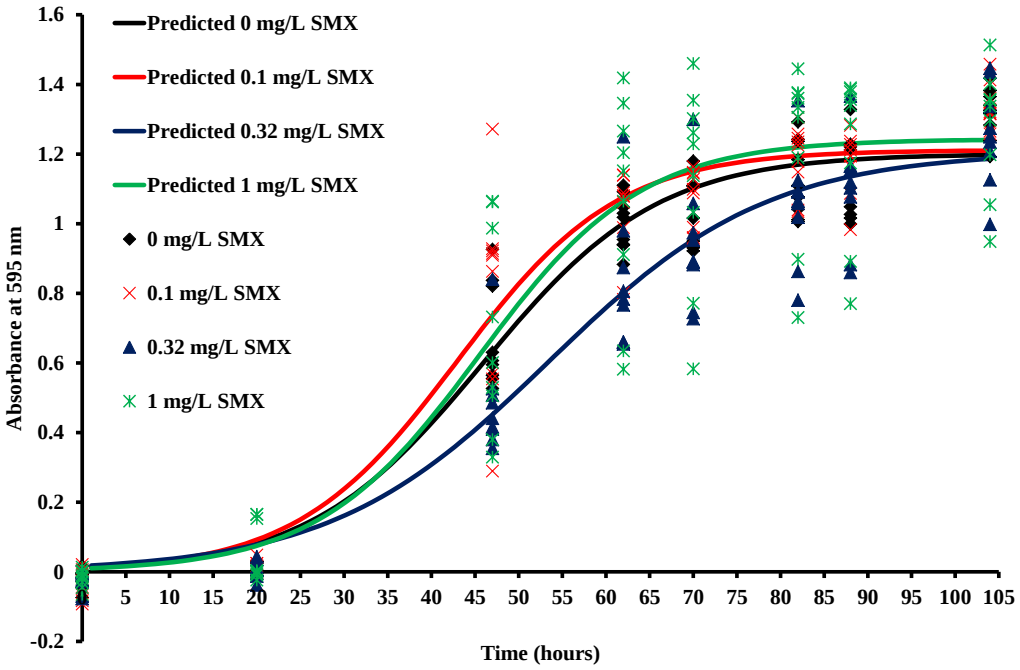
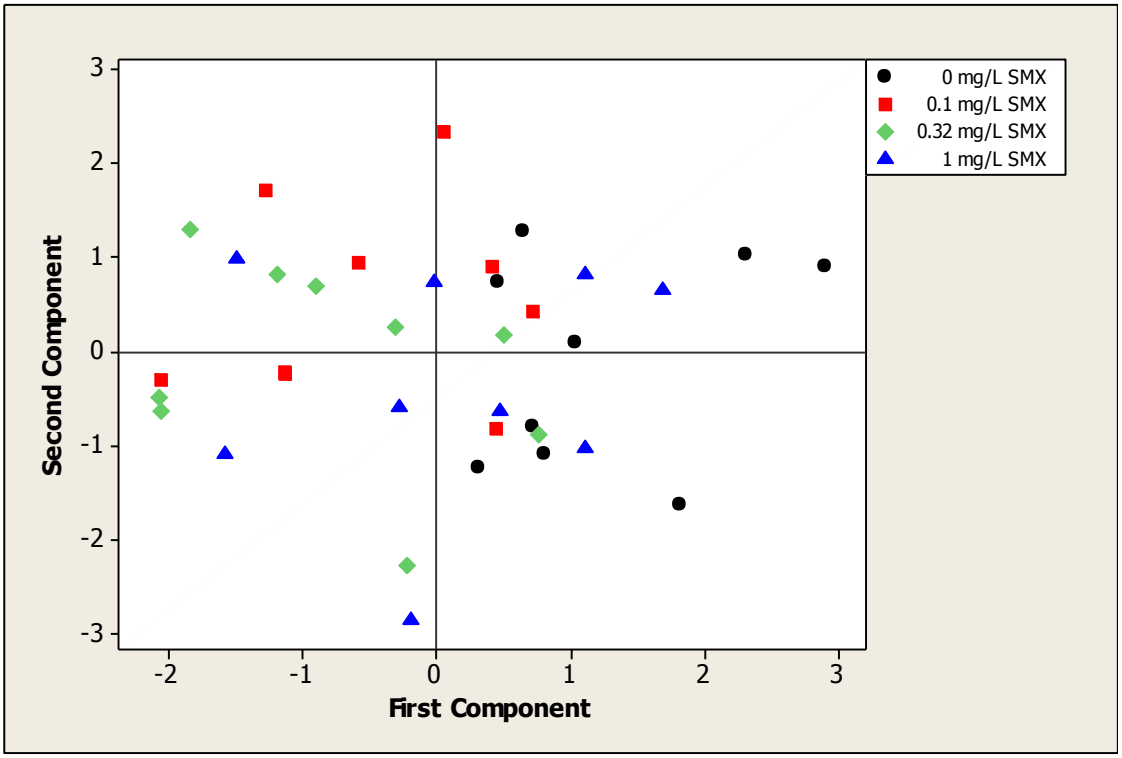


Figure 4.4 (cont).

a)



b)

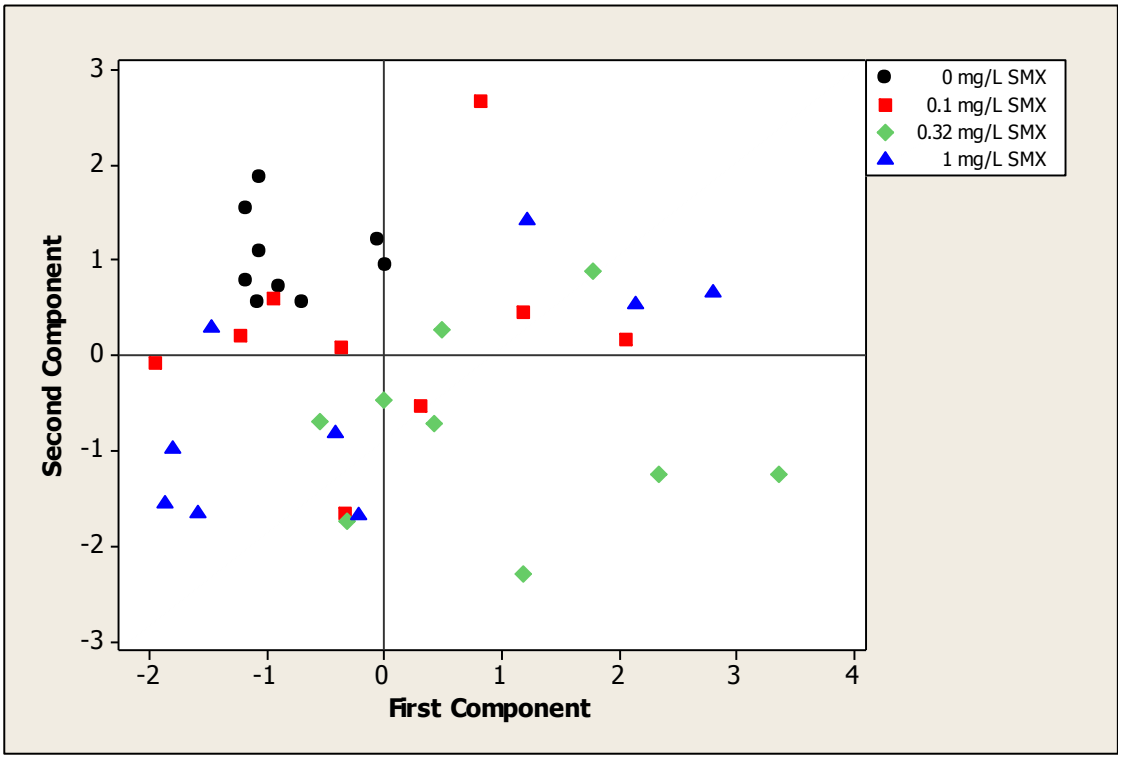
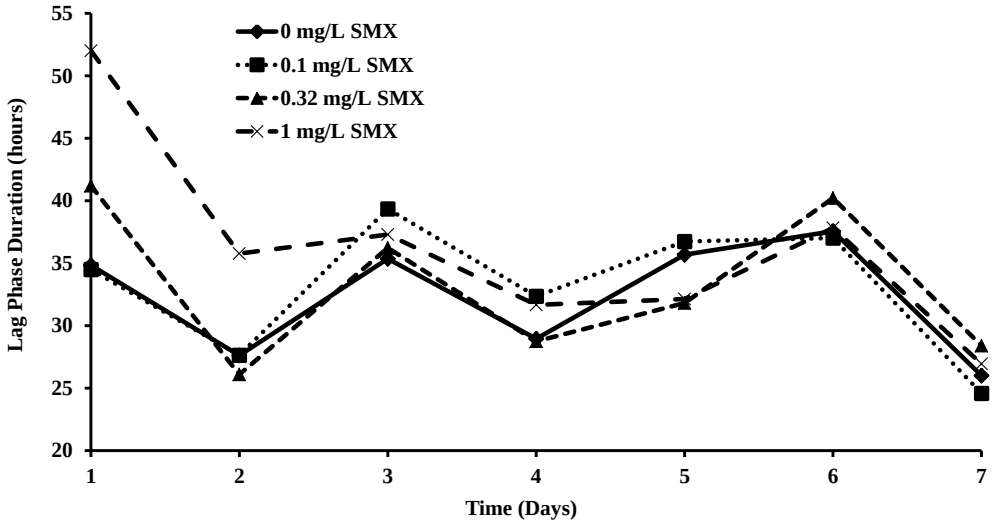


Figure 4.5: Scatter plots of PC scores for the first two principle components for individual replicates from each SMX treatment groups representing amine and amide utilisation on a) day 1 and b) day 7. Day 1 (a) proportion of variation: PC1 = 52.5%, PC2 = 9.2%; Day 7 (b) proportion of variation: PC1 = 20.8%, PC2 = 14.6%.

a)



b)

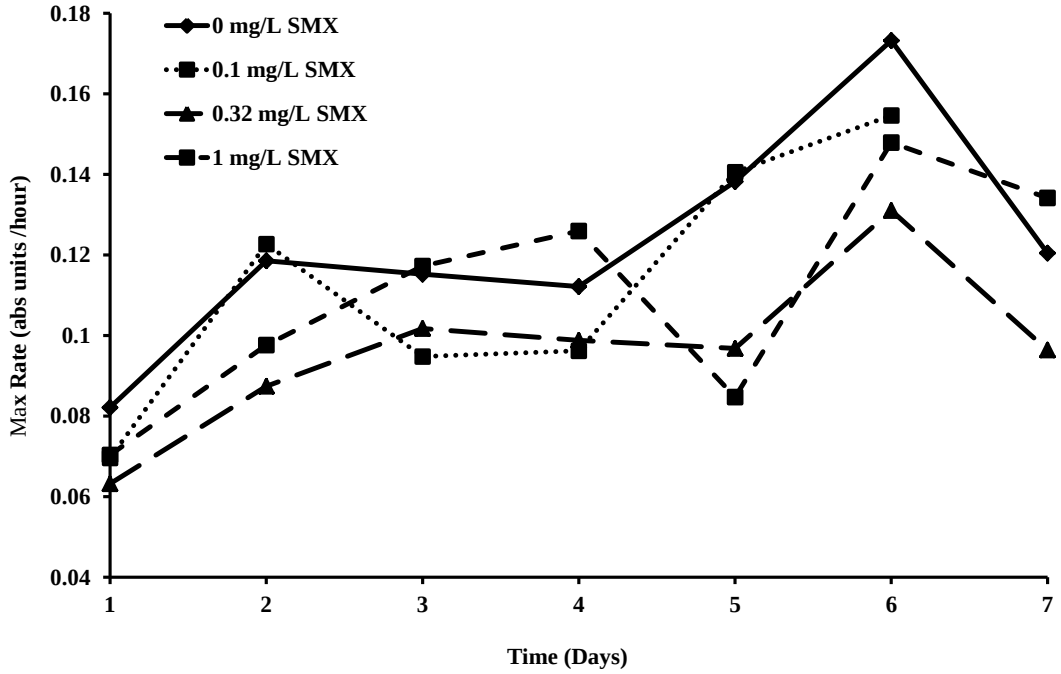


Figure 4.6: a) Lag phase duration versus exposure period time for amine and amide utilisation and b) Max rate of amine and amide utilisation versus exposure period time following exposure to SMX.

Table 4.5: Summary of one-way ANOVA results for lag phase values before amine and amide utilization from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Lag Phase Duration[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns<	ns>	>ns	>ns	>ns	>ns	<ns
0.32	ns>	ns<	<ns	<ns	<ns	>ns	>ns
1	*>	*>	>ns	>ns	<ns	>ns	>ns

Table 4.6: Summary of one-way ANOVA results for maximum rate of amine and amide utilization values from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Max Rate[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns<	=	ns<	*<	=	ns<	ns<
0.32	ns<	ns<	ns<	*<	ns<	ns<	ns<
1	ns<	ns<	ns>	ns>	ns<	ns<	ns>

4.3.3. Effect of SMX on Amino Acid Utilisation

The data in figure 4.7 shows kinetic plots representing amino acid utilisation on days 1 (a), 4 (b) and 7 (c). PCA score plots of amino acid utilisation fingerprints are shown in figure 4.8 a, day 4 and b, day 7. Figure 4.9 (a and b) shows lag phase durations and max rate values of amino acid utilisation for all SMX treatments versus time. Table 4.7 show a statistical analysis of lag phase duration before amino acid utilisation as a result of SMX exposure. Table 4.8 shows a statistical analysis of the max rate of amino acid utilisation as result of SMX exposure.

4.3.3.1. Kinetic Model Plots of Amino Acid Utilisation

Kinetic plots of amino utilisation on day 1 show that SMX had an inhibitory effect at all SMX concentrations. This was evident in terms of the slope of plots as well as an apparent increase in lag phase duration and the maximum absorbance that was reached by plots. Day 4 kinetic plots show a recovery in terms of amino acid utilisation in 1 mg/L SMX-treated microcosms. An inhibition in amino acid utilisation could still be observed at the two lower SMX treatments in terms of lag phase duration and maximum absorbance. By day 7 of the study, maximum absorbances (for all SMX doses) were lower than the control value and the rate of amino acid utilisation seemed to be lower in microcosms treated with 0.32 mg/L SMX.

4.3.3.2. Lag Phase Duration: Amino Acid Utilisation

On day 1 of the investigation all treated microcosms displayed a longer lag phase duration before amino acid utilisation occurred. These increases were shown to be statistically significant for SMX treatments of 0.32 and 1 mg/L. In the case of the 1 mg/L treatment, a further significantly higher lag phase duration was observed on day 2 also. Lag phase durations were generally higher at all SMX doses until day 4, although not significantly so. After day 4, lag phases were reduced to values either near or less than the control.

4.3.3.3. Max Rate of Amino Acid Production

On day 1 of the exposure, a significant reduction in the max rate of amino acid utilisation was observed as a result of exposing the microbial community to 1 mg/L SMX. The only other statistically significant reduction in the max rate of amino acid utilisation could be observed on day 4 of the study at 0.1 mg/L SMX. There was however no clear trend in terms of the max rate of amino acid utilisation over time.

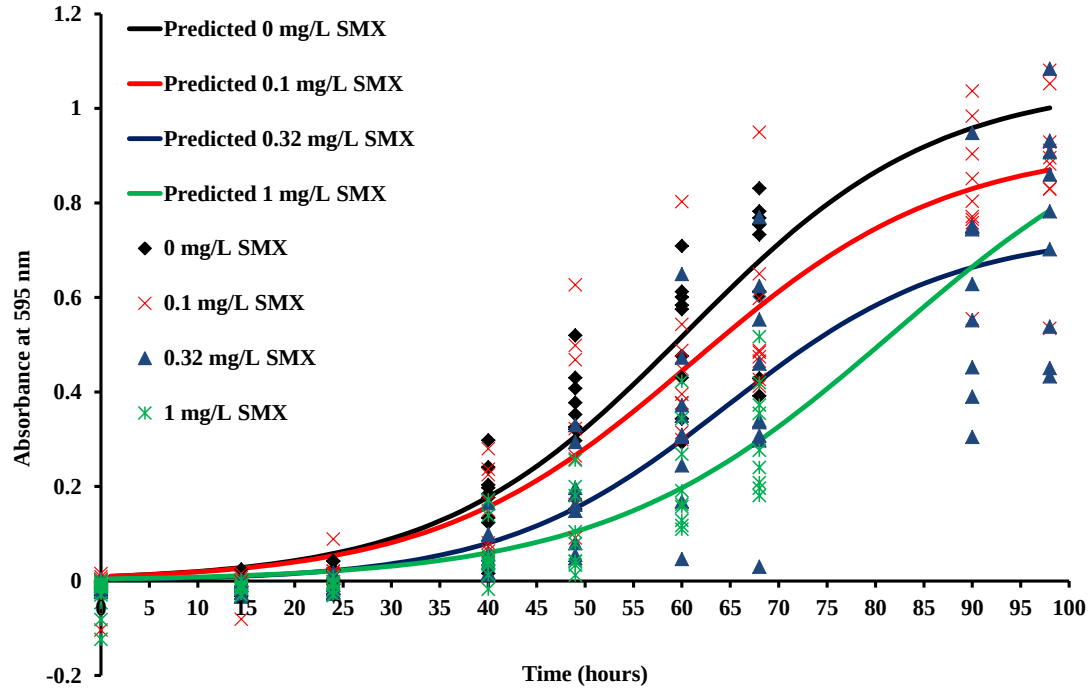
4.3.3.4. PCA Analysis of Amino Acid Utilisation

Day 1 of the exposure period revealed a large degree of mixing between replicates from different SMX treatments, suggesting a degree of similarity in amino acid utilisation between treatments.

This further suggests that the amino acid utilising microbial community structure was also similar between doses. On day 4 however replicates treated with SMX could be separated from control replicates, suggesting a difference in amino acid utilisation between treated and non-treated microbial communities. 0.1 and 1 mg/L SMX treatments could also be separated with mixing of 0.32 mg/L replicates in between. These data suggest that on day 4 of the exposure period the portion of the microbial community that was utilising amino acids had been altered (compared with control communities) in 0.1 and mg/L SMX dosed microcosms and that these communities shared some structural similarity with communities that had been exposed to 0.32 mg/L SMX.

On day 7 of the study, the control and 0.1 mg/L groups were utilising amino acids in a similar manner with some similarity to 0.32 mg/L treated microbial communities as shown by a degree of mixing. Replicates treated with 1 mg/L SMX clustered separately however, indicating that this treatment had resulted in a microbial community that were utilising amino acids differently from the control/0.1 mg/L SMX dosed communities. There was however some mixing with 0.32 mg/L SMX replicates, indicating some similarity between the amino acid utilisation profile (and therefore community structure) of microbial communities exposed to these SMX doses.

a)



b)

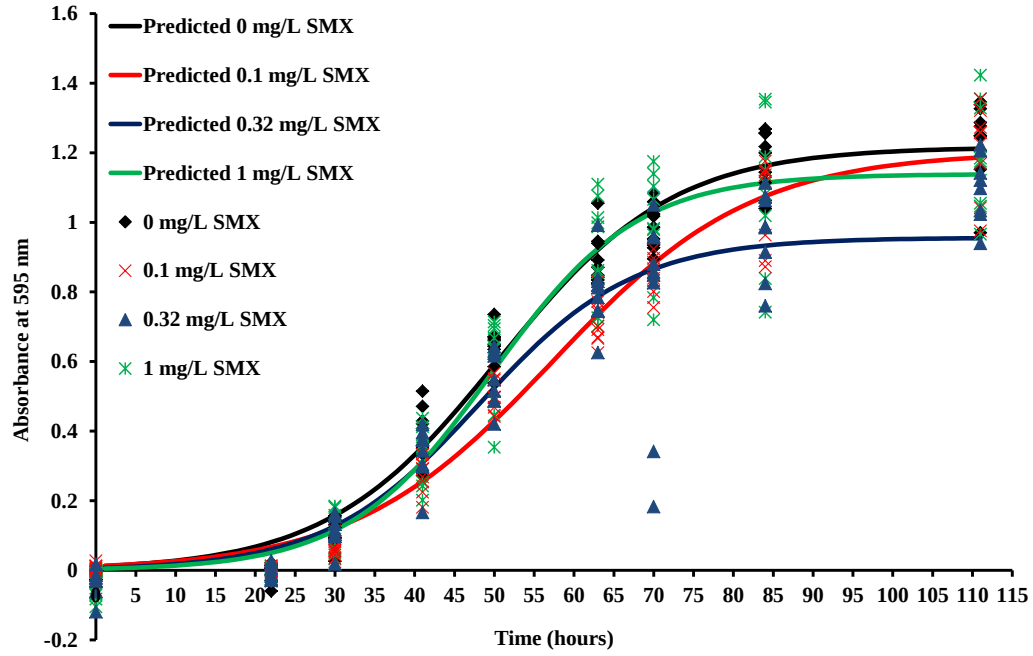


Figure 4.7: Kinetic plots of amino acid utilisation following SMX exposure on a) day 1, b) day 4 and c) day 7.

c)

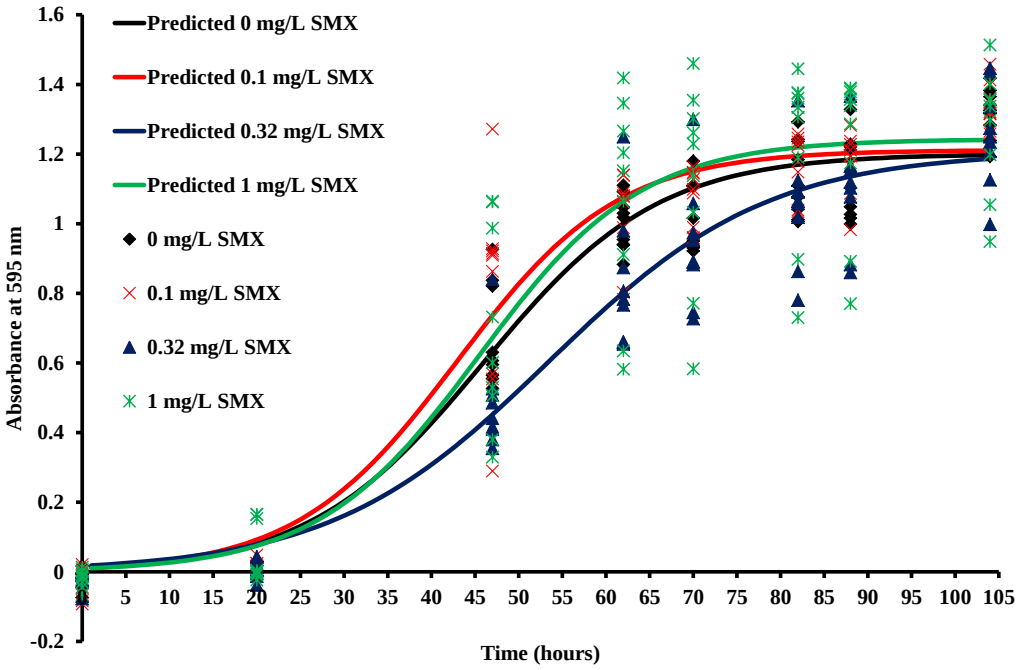
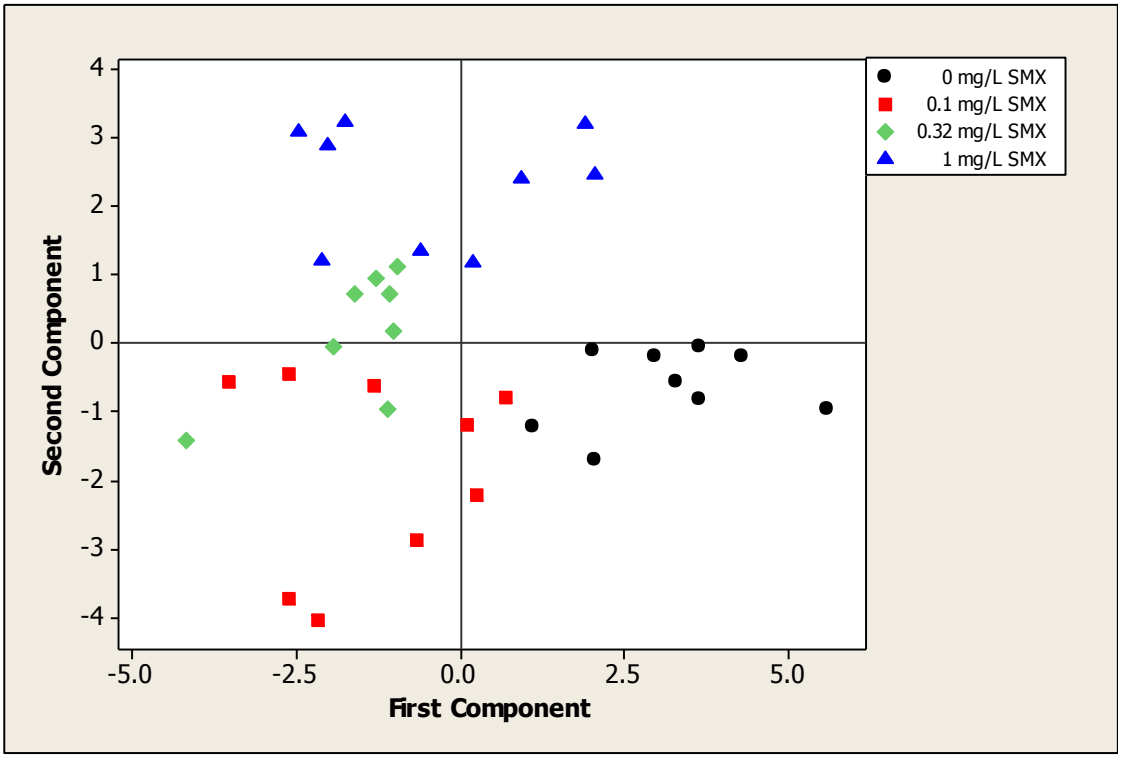


Figure 4.7 (cont).

a)



b)

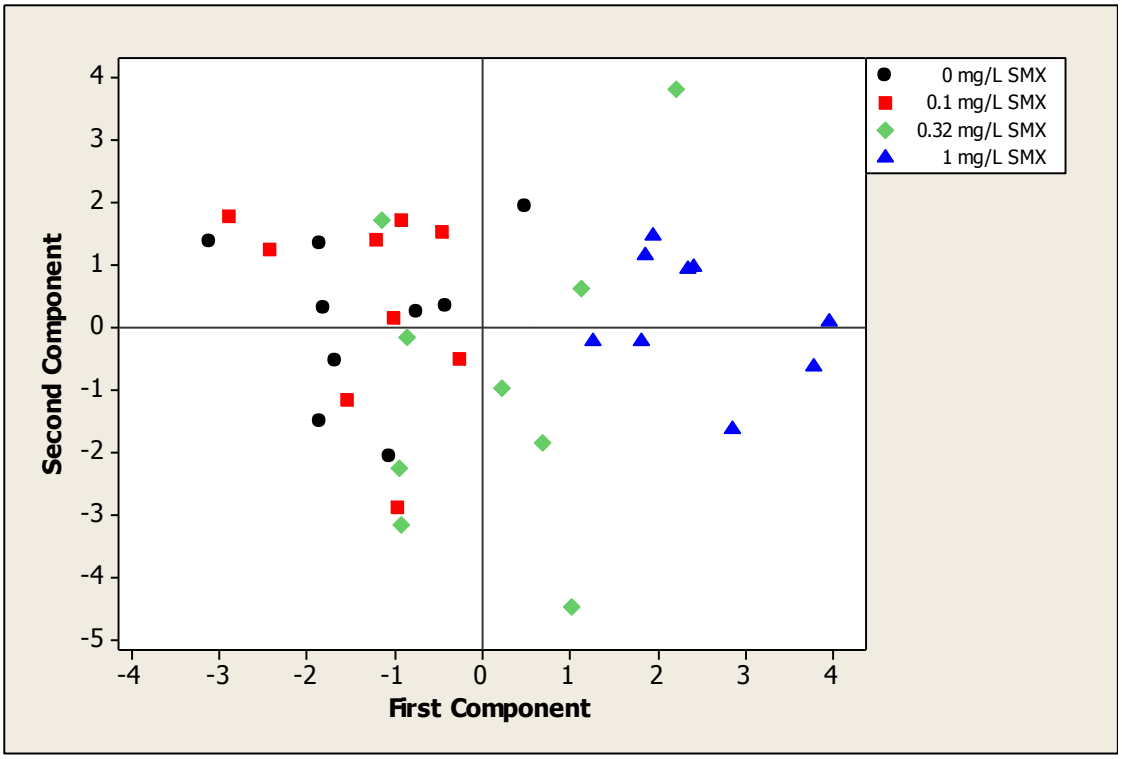
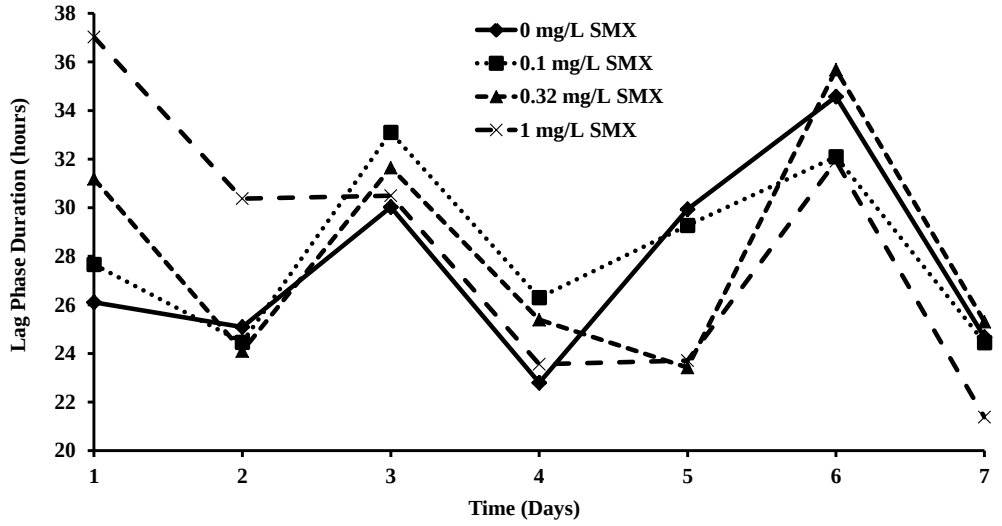


Figure 4.8: Scatter plots of PC scores for the first two principle components for individual replicates from each SMX treatment groups representing amino acid utilisation on a) day 4 and b) day 7. Day 4 (a) proportion of variation: PC1 = 42.3%, PC2 = 18.5%; Day 7 (b) proportion of variation: PC1 = 19.9%, PC2 = 16.7%.

a)



b)

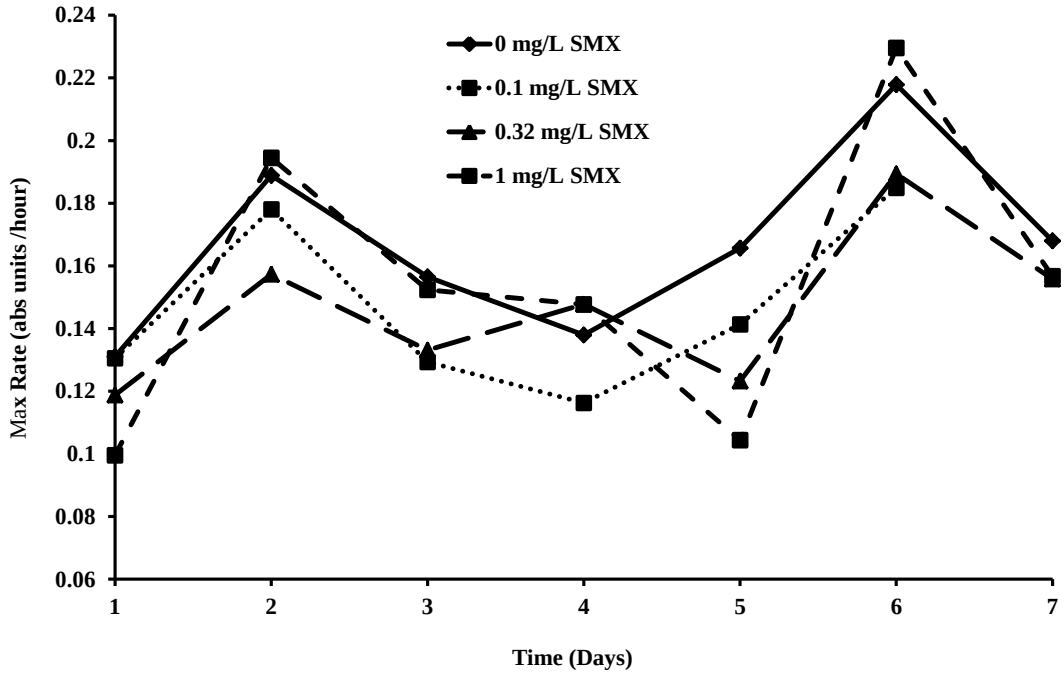


Figure 4.9: a) Lag phase duration versus exposure period time for amino acid utilisation and b) Max rate of amino acid utilisation versus exposure period time following exposure to SMX.

Table 4.7: Summary of one-way ANOVA results for lag phase values before amino acid utilization from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Lag Phase Duration[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns>	ns<	ns>	ns>	ns<	ns<	ns<
0.32	*>	ns<	ns>	ns>	ns<	ns>	ns>
1	*>	*>	ns>	ns>	ns<	ns<	ns<

Table 4.8: Summary of one-way ANOVA results for maximum rate of amino acid utilization values from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Max Rate[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	=	ns<	ns<	*<	ns<	ns<	ns<
0.32	ns<	<ns	ns<	ns<	ns>	ns<	ns<
1	*<	=	ns<	ns<	ns<	ns>	ns<

4.3.4. Effect of SMX on Carbohydrate Utilisation

The data in figure 4.10 illustrate kinetic plots of carbohydrate utilisation on days 1 (a), 4 (b) and 7 (c). Figure 4.11 shows PCA score plots of carbohydrate utilisation on days 1 (a) and 7 (b). The data in figure 4.12 show lag phase duration before carbohydrate utilisation (a) and the max rate of carbohydrate utilisation (b). Tables 4.9 and 4.10 show statistically significant changes in carbohydrate utilisation in terms of lag phase duration and max rate respectively.

4.3.4.1. Kinetic Plots of Carbohydrate Utilisation

Day 1 kinetic plots suggest that carbohydrate utilisation was inhibited by all doses of SMX. The inhibition was visually apparent in terms of lag phase, rate and the maximum absorbance that was reached by plots. On day 4, a similar pattern was visible, with SMX inhibiting carbohydrate utilisation in an almost identical fashion to day 1. On day 7, microcosms treated with 1 mg/L SMX showed a general recovery; kinetic plots representing these microcosms became very similar to control plots. There was still an apparent inhibition of carbohydrate utilisation in microcosms treated with 0.1 and 0.32 mg/L SMX however. This could be visually observed in terms of lag phase duration, steepness of curves and maximum absorbance reached by these plots.

4.3.4.2. Lag Phase Duration: Carbohydrate Utilisation

All lag phase values apart from the highest SMX treatment showed a general increase over time. Day 1 values represented a statistically significant rise in lag phase duration as the result of exposing microbial communities to all SMX concentrations. At the highest SMX treatment, no significant effects could be detected after day 1and a significant reduction was shown on day 5. A recovery in lag phase duration in microcosms treated with 1 mg/L SMX was therefore suggested. In microcosms that had been treated with 0.32 mg/L SMX, significant effects were still being observed on days 3, 4 and 7, suggesting that a recovery had not occurred. There was however intermittent periods (days 2, 5 and 6) when no significant effects on lag phase duration could be shown. An increase in lag phase duration was also observed on day 4 of the study in microcosms that had been treated with 0.1 mg/L SMX, although no significant effects were seen after this time-point. These microcosms were therefore likely to have been showing a recovery in terms of lag phase duration.

4.3.4.3. Max Rate of Carbohydrate Utilisation

Graphs depicting the max rate of carbohydrate utilisation versus time show that max rate values representing all SMX treatments were lower than the control value for the duration of the exposure. The exception to this was observed in microcosms that had been exposed to 1 mg/L SMX. At this dose the max rate of carbohydrate utilisation was either equal to or higher than the control value on day 6 and 7 of the exposure. On day 1 at 0.32 and 1 mg/L SMX, the max rate of carbohydrate utilisation was significantly lower than the control. At the highest SMX treatment, further

significant reductions were witnessed on days 2, 4 and 5. At the lowest SMX concentration, a significant reduction in the max rate of carbohydrate utilisation could be seen on days 2, 3, 4 and 7. In microcosm treated with 0.32 mg/L SMX, the max rate of carbohydrate utilisation was significantly reduced during the entire exposure period.

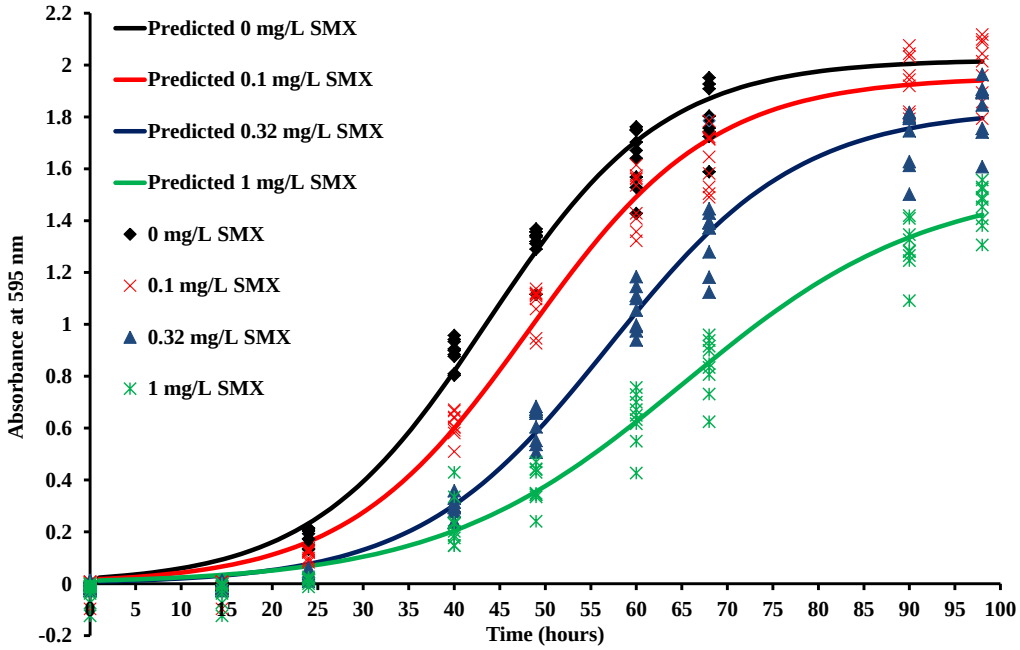
4.3.4.4. PCA Analysis of Carbohydrate Utilisation

On day 1 of the exposure, PCA score plots indicate that the two highest SMX concentrations resulted in distinct clustering of replicates and that 0.1 mg/L replicates were mixed with control replicates. These data would suggest that the microbial community that had been exposed to 0.32 and 1 mg/L SMX utilised carbohydrates in a similar manner as did microbial communities present in 0.1 mg/L SMX-treated and control communities. Based on these data, it is likely that an altered community structure resulted form exposure to 0.32 and 1 mg/L SMX.

Day 4 PCA score plots show that the microbial community that had been exposed to 0.32 mg/L SMX clustered in a distinct manner from other treatments. Other treatments also clustered distinctly; minimal mixing with control replicates was observed. Some mixing was also observed between 0.1 and 1 mg/L replicates. SMX treatment on day 4 had therefore resulted in differential utilisation of carbohydrates by microbial communities exposed to all SMX concentrations. It is likely therefore that the microbial community structures were different in treated and control microcosms also.

Day 7 plots reveal that the exposure of the microbial community to all SMX concentrations resulted in a complete separation of treated and control replicates. There was only minor mixing (1 replicate) between 0.1 and 1 mg/L SMX-treated replicates. These observations would suggest that on day 7 of the exposure microbial communities that had been exposed to all SMX concentrations resulted in differential utilisation of carbohydrates. It is also likely therefore that the microbial community structures were different at these SMX treatments.

a)



b)

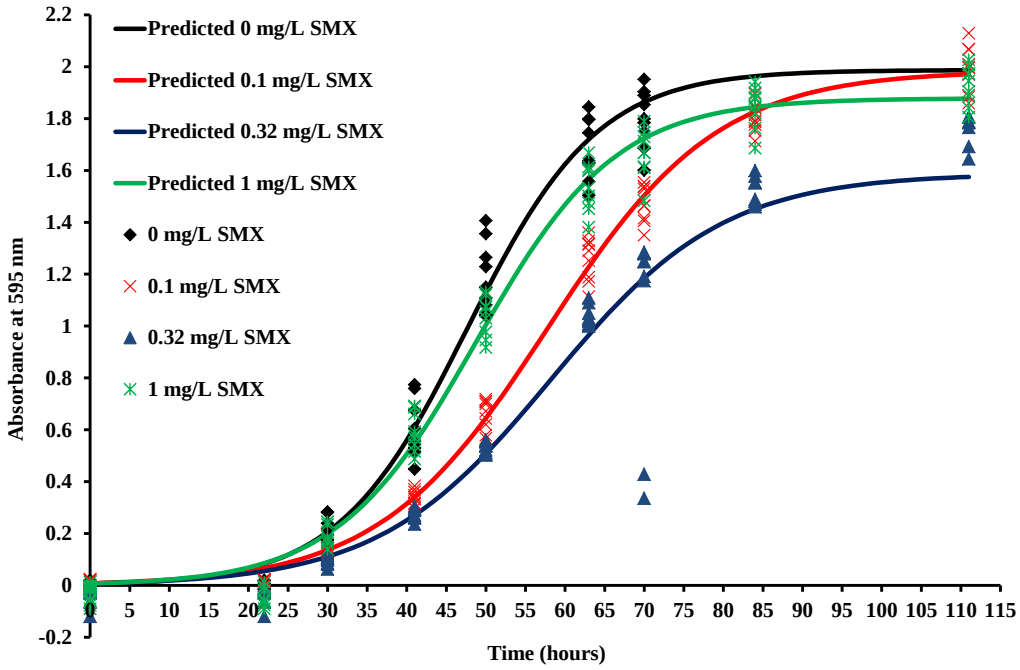


Figure 4.10: Kinetic plots of carbohydrate utilisation following SMX exposure on a) day 1, b) day 4 and c) day 7.

c)

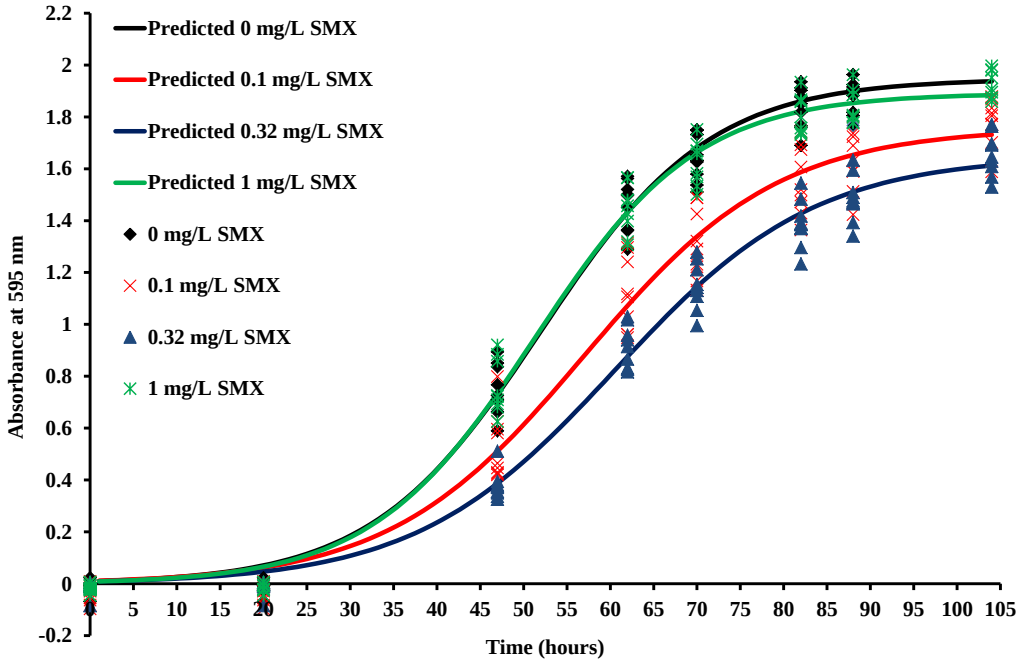
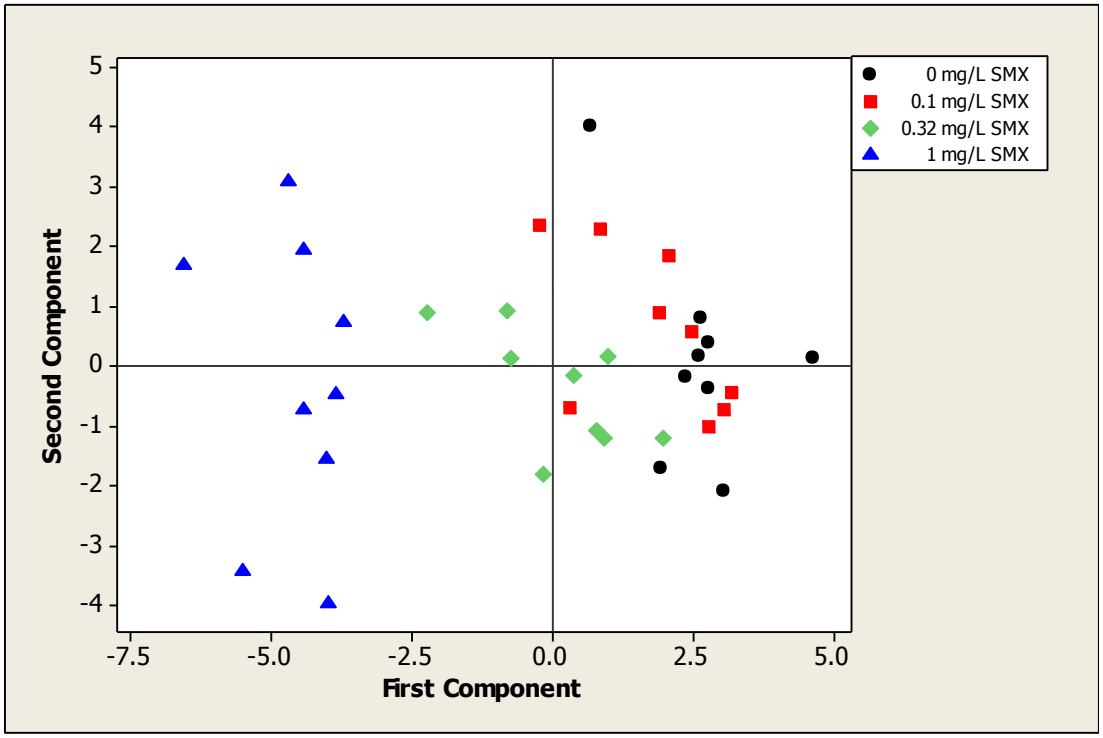


Figure 4.10 (cont).

a)



b)

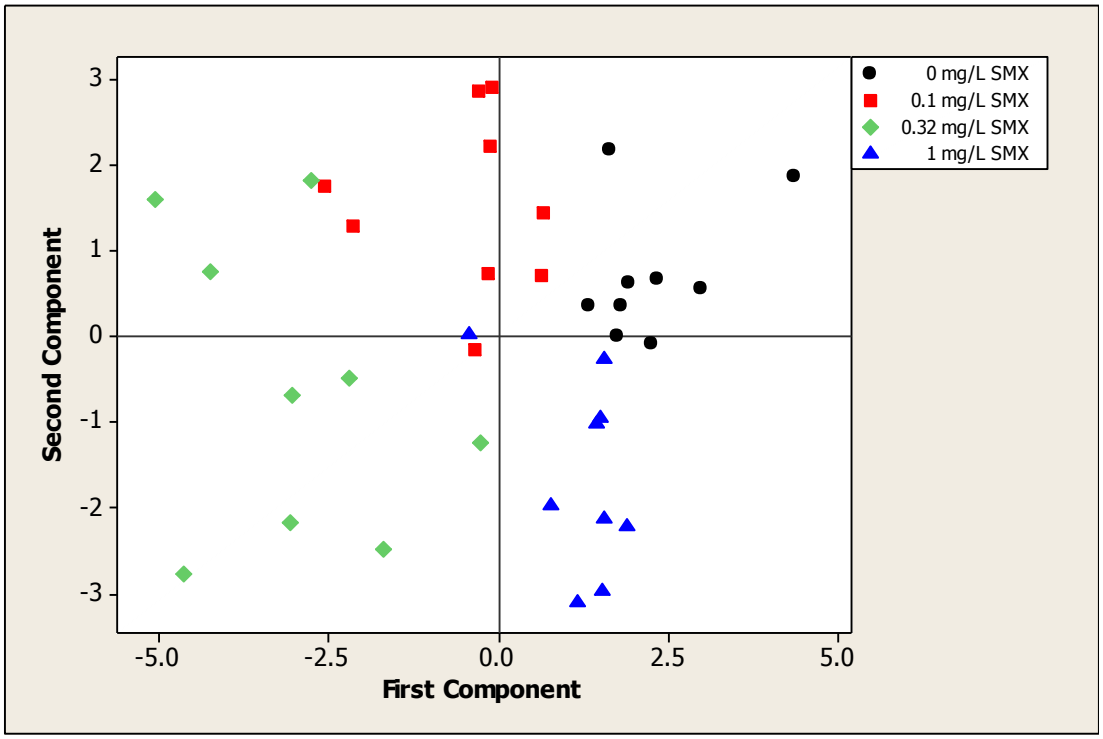
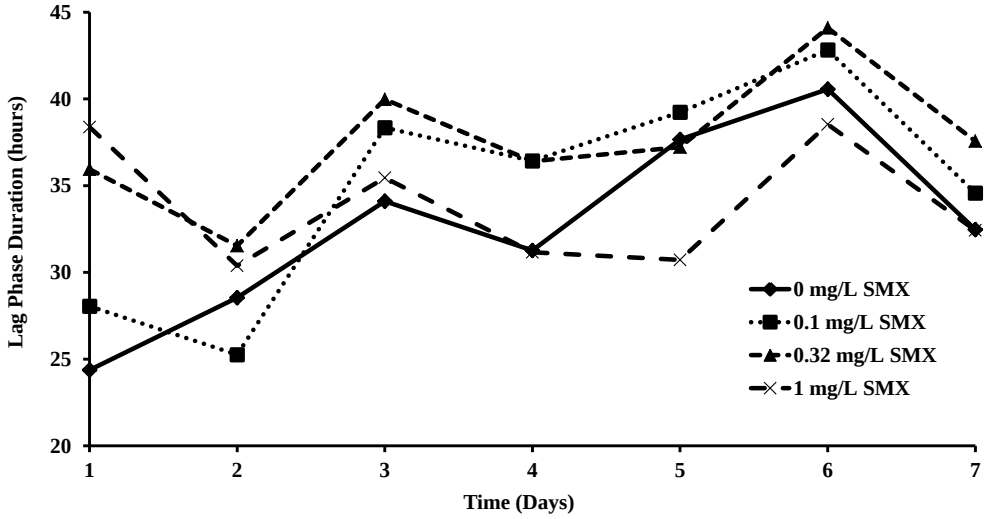


Figure 4.11: Scatter plots of PC scores for the first two principle components for individual replicates from each SMX treatment groups representing carbohydrate utilisation on a) day 1 and b) day 7. Day 1 (a) proportion of variation: PC1 = 72.1%, PC2 = 6.9%; Day 7 (b) proportion of variation: PC1 = 22.3%, PC2 = 21.9%.

a)



b)

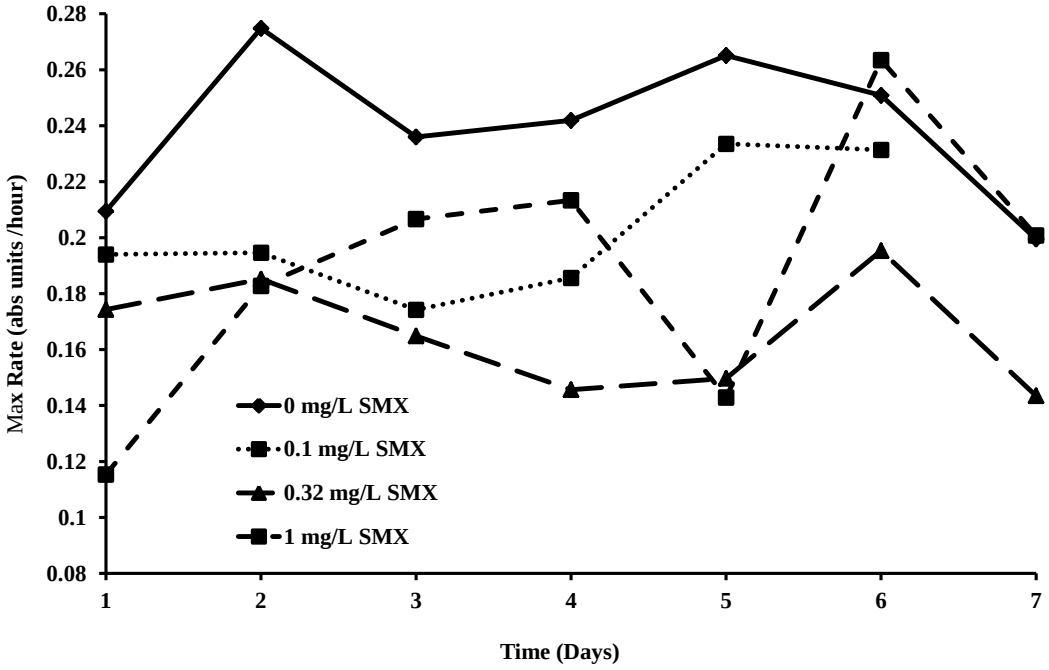


Figure 4.12: a) Lag phase duration versus exposure period time for carbohydrate utilisation and b) Max rate of carbohydrate utilisation versus exposure period time following exposure to SMX.

Table 4.9: Summary of one-way ANOVA results for lag phase values before carbohydrate utilization from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Lag Phase Duration[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	*>	ns<	ns>	*>	ns>	ns>	ns>
0.32	*>	ns>	*>	*>	ns>	ns>	*>
1	*>	ns>	ns>	ns<	*<	ns<	ns<

Table 4.10: Summary of one-way ANOVA results for maximum rate of carbohydrate utilization values from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Max Rate[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns<	*<	*<	*<	ns<	ns<	*<
0.32	*<	*<	*<	*<	*<	*<	*<
1	*<	*<	ns<	*<	*<	ns>	=

4.3.5. Effect of SMX on Carboxylic Acid Utilisation

The data represented by figure 4.13 show kinetic plots for carboxylic acid utilisation on days 1 (a), 4 (b) and 7 (c) of the study. Figure 4.14 shows PCA score plots representing data for days 1 (a) and 7 (b). The data in figures 4.15 show lag phase duration following carboxylic acid utilisation on days 1-7 (a) and max rate of carboxylic acid utilisation on day 1-7 (b). Tables 4.11 and 4.12 show statistically significant changes in lag phase duration and max rate respectively.

4.3.5.1. Kinetic Model Plots of Carboxylic Acid Utilisation

Day 1 kinetic plots show that there was an initial inhibition of carboxylic acid utilisation in terms of the visual increase in lag phase duration, slope and the maximum absorbance that was reached by plots illustrating all SMX doses.

On day 4 of the exposure, inhibition could still be observed at all SMX concentrations, with the lag phase, slope and maximum absorbance still showing signs of inhibition compared with the control kinetic plot. The greatest effects were seen as a result of exposing microcosms to 0.32 mg/L SMX. Plots representing 0.1 and 1 mg/L SMX exposures were more similar in terms of slope, lag phase duration and the maximum absorbance that was reached by the plot.

At the end of the exposure (day 7), there were signs of a slight recovery compared with plots from previous days. There were still some visible signs of the inhibitory effects of SMX however. Plots representing 0.l and 1 mg/L were not as steep as the control plot slope indicating a possible slower rate of carboxylic acid utilisation. The maximum absorbance that was reached by these two plots was below also that of the control. In addition, although the steepness of the curve representing 0.1 mg/L SMX was comparable to the control plot in terms maximum steepness, the maximum absorbance that was reached was not as high.

4.3.5.2. Lag Phase Duration: Carboxylic Acid Utilisation

The data in graphs depicting lag phase duration versus time do not follow a trend at any SMX dose (including the control). However, on days 1, 3 and 4 of the exposure lag phase durations for all exposures were higher than the lag phase duration that was observed in control microcosms. In terms of statistically significant increases in lag phase duration, a significant increase was observed on day 1 at 0.32 and 1 mg/L SMX. After this, there was an apparent recovery at these SMX concentrations. A significant increase in lag phase duration was also observed on day 4 as a result of exposing the microbial community to 0.1 mg/L SMX.

4.3.5.3. Max Rate of Carboxylic Acid Utilisation

On all days of the exposure period every treatment of SMX caused a reduction in the max rate of carboxylic acid utilisation, with the exception of 0.1 mg/L SMX, which didn't cause any inhibition on day 2. There was no apparent trend to each plot over time.

Statistically significant reductions in the max rate of carboxylic acid utilisation were observed on days 1 and 2 as a result of exposing microcosms to 1 mg/L SMX. There was no further inhibition after this, suggesting that a recovery had taken place after day 2. Although no effects on the max rate of carboxylic acid utilisation were observed on day 1 of the study in microcosms spiked with 0.32 mg/L, a significant reduction was observed on all subsequent days. No significant effects on the max rate of carboxylic acid utilisation were observed in microcosms spiked with 0.1 mg/L on days 1, 2, 5 or 6, although significant effects were observed at this SMX concentration on days 3, 4 and 7.

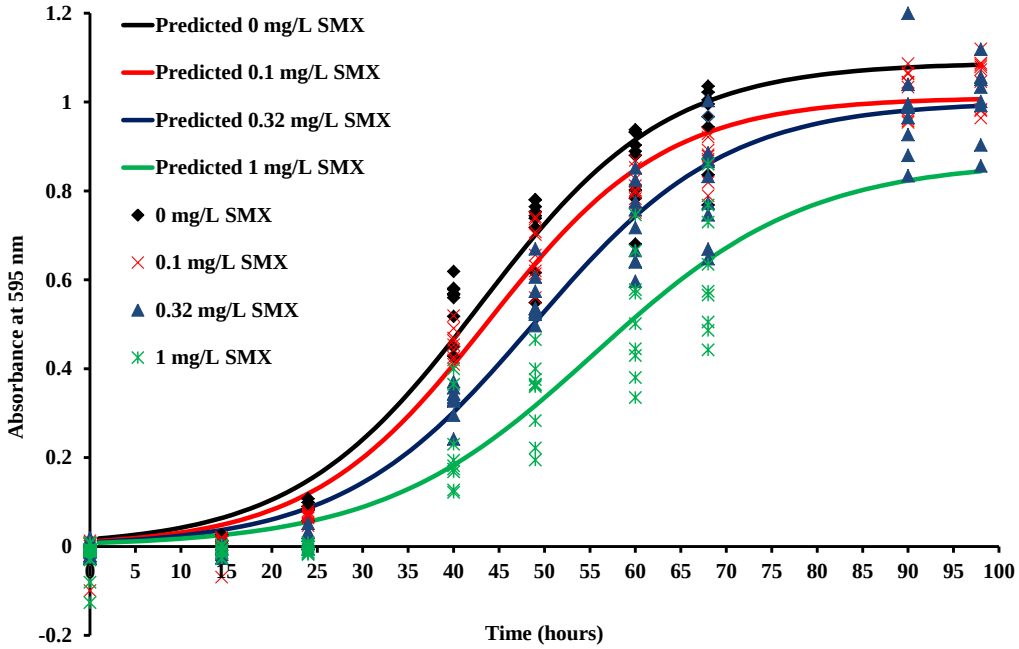
4.3.5.4. PCA Analysis of Carboxylic Acid Utilisation

On day 1 of the exposure, PCA score plots show that there was a clustering of 1 mg/L SMX replicates with some mixing of 0.32 mg/L replicates. There was considerable mixing of all other replicates with control replicates. The results of day 1 PCA analysis would therefore suggest that SMX treatments of 0.32 mg/L and, to a greater extent, 1 mg/L caused a change in the carboxylic acid utilisation profile. It can also be suggested that microbial communities that had been exposed to these SMX concentrations had altered community structures compared with control microcosms.

On day 4 of the exposure there was considerable mixing of replicates representing SMX treatments of 0, 0.1 and 1 mg/L and replicates representing 1, 0.1 and 0.32 mg/L SMX also. These data would suggest that there were some changes in carboxylic acid utilisation as a result of exposing microbial communities to SMX, especially 0.32 mg/L SMX. It is possible therefore that an SMX concentration of 0.1 mg/L and above caused a shift in microbial community structure compared with unexposed communities.

On day 7 PC score plots there were two clusters containing replicates from microcosms treated with all SMX concentrations. All replicates from microcosms treated with 0.32 mg/L were in one of these two groups. The majority of the replicates representing SMX treatments of 0.1 and 1 mg/L clustered away from control replicates. This would suggest that on day 7 of the exposure period SMX concentrations of 0.1 and 1 mg/L were still causing differential carboxylic acid metabolism compared with control microbial communities. These SMX concentrations were likely to be causing a shift in microbial community structure compared with control community structures.

a)



b)

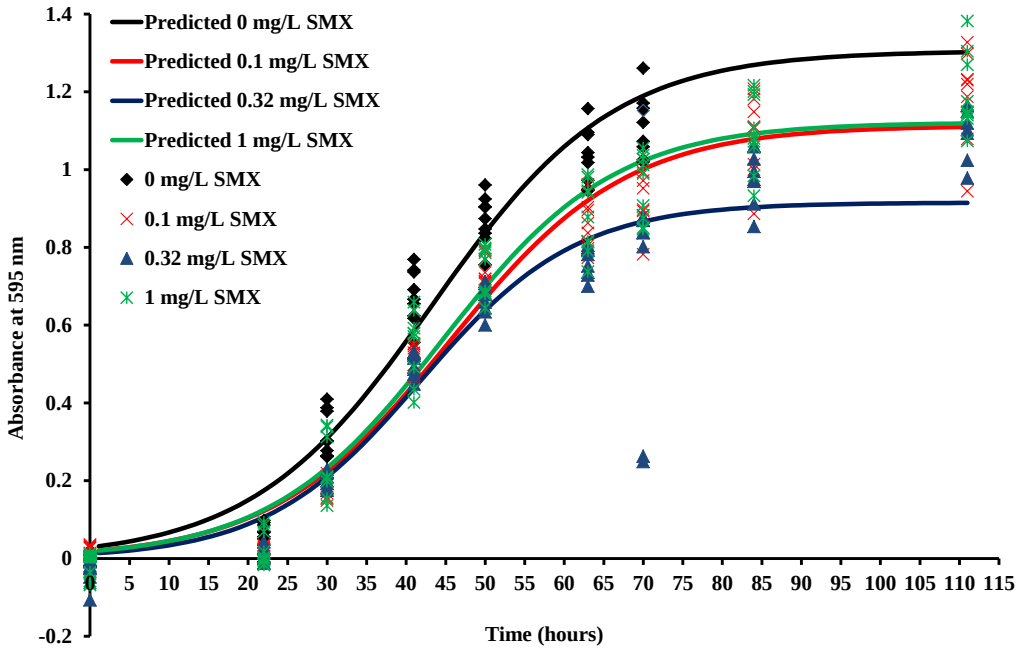


Figure 4.13: Kinetic plots of carboxylic acid utilisation following SMX exposure on a) day 1, b) day 4 and c) day 7.

c)

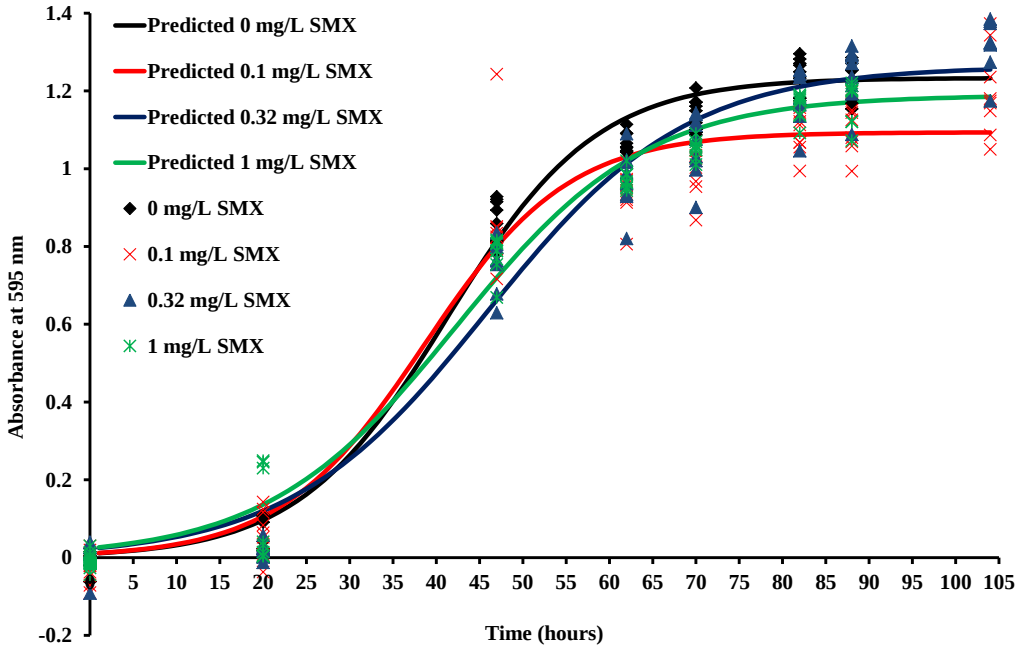
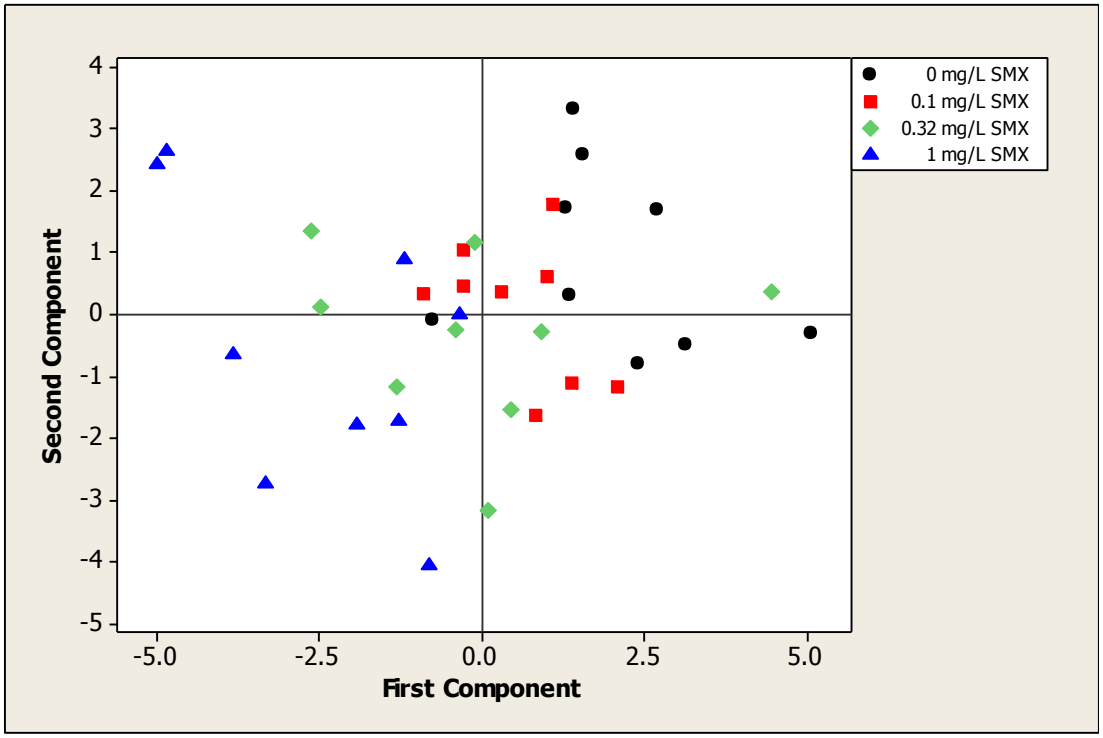


Figure 4.13 (cont).

a)



b)

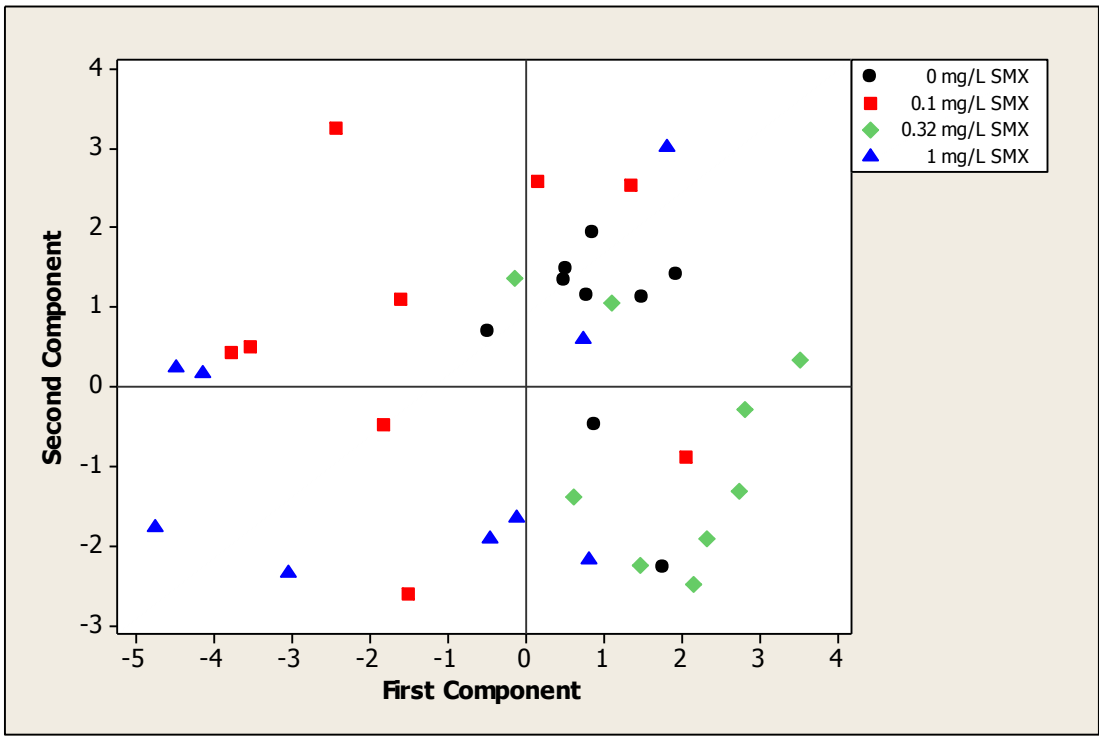
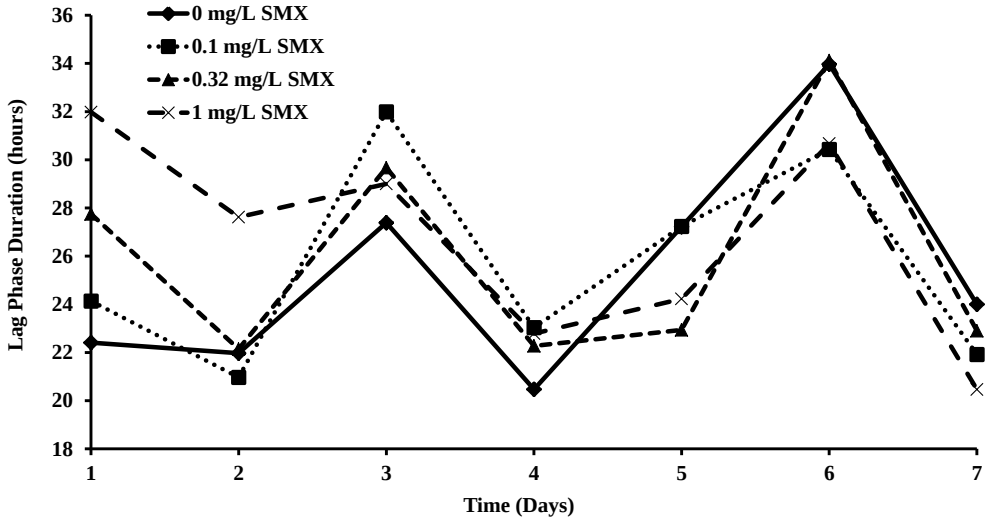


Figure 4.14: Scatter plots of PC scores for the first two principle components for individual replicates from each SMX treatment groups representing carboxylic acid utilisation on a) day 1 and b) day 7. Day 1 (a) proportion of variation: PC1 = 51.2%, PC2 = 22.8%; Day 7 (b) proportion of variation: PC1 = 22.4%, PC2 = 14.7%.

a)



b)

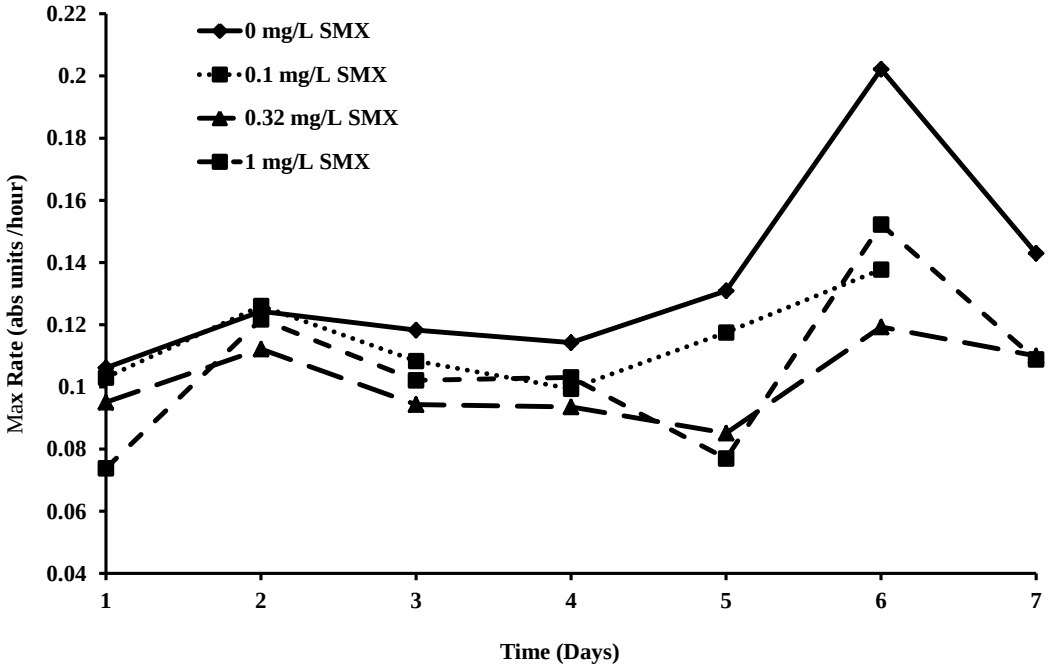


Figure 4.15: a) Lag phase duration versus exposure period time for carboxylic acid utilisation and b) Max rate of carboxylic acid utilisation versus exposure period time following exposure to SMX.

Table 4.11: Summary of one-way ANOVA results for lag phase values before carboxylic acid utilization from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Lag Phase Duration[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns>	ns<	ns>	*>	ns>	ns<	ns<
0.32	*>	ns<	ns>	ns>	ns<	ns>	ns>
1	*>	ns>	ns>	ns>	*<	ns<	ns<

Table 4.12: : Summary of one-way ANOVA results for maximum rate of carboxylic acid utilization values from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

Day of Exposure / Statistical Significance of Max Rate[1]							
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns<	ns<	*<	*<	ns<	ns<	*<
0.32	ns<	*<	*<	*<	*<	*<	*<
1	*<	*<	ns<	ns<	ns<	ns<	ns<

4.3.6. Effect of SMX on Polymer Utilisation

The data in figure 4.16 show kinetic plots of polymer utilisation on days 1 (a), 4 (b) and 7 (c). Figure 4.17 shows PCA score plots of polymer utilisation on days 1 (a) and 7 (c). Figure 4.18 shows the lag phase duration before polymer utilisation (a) and the max rate of polymer utilisation (b). Tables 4.13 and 4.14 show statistically significant changes in lag phase duration and max rate of polymer utilisation respectively.

4.3.6.1. Kinetic Plots of Polymer Utilisation

Day 1 kinetic plots of polymer utilisation show that polymer utilisation was inhibited at all SMX concentrations. This trend was visible in terms of lag phase duration, slope and the maximum absorbance that was reached by plots representing each SMX treatment.

On day 4 of the exposure, inhibition of polymer utilisation was still visible in terms of lag phase duration, slope and the maximum absorbance that was reached by plots, although the slope for each plot had exhibited some visual recovery. The maximum absorbance that was reached by each plot had increased also, which also suggests that a recovery was occuring in terms of polymer utilisation.

By day 7, kinetic plots representing microcosms exposed to 1 mg/L were visually more similar to control plots in terms of curve shape. These data would suggest that a full recovery of the microbial community in terms of polymer utilisation had occurred at this SMX concentration. There was a degree of recovery apparent in curves representing microcosms that had been exposed to 0.l and 0.32 mg/L SMX in terms of the maximum absorbance that was reached by kinetic plots. Different slopes still suggested that an effect on polymer utilisation was still taking place however.

4.3.6.2. Lag Phase Duration: Polymer Utilisation

Graphs representing lag phase durations following carboxylic acid utilisation as a result of exposing microcosms to SMX reveal no temporal trend at any SMX treatment. On day 1 however lag phase durations were significantly higher at all SMX treatments compared with control values. There were no further statistically significant increases as a result treating microcosms with 1 mg/L during the exposure. These data suggest that a recovery in terms of lag phase duration was occurring in microcosms that had been exposed to 1 mg/L from day 2 onwards. There were significant increases in lag phase durations on day 3 as a result of exposing microcosms to 0.1 and 0.32 mg/L SMX. After this there were no further increases in lag phase duration suggesting that these microcosms were showing a recovery in terms of lag phase from day 5 onwards.

4.3.6.3. Max Rate of Polymer Utilisation

Although graphs illustrating the max rate of polymer utilisation over time do not show any temporal trends, they do show that there was an inhibition of the max rate of polymer utilisation. In general, the max rate of polymer utilisation was lowered as a result of exposing microcosm to all SMX concentrations. The only exceptions to this were seen on days 1 and 5, when microcosms exposed to 0.1 mg/L SMX were not inhibited in terms of the max rate of polymer utilisation.

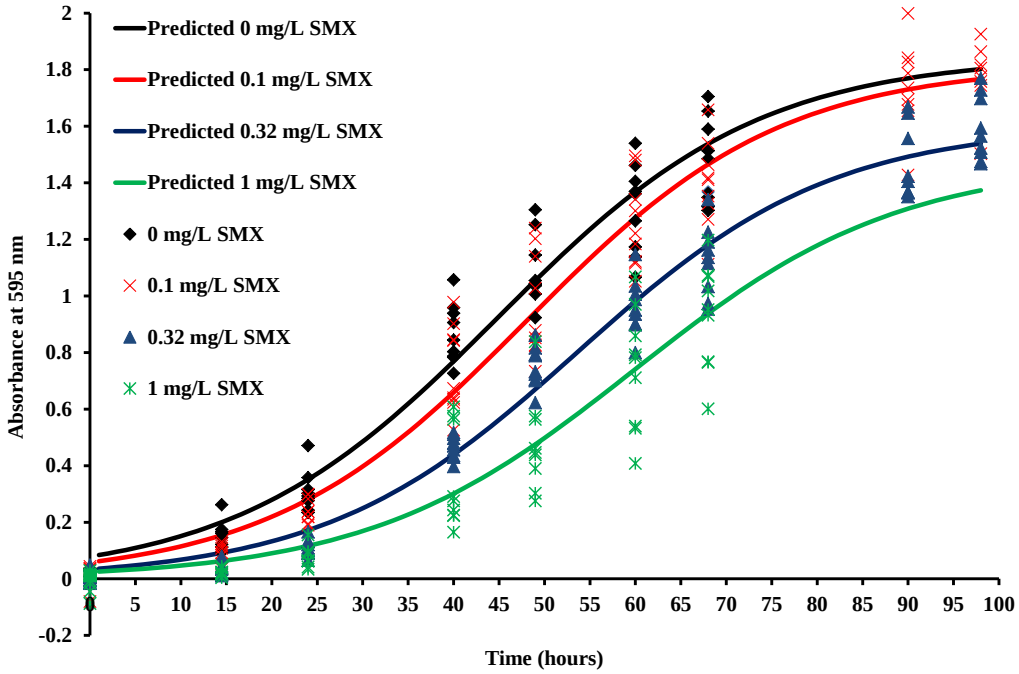
Statistically significant decreases in the max rate of polymer utilisation were seen at 1 mg/L SMX on days 1, 2 and 4 of the exposure, after which no more significant effects were observed. On days 2, 3, 4, 6 and 7 a significant reduction of the max rate of polymer utilisation was observed at 0.32 mg/L SMX. An SMX concentration of 0.1 mg/L caused significant changes in the max rate of polymer utilisation on days 2 and 4.

4.3.6.4. PCA Analysis of Polymer Utilisation

PCA analysis of polymer utilisation on day 1 of the exposure showed that control replicates tended to cluster near replicates from microcosms that had been treated with 0.1 mg/L SMX, although some replicates from the other two SMX treatments clustered with control replicates also. However most replicates representing microcosm treated with 0.32 and 1 mg/L clustered separately from control replicates. These data suggest that the top two SMX concentrations had resulted in the microbial community utilising polymers differently on day 1 of the study. It can therefore be inferred that exposure to 0.32 and 1 mg/L resulted in changes in the microbial community structure that was present in corresponding microcosms.

This trend continued into day 4 of the exposure period, with the most pronounced separation apparent for replicates from microcosms treated with 0.32 mg/L SMX. By day 7 of the study, PCA plots were much more mixed. As a result, separate clusters were hard to visualise indicating that the microbial communities present in all microcosm were behaving in a similar manner in terms of polymer utilisation. It is therefore likely that, by day 7, microbial community structure was similar in all microcosms (treated and control).

a)



b)

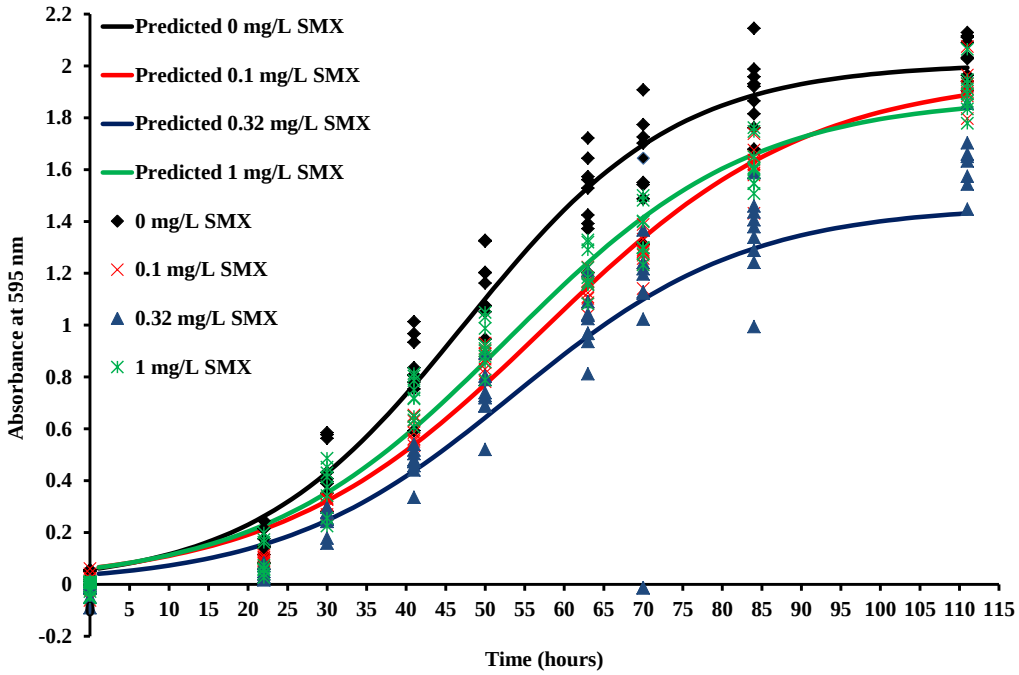


Figure 4.16: Kinetic plots of polymer utilisation following SMX exposure on a) day 1, b) day 4 and c) day 7.

c)

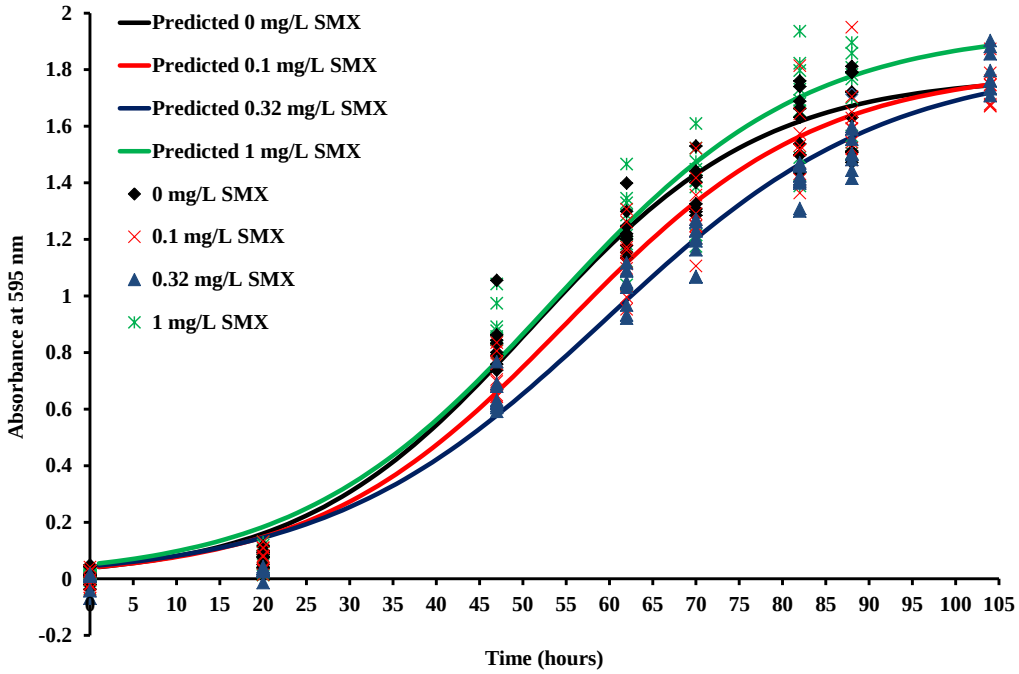
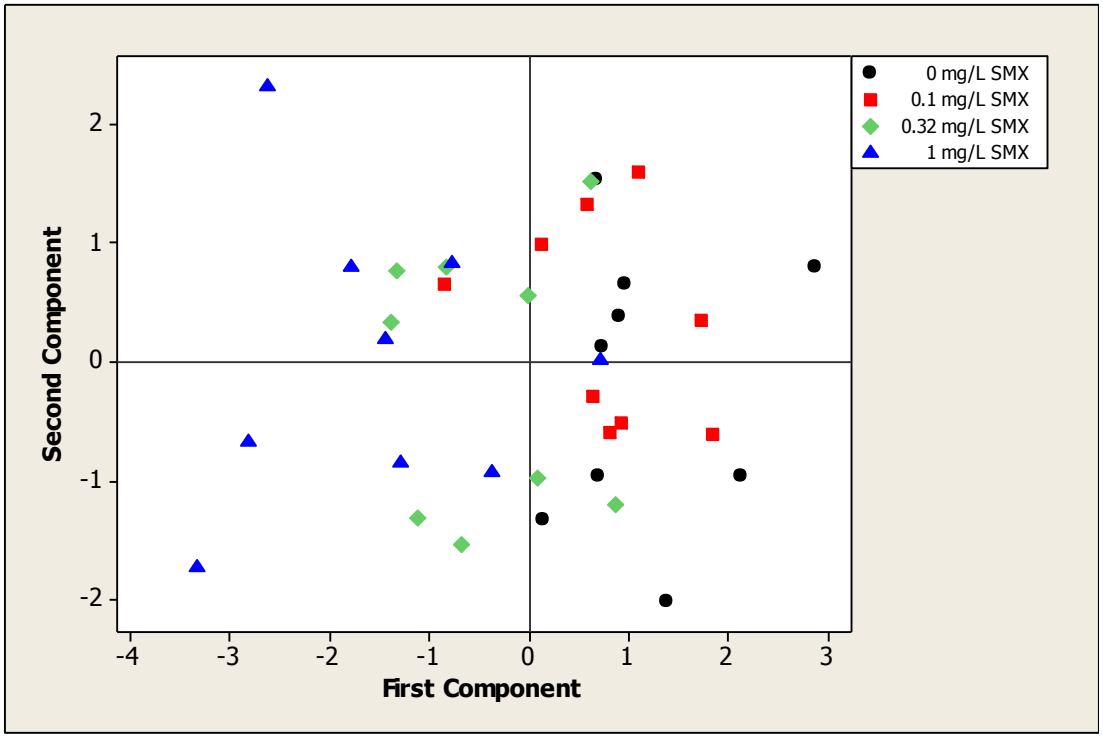


Figure 4.16 (cont).

a)



b)

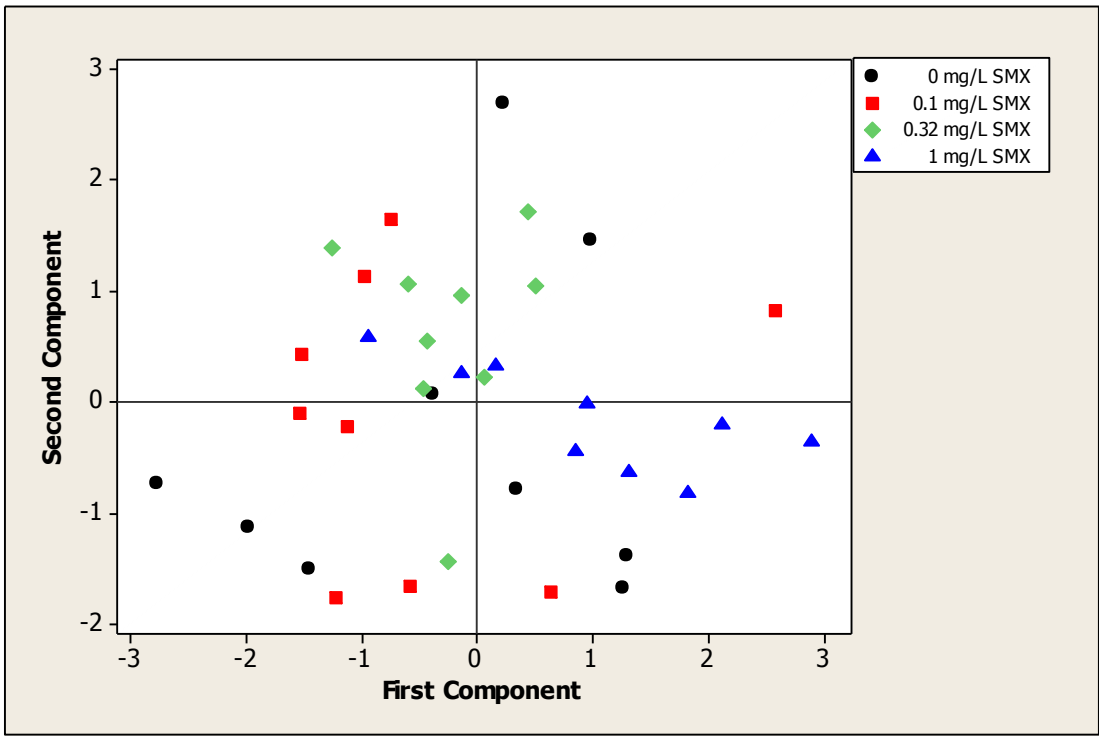
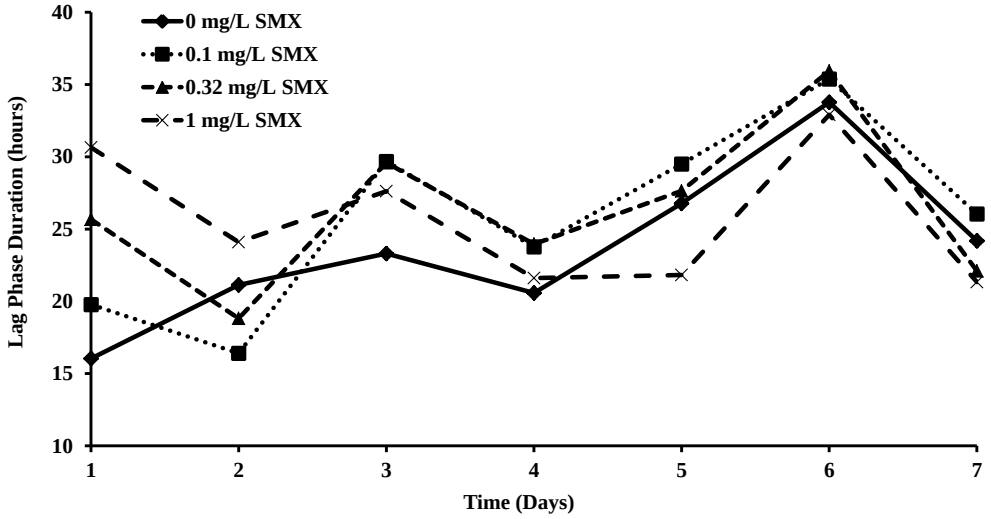


Figure 4.17: Scatter plots of PC scores for the first two principle components for individual replicates from each SMX treatment groups representing polymer utilisation on a) day 1 and b) day 7. Day 1 (a) proportion of variation: PC1 = 80.2%, PC2 = 12.3%; Day 7 (b) proportion of variation: PC1 = 43.4%, PC2 = 35.6%.

a)



b)

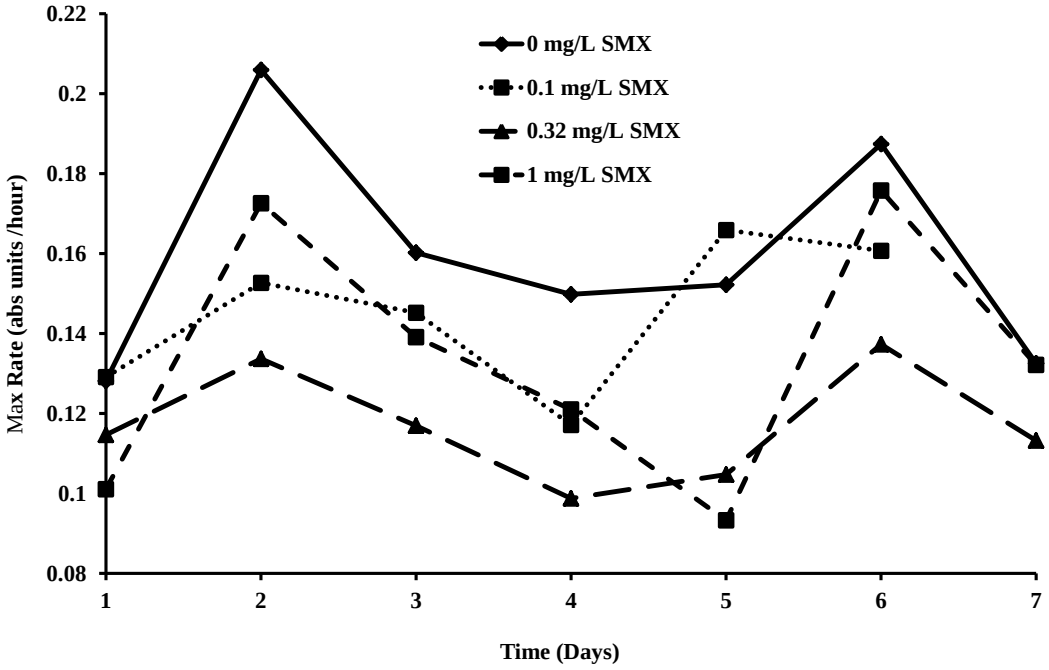


Figure 4.18: a) Lag phase duration versus exposure period time for polymer utilisation and b) Max rate of polymer utilisation versus exposure period time following exposure to SMX.

Table 4.13: Summary of one-way ANOVA results for lag phase values before polymer utilization from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Lag Phase Duration[1]						
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	ns>	ns<	*>	ns>	ns>	ns>	ns>
0.32	*>	ns<	*>	ns>	ns>	ns>	ns>
1	*>	ns>	ns>	ns>	ns<	ns<	ns<

Table 4.14: Summary of one-way ANOVA results for maximum rate of polymer utilization values from Biolog data for inocula from microcosms following different exposure times to SMX in comparison with unexposed inocula.

	Day of Exposure / Statistical Significance of Max Rate[1]						
[SMX] / mg/L	**1**	**2**	**3**	**4**	**5**	**6**	**7**
0.1	=	*<	*<	*<	ns>	ns<	<ns
0.32	*<	*<	ns<	*<	ns<	*<	*<
1	*<	*<	ns<	*<	ns<	ns<	=

4.3.6. Effect of SMX on Total CFU Counts

There were no statistically significant changes in CFU counts compared with control values as a result of exposing microbial communities to any SMX dose on day 1 or day 7 (figure 4.19). These data would suggest that the total number of cells that were being produced by the microbial community was not significantly affected by exposure to any SMX concentration. It can therefore be concluded that microbial communities in general exhibited similar growth regardless of which SMX treatment (including control microcosms) they were exposed to.

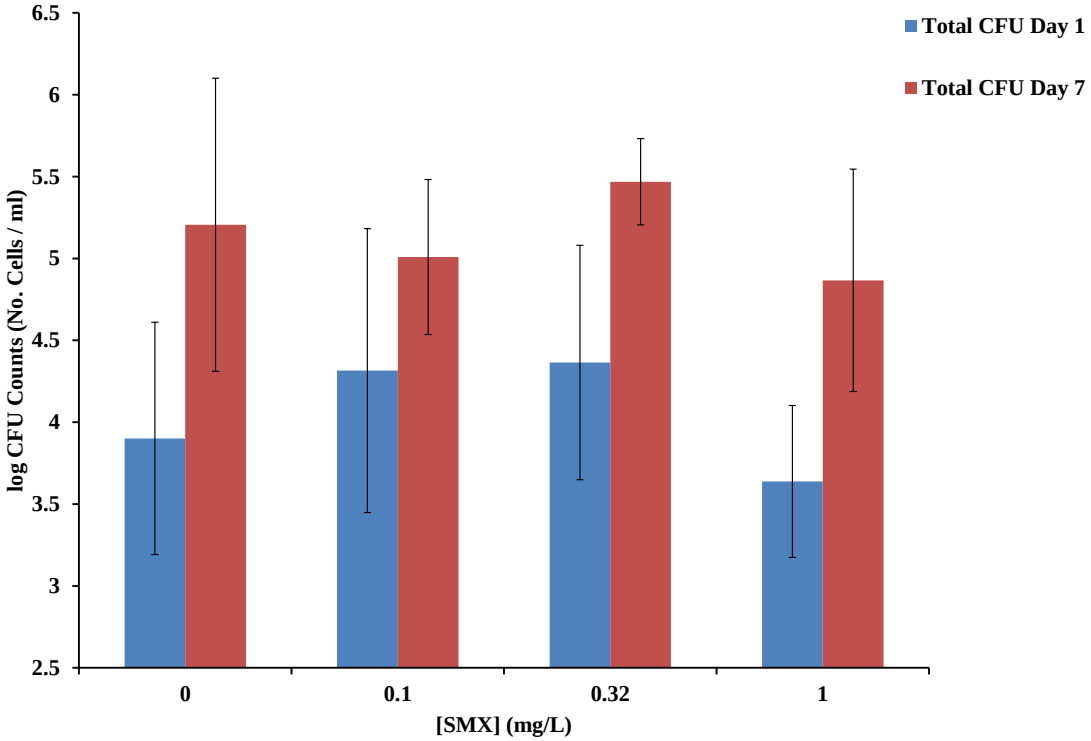


Figure 4.19: Bar graph representing total log CFU counts versus [SMX] on days one and seven of the exposure period. Any statistically significant changes are indicated by asterisk notation (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

4.3.7. Effect of Sulfamethoxazole on the Functional Diversity of Multisubstrate Utilisation

The data shown by figure 4.20 indicates changes in the functional diversity of substrates that were being utilised by microbial communities, as shown by changes in the SWDI value. The data show the effect that exposure to various concentrations of SMX had on this on each day of the exposure period. It can be seen that the functional diversity of substrate utilisation (as shown by SWDI values) was significantly reduced at all SMX concentrations ($P < 0.001$) on day 1 of the exposure. This trend continued throughout the exposure period, with a lower SWDI value being observed at all SMX concentrations on every day of the study. Significantly lower effects were observed in at least one SMX dose group on every exposure day. On day 4, every SMX concentration had again caused a statistically significant reduction in the SWDI value (0.1 mg/L, $P < 0.001$; 0.32 mg/L, $P < 0.001$; 1mg/L, $P < 0.05$). On day 7 of the exposure a recovery (in terms of the functional diversity of substrate utilisation) could still not be observed. Indeed a significant reduction could still be detected at the two lower SMX concentration (0.1 and 0.32 mg/L; $P < 0.01$). These lower SWDI values at each SMX concentration (compared to control values) on each day of the exposure period therefore indicate that the functional diversity of substrate utilisation was severely impacted with no sign of recovery.

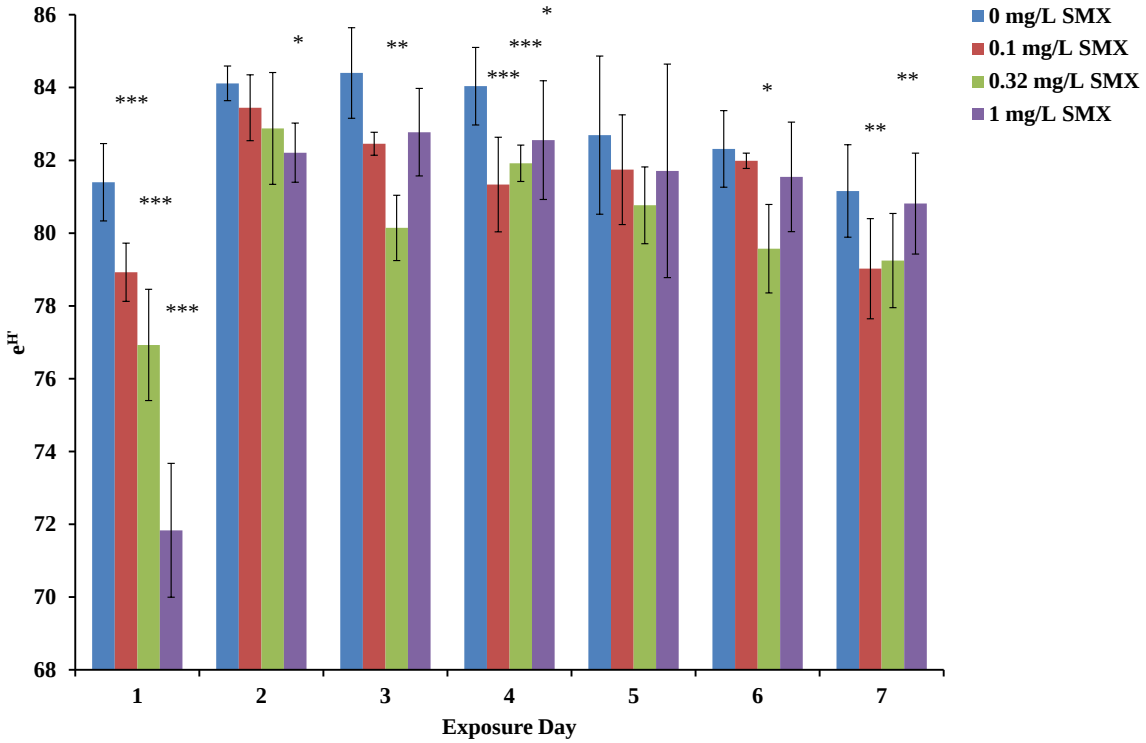


Figure 4.20: Bar graph showing values of the Shannon-Weaver Diversity Index ($e^{H'}$) on each day of the exposure period for each SMX treatment group. Statistical significance is indicated by asterisk notation (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

4.4. Discussion

Sulfonamide antibiotics are widely used chemotherapeutic agents in both human and veterinary medicine. The parent compound is excreted (90%) and can enter the aquatic environment via a number of exposure routes. When they enter the environment, sulfonamide antibiotics have been shown to cause a number of effects on environmental bacteria. There are very few standard tests that offer protection to environmental microbes in terms of ecologically relevant end-points however. Moreover, there is a gap in knowledge as to what effects sulfonamide antibiotics may have on the ecological function of microbial communities in the environment. The data described in this chapter therefore aim to use techniques and systems that had been developed earlier in the study to answer some of these questions.

4.4.1. Effects of Sulfamethoxazole on Total Organic Substrate Utilisation

The first part of work in this chapter aimed to evaluate the temporal effects of a candidate sulfonamide, sulfamethoxaole (SMX) on the ability of the aquatic microbial community to utilise a wide array of distinct metabolically diverse carbon and nitrogen sources. The data shown in kinetic plots on day 1 of the exposure (figure 4.1a) show that total substrate utilisation was inhibited at all SMX concentrations. Statistical analysis revealed that a significant increase in the lag phase duration had occurred as a result of exposing microbial communities to 0.32 and 1 mg/L SMX. There was also a significant decrease in the max rate of total substrate utilisation at these SMX concentrations on day 1 of the exposure. It is likely that these effects were caused by the specific mode of action of SMX. The compound inhibits the biosynthesis of folate by the microbial community (Brown, 1962). Folate is an integral part of many biochemical systems in bacteria, acting as both a promoter of growth and as a micronutritive compound (Jenkins and Spector, 1976; Shane and Stockstad, 1975).

More specifically, in the form of 5, 6, 7, 8-tetrahydrofolic acid/tetrahydrofolate (THFA or THF), folate acts as a co-factor in one carbon transfer reactions related to growth. THFA has been shown to play an important role as a co-factor in several metabolic reactions. Many of these concern amino acid catabolism and biosynthesis. THF donates carbon atoms during the synthesis of serine to glycine. In addition THF is also required for the catabolism of histidine and glycine. Importantly, THF plays a crucial role in the formation of the amino acid methionine and consequently S-adenosyl methionine (SAM). Methionine is an important molecule in its own right, most notably in the formation of *N*-Formylmethionine (fMet) start codons during protein synthesis. SAM is also an important molecule; the synthesis of creatine, polyamines, glutathione, and proteoglycans are all SAM-dependent. SAM also provides the methyl group on DNA molecules that comprise its methylated cap. This is an important process in gene expression and DNA repair. THF also plays an important role in the biosynthesis of deoxyuridine monophosphate, dUMP, into TMP (Thymidylate Synthetase). TMP is important in DNA replication, playing a key role in the

biosynthesis of the pyrimidine ring that is a vital structural component of purine amino acids; carbons 2 and 8 are transferred to the structure via a single carbon transfer reaction (Stockstad and Jukes, 1987; o'Brien, 1966).

Bearing these facts in mind, the mechanisms by which the growth and cellular function of the microbial community were likely to have been inhibited become apparent. Firstly, a number of factors relating to cell division (mitosis) would likely have been repressed. The main factor in this would have been the inability of intoxicated bacteria to produce purine nucleic acids. Purine nucleic acids (adenine and guanine) play a key role in the formation of DNA bases. In addition to this, the reduced ability of the microbial community to methytlate DNA has several implications. Firstly, methylated DNA is a mechanism by which bacteria recognise native DNA. Foreign DNA that has not been methylated is degraded so that phage DNA for example is not expressed. Inhibition of this process could therefore result in expression of foreign DNA and degradation of host bacterial DNA. Secondly, the timing of DNA replication is affected by methylation in relation to other cell-cycle events. A lack of methylation therefore results in cell cycling becoming out of sync. Thirdly, DNA methylation plays a crucial role in DNA strand repair. Inhibition of this process has been shown to result in an increased rate of DNA strand mis-matches. In mutants that lack DNA adenine methyl transferases (DAM, the main enzymes that methylated bacterial DNA) cell viability has been shown to have been lowered (Reisenauer *et al.*, 1999).

There are also implications for the microbial community if the ability to synthesise methionine is reduced. fMet molecules signal the start of translation during protein synthesis in bacteria. Without this "translation initiation codon" the first peptide in a newly forming nascent polypeptide chain cannot form (Sherman *et al.*, 1985). In addition to this, methionine is an amino acid constituent in polypeptide chains and is therefore required for the production of certain proteins. Recent evidence has suggested that methionine-containing proteins play a crucial role in the cellular defense against oxidative stress, indicating that that an inhibition of this could also have affected the microbial community that were exposed to SMX in the current study (Luo and Levine, 2009).

The microbial communities that were exposed to 0.32 and 1 mg/L SMX on day 1 could also have experienced a reduction in the pool of available polyamines due to the inhibition of SAM. The role of polyamines in microbial cells is cell wall incorporation, siderophore biosynthesis and functioning relating to microbial growth (Wortham *et al.*, 2007). If the microbial community cannot form key components of the cell wall then wall integrity could potentially be reduced. The production of siderophores by the microbial community is crucial to cellular biochemistry since siderophores are the main mechanism by which Gram negative bacteria scavenge metal ions (especially iron) from their environment (Neilands, 1995). Metals often form key prosthetic groups within bacteria enzymes that are crucial for cellular function such alcohol dehdrogenases, a group of enzymes that are involved in a multitude of catabolic microbial pathways. These metals are usually zinc, iron or magnesium (Clark, 1992). The inhibition of polyamine synthesis therefore

provides another potential explanation for why the utilisation of total substrates may have been affected in terms of growth of and the retardation of key cellular biochemical function.

The inhibition of SAM production as a result of SMX exposure could also have reduced bacterial glutathione production. Glutathione is the main thiol-containing compound present in bacteria (Fahey *et al.,* 1978). For years their role in cellular function was a mystery until Chesney *et al*. (1997) implicated their role in the sacrificial defence against intracellular chlorinated compounds and hydrogen peroxide. It can be suggested therefore that the reduced ability of the microbial community to synthesise glutathiol may potentially result in free radical intoxication. Free radicals have been shown to cause DNA damage in microbial cells (Ross, 1988). This may result in the death of some members of the microbial community via apoptosis (cell death) which often follows DNA damage (Rich *et al.,* 2000). Indeed Chesney *et al*. (1997) reported that glutathione-deficient *E. coli* were twice as likely to die as a result of chlorine and hydrogen peroxide poisoning. It is possible therefore that the growth of the microbial community in microcosms exposed to 0.32 and 1 mg/L on day 1 of the exposure may have had a reduced growth rate and therefore reduced total substrate utilisation as a result of free radical intoxication (due to metabolic free radicals not being scavenged efficiently).

In terms of looking at the growth of the microbial community, CFU counts were performed. These did not yield any significant differences in cell density (and therefore microbial growth) by dose group. It may be the case that this is because CFU counts are not always the best measure of microbial growth or, in the case of the current investigation may not be a reliable measure of what was occurring due to the use of different growth media in agar plates and Biolog GN2 plates. These have been discussed previously in section 3.4.1.

A further increase in lag phase duration was observed on day 2 at 1 mg/L SMX and on day 4 at 0.1 mg/L SMX. In the microcosms that were exposed to the top two SMX concentrations, a recovery was suggested by the lack of further inhibition from days 2 and 3 onwards (for 0.32 and 1 mg/L SMX respectively). However, a significant effect on the max rate of total substrate utilisation was still occurring in microcosms exposed to these SMX concentrations up to day 3 (1 mg/L) and day 7 (0.32 mg/L). In microcosms exposed to 0.1 mg/L SMX significant effects on the max rate of total substrate utilisation were observed on days 2, 3, 4 and 7. These data would suggest three things. Firstly, there appears to have been a recovery in total substrate utilisation from day 4 onwards in microcosms treated with 1 mg/L SMX. Secondly, there didn't appear to be any sort of recovery in microcosms treated with 0.32 mg/L SMX. Thirdly, there would appear to have been an intermittent recovery in microcosms treated with 0.1 mg/L SMX followed by another period of effects.

Data was also analysed in terms of looking at the SWDI for each dose group over the exposure period. SWDI values were lower than control values at all SMX concentrations over all days of the exposure. On each day of the exposure, SWDI values were statistically significant in at least one dose group, apart from on day 5, when no significant changes could be observed. These data

indicate that SMX was having a negative impact on the functional diversity of total substrate utilisation over the entire exposure period. As such, SMX was resulting in SMX-exposed microbes not being able to utilise the same range of substrates that those present in control (unexposed) microcosms were able to. With the possible exception of a temporary recovery on day 5, no real recovery in terms of the functional diversity of substrate utilisation could be observed. The main reason for this is likely to be due to the reduced ability of the microbial community to synthesise folate. As discussed previously in this section, a number of consequences of impaired folate biosynthesis (such as reduced levels of SAM and methionine) include reduced growth and the inability of microbes to produce catabolic enzymes, such as alcohol dehydrogenases. It is therefore likely that certain bacteria which utilise a specific substrate or substrates are unable to grow to a sufficient abundance such that their substrate utilisation would be observable. In control microcosms, such members of the microbial community would be free to thrive and utilise a wider range of substrates. It is likely that a number of bacteria that utilise a wide array of substrates would have been inhibited in exposed microcosm. In addition, the reduced ability of the microbial community (or at least a portion of the microbial community) to produce essential catabolic enzymes (and other proteins vital for cellular function) would likely have resulted in the reduced ability of microbes to utilise a range of substrates. Kong *et al*. (2006) investigated the effect of exposing a soil microbial community to an antibiotic (CTC) at similar concentrations to the present investigation. They observed a general decrease in the diversity of Biolog substrates that could be utilised by the microbial community (or at least a portion of the microbial community). The results are however in general agreement with the current study, suggesting that antibiotics from different classes can have a negative impact on the range of substrates that both soil and aquatic microbial communities are able to utilise.

PCA data (figure 4.2a) show that on day 1 all SMX-treated replicates showed distinct substrate utilisation profiles, suggesting that the way in which substrates were being utilised differ at all SMX concentrations. Day 4 PCA plots generally show the same trend. Day 5 PCA plots show that 0.1 mg/L SMX-treated replicates clustered independently from control replicates. By day 7 (figure 4.2b), PCA plots show that significant mixing (of replicates on PC score plots) had occurred between 0.1 mg/L SMX and control replicates, but 0.32 and 1 mg/L replicates were still clustering distinctly from control replicates. These data suggests several things. Firstly, no overall temporal recovery occurred in microcosms exposed to 0.32 or 1 mg/L SMX, which is contrary to what was detected by kinetic analysis. Secondly, 0.1 mg/L SMX was altering total substrate utilisation, which is also contrary to what kinetic data had shown. This could mean several things. Firstly, it could be the case that the kinetic and multivariate data show different things and that the kinetic response may be different from the actual "metabolic fingerprint." As such, the actual pattern of substrate utilisation (PCA) and kinetic response could be considered as two different end-points. To expand on this, PCA data can be considered a measure of how differential metabolism occurs in the microbial communities and kinetic data may be an indicator as to the extent that specific substrate

utilisation occurred. This means that PCA data is a good indicator of how the microbial community structure is changing over time as the result of exposure to SMX. PCA data would therefore indicate that the microbial community structure (in terms of total substrate utilisation) was altered at all SMX concentrations throughout the vast majority of the exposure, with only a slight recovery being observed at the lower SMX dose group towards the end of the experiment. It would therefore be an interesting future study to validate PCA data with molecular community analysis such as DGGE or TRFLP analysis to see if results from these analyses matched the finding of the present study.

Assuming that the kinetic analysis did observe a recovery in microcosms exposed to 1 mg/L SMX but not 0.32 mg/L SMX (and to a lesser extent 0.1 mg/L SMX), this could potentially be explained by the selection of sulfonamide resistance. Sulfonamide resistance occurs via changes in the folate biosynthetic pathway (Perreten and Boerlin, 2002; Vedantam *et al.*, 1998; Scholz *et al*., 1989). Moreover, sulfonamide resistance has been extensively detected in the aquatic environment (Zhang *et al*., 2009). The reason that this formed at the highest SMX concentration but not at other SMX concentrations could be that 0.32 mg/L SMX did not exert a strong enough selective pressure but 1 mg/L SMX did. Heuer *et al.* (2008) found that a sulfadiazine concentration of 0.15 mg/kg selected for sulfonamide resistance, although this was investigated in the soil environment and aquatic formation of resistance could well be selected by different sulfonamide concentrations. In addition, selection of sulfonamide resistance may be different with each sulfonamide type. It is not clear therefore if resistance formation is the key to explaining these particular results. Chapter 5 shall investigate the temporal formation of antibiotic resistance however, which may clarify what is occurring within the test systems.

The tendency for intermittent effect and recovery in microcosms exposed to 0.1 mg/L may have been due to the kinetic model not being sensitive enough to detect smaller changes in the max rate of substrate utilisation. Alternatively, it may be the case that transient communities with varying degrees of sulfonamide susceptibility were present. For example, on day 1 of the exposure period there could have been a large portion of the microbial community that exhibited resistance to sulfonamides. The presence of resistant bacteria in both natural rivers has been detected (Zhang *et al*., 2009), so it is entirely possible that resistant members of the microbial community were present at the start of the exposure. In addition, there may have been sulfonamide-resistant bacteria in the system that came from the treated sewage that was added to the microcosms (Zhang *et al.*, 2009; Silva *et al.*, 2006; Szczepanowski *et al.*, 2004; Selvaratnam and Kunberger, 2004).

It is also possible that there were transient portions of the microbial community that exhibited differential control of their internal pH. Tappe *et al.* (2008) demonstrated that bacteria that are poor regulators of pH will be less affected by sulfonamides at a pH of 7 or 8. The microcosm system used in the present study had an initial pH of 7.2. It is likely that the system was kept at this pH throughout the study by the presence of phosphate buffer (pH 7.2) in OECD synthetic sewage.

Therefore if transient communities of poor pH-regulating bacteria were present during the exposure period then the antimicrobial effects of SMX would have been reduced on days when such members of the microbial community were present at relatively higher densities.

Other studies have also shown effects of sulfonamides on the growth of environmental bacteria. Isidori *et al.* (2005) and Kim *et al.* (2007) observed that various sulfonamide antibiotics inhibited the growth of the marine bacterium *Vibrio fischeri*. They observed EC_{50} values ranging from 23.3-78.1 mg/L. Ando *et al*. (2007) investigated the effect of sulfonamides on cyanobacterial growth. EC_{50} values of between 2.3 and > 2000 mg/L were recorded. Halling-Sorenson *et al.* (2002) found that sulfonamide antibiotics reduced CFU counts in sludge and soil studies. These results corroborate the findings of the current study which also observed effects of sulfonamides that may be linked (or partially linked) to the reduced growth of the microbial community as a result of exposing environmental bacteria to sulfonamides. In general however, the current study has observed that significant effects have occurred at lower concentrations compared with previous studies.

Studies that have observed changes in total substrate utilisation as result of exposure to other antibiotics have also been reported. Maul *et al*. (2006) exposed leaf-bound microbial communities to the antibiotic ciprofloxacin. A significant variation in PC1 (principal component 1) and PC2 (principal component 2) scores were observed at an exposure concentration of 0.1 mg/L, suggesting a change in total carbon utilisation profiles compared with controls. Schmitt *et al*. (2005) reported a shift in PCA scores on the second axis, suggesting a change in the total substrate utilisation profile had occured as a result of exposing soil microbial extracts to the antibiotic sulfachloropyridazine. The results of these and the present study suggest that a range of antibiotics can affect the metabolic physiology of microbial communities in a range of environments. As such, both the current and previous investigations have shown that sulfonamides can cause changes in microbial community structure in both soil and aquatic microbial communities and at similar concentrations. Landi *et al.* (1993) had already reported a significant decrease in soil respiration rates as a result of exposure to streptomycin. As the substrate being respired was SOM (soil organic matter), this pointed to the inhibition of multi substrate utilisation in soil microbial communities.

The results of the present study therefore suggest that the presence of SMX may inhibit the ability of aquatic microbes to utilise and degrade this broad range of molecules contained within DOC. Tranvik and Hofle (1987) commented on the high efficiency with which microbes normally achieve this. If SMX inhibits the ability of aquatic microbes to utilise DOC then this could result in a reduced microbial biomass in aquatic environments. Although this may potentially increase oxygen levels in the aquatic environment (potentially making more oxygen available to aerobic organisms), the absence of certain bacteria may also result in key processes within nutrient cycles not being performed as efficiently. For example, a reduction in key nitrifying, denitrifying and xenobiotic-degrading bacteria may have adverse effects on nitrogen cycling and on the removal of

pollutants from the aquatic environment. In addition to this, bacteria provide a key food source for aquatic organisms such as heterotrophic flagellates and protozoa. In turn, these organisms are preyed upon and so on until energy is ultimately transferred to an apex predator (an organism that has no predator of its own). A reduction in bacterial numbers may therefore affect the normal transfer of energy through aquatic food chains. Blomqvist *et al*. (2001) demonstrated the importance of DOC in terms of its conversion into microbial biomass. This also means that, *via* the incorporation of DOC into bacterial biomass, aquatic bacteria act as important carbon sink. Carbon sinks play an important role in carbon sequestration from the atmosphere (removal or reduction in carbon dioxide levels). As such, bacterial biomass plays an important biogeochemical role in regulating the earth's climate.

The degradation of a wide array of autochthonous and allochtonous organic matter (such as humus, undigested food and dead animal material) in the aquatic environment requires microbial communities to be efficient organic multi substrate utilisers. Within the carbon cycle, bacterial breakdown of DOC can be liberated back into the environment via the release of carbon dioxide, which is commonly regarded as being the most critical greenhouse gas on the planet (Siegenthaler and Sarmiento, 1993). DOC degradation therefore plays an important role in releasing gasses which regulate the planet's temperature over time (Berner, 1990). Other inorganic nutrients (such as nitrates and phosphates) are also liberated during microbial degradation of DOC. These may be taken up as nutrients by, for example, aquatic plants, or they may enter another nutrient cycle (such as the nitrogen cycle) for further processing. Any effect on DOC degradation in the aquatic environment therefore has the potential to interfere with climate and nutrient cycling.

Previous studies have shown that antibiotics may inhibit the ability of bacteria to break down the broad range of volatile organic substances in manure. Biogass production was therefore reduced. Inhibition of biogass production (both aerobic and anaerobic) as a result of exposing microbial communities to oxytetracycline ranged from 27-50% across three studies (Sankvist, 1984; Gamal-El-Din, 1986; Arikan Rital *et al*., 2006). Landi *et al*. (1993) also reported a significant decrease in soil respiration rates as a result of exposure to streptomycin. As the substrate being respired was SOM (soil organic matter), this pointed to the inhibition of multisubstrate utilisation by soil microbial communities. The inhibition of organic substrate utilisation by different antibiotics has therefore been reported in both the aquatic and terrestrial environment.

4.4.2. Effect of Sulfamethoxazole on Substrate Guild Utilisation

The present study also investigated the effect of three concentrations of sulfamethoxazole (0.1, 0.32 and 1 mg/L) on the utilisation of 5 distinct substrate "guilds"; amines and amides, amino acids, carbohydrates, carboxylic acids and polymers (Preston-Mafham *et al*., 2002). The following sections shall discuss these effects and the implications of these effects with regards to the available literature.

4.4.2.1. Amines and Amides Utilisation

Kinetic model plots show that amine and amide utilisation was significantly inhibited on days 1 and 2 in terms of lag phase duration before utilization at the highest SMX concentration (1 mg/L). There were no effects on amine and amide utilisation in terms of lag phase duration at any other SMX treatments on any other day of the exposure. Although there was no significant effects of SMX on the max rate of amine and amide utilisation on any day of the study at the higher SMX concentration a significant reduction in the max rate of amine and amide utilisation was observed on day 4 as a result of exposing microbial communities to 0.1 and 0.32 mg/L.

In terms of PCA analysis, there was considerable mixing of replicates (on PC score plots) from all SMX treatments on day 1. There was also significant mixing of replicates from microcosms exposed to all SMX concentrations. There was also significant separation of replicates from all SMX treatments from control replicates. This trend continued up until the end of the exposure period, suggesting that all concentrations of SMX caused differential amine and amide utilisation across the exposure period. Once again the results obtained using multivariate analysis show different effects data from kinetic analysis. These data show that the community structure of amine and amide utilizing bacteria was different from control communities in microcosms exposed to all SMX concentrations. As discussed in section 3.4.2, these results suggest that the compositions of certain aquatic nitrogen cycling communities are altered as a result of SMX exposure. Specifically, it is highly likely that communities involved in ammonification will be different (see also section 3.4.2).

Although there are no studies from the literature that have investigated amine and amine utilization by microbial communities that been exposed to SMX, Kong *et al.* (2006) observed a decrease in amine and amide utilisation (quantified by maximum colour development) as a result of exposing Biolog GN2 plates (inoculated with soil extract) to different oxytetracycline concentrations. Although the study exposed microbial communities to a tetracycline, the present study and this study both show effects of bacteriostatic antibiotics on amine and amide utilisation.

Amines and amides can be utilised in a number of ways by environmental bacteria. They may enter the nitrogen cycle where they are first subject to the process of ammonification. When ammonia has been produced via this reaction it is then subject to further biogeochemical processing in the process of nitrification, whereby nitrite and nitrate are formed. Nitrogen in these forms can be utilised by bacteria (Paul and Clark, 1996). Amines and amides can therefore act as an important nitrogen source for environmental microbes.

Inhibition of amine and amide utilisation as a result of SMX exposure could indicate a potential inhibitory effect on the processes of ammonification and nitrification in the aquatic environment. Although no other previous work has detected inhibitory effects of specific aspects of the nitrogen

cycle as a result of exposure to sulfonamides, work has been conducted on other antibiotics. Halling-Sorensen *et al.* (2000) detected an inhibition of the growth of the aquatic nitrifying cyanobacterial species *Microcystis aerugenosa* as a result of exposure to CTC and tetracycline. In the case of both compounds an EC_{50} value of < 0.1 mg/L was observed. Halling-Sorensen (2000) also detected a significant inhibition of nitrification as a result of exposing activated sludge communities to CTC, oxytetracycline and tetracycline. The data in these studies are a link between inhibition of nitrogen utilisation and exposure to antibiotics, providing a degree of corroboration with the present study.

4.4.2.2. Amino Acid Utilisation

Kinetic analysis showed that on day 1 of the exposure period amino acid utilisation was significantly inhibited by 0.32 and 1 mg/L SMX in terms of an increased lag phase duration. This trend continued into day 4 in microcosms treated with 1 mg/L. A significant inhibition of the max rate of amino acid utilisation was only observed on day 1 of study at an SMX concentration of 1 mg/L and on day 4 at a lower SMX concentration of 0.1 mg/L.

Day 1 PCA analysis revealed much mixing of replicates from different microcosms, although replicates from microcosm spiked with 1 mg/L SMX tended to separate from control replicates, suggesting that these replicates were utilising amino acids in a distinct manner than compared with control replicates. On day 4 all replicates could be separated almost by dose, while on day 7 only replicates from microcosms exposed to 0.32 and 1 mg/L could be separated from controls. This would again suggest that effects in amino acid utilisation showed different results from what the kinetic response displayed. The data also suggest that the structure of the microbial community that is composed of bacteria which utilise amino acids was different in control communities than compared with exposed microcosms throughout the exposure. The lowest SMX concentration to have caused this change was 0.1 mg/L. Specifically, these data indicate that exposure to SMX can cause changes in the community structure of bacteria which are involved in deamination, a key step in the nitrogen cycle (see also section 3.4.3).

Other studies have also observed the inhibition of amino acid uptake by sulfonamides. Some of these studies were conducted on pure cultures in the mid-20th century when a number of research teams were investigating the sulfonamide mechanism of resistance and mode of action. Sevag and Green (1944) for example demonstrated that tryptophan utilisation by sulfonamide susceptible *Staphylococcus aureus* was inhibited by 6888 mg/L sulfanilamide and 1699 mg/L sulfathiazole. Although the doses used in these studies were much higher than those used in the current investigation, the results still infer that these mechanisms may occur, possibly at lower doses. This would require further experimentation however. These studies could investigate relevant biochemical pathways by techniques such HPLC or GC analysis of biochemical.

Other studies have looked at the inhibition of amino acid uptake in the environment. Brandt *et al.* (2009) also showed a reduction in bacterial growth using ^{3}H leucine incorporation. This was significantly reduced by exposing a soil microbial community to 0.1 µg sulfadiazine/g. The order of magnitude of this environmental study is therefore within the order of magnitude of the LOEC (lowest observable effects concentration) that was observed during the present study. Effects on amino acid uptake have also been seen as a result of exposing environmental bacteria to other antibiotics. Wheeler and Kirchman (1986) had previously reported a > 58% decrease in amino acid uptake as a result of exposing marine bacteria to the protein synthesis inhibitor chloramphenicol. Verma *et al.* (2007) observed a significant reduction in leucine incorporation rates by river water microbial communities as result of exposure to 5 µg/L tetracycline. In another study, Kong *et al.* (2006) observed a significant reduction in amino acid utilisation as a result of exposing soil microbial communities to oxytetracycline. Exposure concentrations were similar to those that were employed during the present study. These results are in agreement with the current study.

In the case of amino acid utilisation it is possible to make some tentative judgments that link the sulfonamide mode of action with inhibition of amino acids. Amino acids are metabolised by a range of dehydrogenase enzymes. The most documented of these is glutamate dehydrogenase, which catalyses the reaction that converts glutamate into α-ketoglturate and ammonia. Ammonia can be then be easily utilized as a nitrogen source by environmental bacteria. These reactions rely heavily upon using NADP (nicotineamide adenine phosphate dinucleotide) as a cofactor. However since NADP is composed of the purine base adenine, it is likely that there were reduced levels present in intoxicated microbial communities. This is because the synthesis of adenine is dependent on the one carbon transfer capabilities of THF, as discussed earlier. The inhibition of NADP by sulfonamides (sulfanilamide) has been observed previously in cell free microbial enzyme systems as far back as 1946, providing evidence that reactions involving NADP can be significantly inhibited by sulfonamides (Altman, 1946).

4.4.2.3. Carbohydrate Utilisation

Kinetic analysis showed that carbohydrate utilisation was significantly inhibited in terms of an increased lag phase duration on day 1 of the study at all SMX doses. Lag phase was also significantly increased on day 3 in microcosms exposed to 0.32 mg/L. On day 4, significant lag phase increases were observed as a result of exposing the microbial community to 0.1 and 0.32 mg/L SMX. An increase in the max rate of carbohydrate utilisation was observed in microcosms exposed to 0.32 and 1 mg/L on day 1 and at all SMX concentrations on day 2. Indeed, the max rate of carbohydrate utilisation was significantly inhibited for the duration of the study in microcosms exposed to 0.32 mg/L SMX. Further significant reductions in the max rate of carbohydrate duration were observed on days 4 and 5 (1 mg/L) and on days 3, 4 and 7 (0.1 mg/L).

Multivariate analysis also showed that replicates exposed to 0.1 mg/L SMX were similar to control replicates on day 1 of the exposure period. However by days 3 and 4 replicates were clustering almost by dose; this continued until day 7. It can therefore be suggested that the microbial community were utilizing carbohydrates in a different manner in treated replicates compared with control replicates. In the case of carbohydrate utilisation therefore kinetic and multivariate analysis was more similar than had been seen with other utilisation scenarios. From these data, it can be suggested that exposure of aquatic microbial communities to SMX results in changes to certain portions of the microbial community that are able to utilise/metabolise carbohydrates. These data suggest that all doses of SMX were causing a change in the structure of the portion of the microbial community that was utilizing carbohydrates. The influence of SMX on the community structure of carbohydrate utilisers lasted throughout the exposure period. It is likely therefore that microbes involved in the cycling of carbon are affected in terms of having an altered community structure compared with unaffected (control) communities. As mentioned previously, these community shifts could be confirmed by molecular techniques such as PCR-DGGE.

Several laboratory experiments have also found that sulfonamides inhibit carbohydrate utilisation. These have mainly found that sulfonamide compounds inhibited the utilisation of glucose (Clinton and Loeuringer, 1942; Dorfman and Koser, 1942; Sevag and Shelburne, 1942).

Other studies have also looked at the effect of sulfonamides on microbial respiration in the environment. Thiele-Bruhn and Beck (2005) for example tracked the effect of sulfapyridine on substrate induced respiration (SIR). They observed EC_{50} values of 6.2 and 11.5 μg/L for two soils. In another experiment, Zielezny *et al*. (2006) tracked SIR rates in soils spiked with sulfadiazine. They also observed an inhibition in respiration rates. Vaclavik *et al.* (2004) recorded a 0.8 fold decrease in respiration rate as a result of exposing soil mesocosms to sulfachloropyridazine. The results of these studies are therefore in agreement with the present study.

Work has also been completed that shows the effects of other antibiotics on carbohydrate utilisation. Kong *et al.* (2006) tracked carbohydrate utilisation in Biolog plates and observed an inhibition in the maximum level of carbohydrates utilised as a result of increasing CTC concentration. Maul *et al*. (2006) observed a 2.7-3.5-fold reduction in carbohydrate utilisation as a result of exposing leaf-bound microbial communities to the antibiotic ciprofloxacin (at 0.1 mg/L).

The main consequence of a reduction in carbohydrate utilisation is the reduced ability of the microbial community to gain energy from a range of preferential sources. In the absence of a suitable energy source, the growth of certain portions of the microbial community will be inhibited. This has been demonstrated by whole-lake DOC addition experiments, in which a significant increase in aquatic microbial biomass was observed as a result of adding increased DOC (Blomqvist *et al*., 2001). The high degree with which aquatic microbes are able to convert glucose into biomass (20% of total DOC) highlights the importance of carbohydrate utilisation to aquatic

microbial communities (Tranvik and Hofle, 1987). In addition, a wide range of biochemical processes are reliant upon the energy that microbial communities gain from carbohydrates, such as active transport of molecules in and out of cells and maintenance of buoyancy systems in cyanobacterial cells (Chu *et al.*, 2007).

The effect that SMX exerted on bacterial utilisation of carbohydrates can potentially be explained by the reduced ability of aquatic microbial cells to produce the metabolic cofactor NAPD as a direct result of the cells not being able to produce the purine base adenine. NADP is an essential cofactor in carbohydrate metabolism, ultimately acting as an electron acceptor during their oxidative metabolism. At the end of this process electrons are passed onto cytochrome c where energy from carbohydrates is ultimately liberated in mitochondria (Krebs, 1953).

4.4.2.4. Carboxylic Acid Utilisation

Kinetic results showed a significant increase in lag phase duration on day 1 (at 0.32 and 1 mg/L SMX) and on day 4 (0.1 mg/L SMX). A significant decrease in the max rate of carboxylic acid utilisation was observed on days 1 and 2 in microcosm exposed to 1 mg/L SMX. From day 2 onwards there were no further effects. In microcosms exposed to 0.32 mg/L there was a significant reduction in the max rate of carbohydrate utilisation from day 2 until the end of the exposure. Significant reductions in the max rate of carboxylic acid utilisation in microcosms spiked with 0.1 mg/L SMX were observed on days 3, 4 and 7.

PCA analysis of carboxylic acid utilisation shows that there was significant mixing of replicates on day 1 of the study, although replicates from microcosms spiked with the two highest concentrations of SMX (0.32 and 1 mg/L) clustered more distinctly. This would suggest that the microbial community was utilizing carboxylic acids differently from the control at these SMX doses on day 1. By day 7 replicates from 0.1 and 1 mg/L displayed distinct clustering from control replicates suggesting that the microbial communities were utilizing carboxylic in a different manner from control communities. These data suggest that SMX concentrations as low as 0.1 mg/L were causing changes in the microbial community structure of microbes that were utilizing carboxylic acids. These effects were apparent throughout the duration of the exposure. It is likely therefore that a portion of the microbial community that utilise carboxylic acids had a different community structure due to exposure to all SMX doses throughout the exposure period. Such communities are likely to preform biogechemical processes within the carbon cycle.

Previous studies have demonstrated the inhibition of carboxylic utilisation by sulfonamide antibiotics in laboratory studies. Specifically, sulfonamides have been shown to inhibit the microbial utilisation of pyruvate (Sevag *et al.*, 1942), lactate, (Macleod, 1939) and succinate (Gerundo, 1950) in pure culture studies involving various susceptible bacterial species. There have not been any studies exploring the effects of sulfonamides on carboxylic utilisation in the

environment though. Kong *et al.* (2006) did however detect the inhibitory effect of oxytetracycline on soil carboxylic acid utilising microbial communities, indicating the potential for a number of antibiotics to affect carboxylic acid utilisation, as well as sulfonamides.

Carboxylic acids represent an important energy source for environmental microbes. Molecules such as citric acid, pyruvic acid, succinic acid and lactic acid can enter energy liberating biochemical pathways such as the tricarboxylic acid cycle directly (Reactome, 2010). Carboxylic acids are also formed from the cleavage of fatty materials in the environment in the form of fatty acids. These reactions are dependent on adenine based electron transfer systems (NADP).

In addition, sulfonamides have been shown to reduce microbial carboxylase activity in pure cultures of *Staphylococcus aureus* and *E.coli* (Sevag *et al.*, 1942). Carboxylase enzymes (such as carboxyl transferase) play a key role in metabolising carboxylic acids so that they can enter the energy-liberating citric acid cycle. For example, pyruvate decarboxylase catalises the reaction that transforms pyruvate into oxaloacetate. Oxaloacetate can then enter the citric acid cycle where energy is ultimately liberated via electron transfer systems.

Naumann (1918) first showed the utilisation of humic materials by aquatic microorganisms. Humic acids contain high carboxylic acid content (Kawahigashi and Sumida, 2006). These form an important part of the carbon cycle in terms of the recycling of dead animal and plant material. Inhibition of carboxylic acid utilisation as result of SMX exposure in the environment could therefore inhibit the ability of environmental microbes to produce energy and degrade certain molecules within the carbon cycle.

Guven *et al.* (2005) demonstrated the ability of certain Archaea (such as *Candidatus* Brocadia and *Canditus* Kuenemia) to utilise organic acids (such as propionate) using nitrite/nitrate instead of oxygen as a terminal electron acceptor. The important role of these organisms in the nitrogen cycle was therefore shown. Inhibition of carboxylic acid utilisation could possibly affect this part of the nitrogen cycle, although it is unclear if these types of reactions are taking place on Biolog GN2 plates.

4.4.2.5. Polymer Utilisation

Kinetic analysis revealed that the lag phase prior to polymer utilisation was significantly increased on day 1 in microcosms exposed to 0.32 and 1 mg/L. On day 3 of the exposure, lag phase duration was also significantly at these SMX concentrations. Max rate values were significantly decreased on days 1, 2 and 4 at an SMX concentration of 1 mg/L. Significant reductions in the max rate of polymer utilisation were also observed on days 2, 3, 4, 6 and 7 in microcosm that were exposed to 0.32 mg/L SMX. In microcosms exposed to 0.1 mg/L SMX, a significant reduction in the max rate of polymer utilisation was shown to have occurred on days 2 and 4 of the exposure.

PCA analysis of polymer utilisation data on day 1 showed that the microbial community present in microcosms spiked with 0.32 and 1 mg/L SMX generally clustered separately from control replicates whereas replicates from microcosms spiked with 0.1 mg/L SXM clustered more similarly to control replicates. This would suggest differential polymer utilisation was occurring as a result of exposure to 0.32 and 1 mg/L SMX. By day 7 replicate clustering was more mixed, suggesting replicates from all treatments were utilising polymers more similarly. These data would suggest that exposure of microcosms to 0.32 and 1 mg/L SMX were causing changes in the microbial community structure that was utilising polymeric compounds. These effects were seen until day 6 at an SMX concentration of 0.32 mg/L. These results suggest that the portion of the microbial community that is able to utilise/biodegrade polymeric compounds has a different microbial community structure at SMX concentrations as low as 0.1 mg/L SMX. The structure of these communities is also different as the result of exposure to 0.32 mg/L SMX when the community is exposed for a slightly longer time period (6 days).

Although no environmental studies have investigated the effect of sulfonamides on polymer utilisation, Kong *et al.* (2006) used Biolog plates to show an inhibitory effect of oxytetracycline on polymer utilisation by soil microbial communities at concentrations similar to those that were used in the current investigation.

The main source of the inhibitory effect of sulfonamides on polymer utilisation may be an artefact of the inhibition of glutathione production by the microbial community. Although no literature is available on the effect of glutathione, it is clear that the enzyme glutathione-s-transferase plays a key role in the degradation of many polymeric compounds. Perhaps the best example of this is the role glutathione-s-transferases in the biodegradation of lignin and atrazine. In fact, glutathione-s-tranferases are important in the microbial degradation of a huge array of unrelated polymeric and often xenobiotic compounds (Allocati *et al.*, 2009). In addition to this, the degradation of polymeric compounds is heavily reliant on electron transfer mechanisms such as NADP which, as discussed earlier in this chapter, is likely to be present at much reduced concentrations in sulfonamide-inhibited microbial communities. An example of the role NADP in polymer utilisation is biodegradation of lignin (Kirk and Farrell, 1987).

Environmental microorganisms are responsible for the degradation of polymers within ecosystems as result of biosynthesis of lipases, ureases, esterases and proteases (Fleming, 1998; Lugauskas, 2003). Microorganisms also secrete enzymes into their environment to progressively reduce their molecular weight, producing oligomers, dimmers and monomers (Lucas *et al*., 2008). They have also been shown to be capable of degrading polymers that are widely regarded as being recalcitrant (Shimano, 2001; Howard, 2002; Szostac-Kotowa, 2004; Shah *et al*., 2008). Microrganisms ultimately assimilate polymers as cellular biomass and energy (Lucas *et al*., 2008). Any inhibition of the microbial community in terms of polymer utilisation could therefore result in the reduced functioning of such processes within the aquatic environment.

4.4.3. CFU Counts

In the present study no significant effects were seen on CFU counts as a result of exposing aquatic microbial communities to three concentrations of SMX. In other studies, Halling-Sorensen *et al*. (2002) observed an EC_{50} value of 0.03 mg/L for CTC and 5a, 6-CTC when studying the effect of these compounds on CFU counts. In this study activated sludge microbial communities were exposed to antibiotics, indicating that the growth of activated sludge communities may be more sensitive to antibiotics than river water communities. Colinas *et al*. (1993) also showed a significant decrease in CFU counts in soils exposed to oxytetracycline, with a 20% reduction in CFU count being observed. It is possible that the growth of soil microbial communities is more sensitive to tetracyclines than aquatic communities are to sulfonamides.

Alternatively, it may be the case that the portion of the aquatic microbial community that is affected by SMX is not culturable using traditional culturing techniques. A final possibility for not seeing any inhibition of microbial growth in the present study could be the presence of more resistant members of the aquatic microbial community than in other studies. One piece of evidence that supports this view is the high increase in CFU between days 1 and 7 as a result of exposure to 1 mg/L SMX. Although this change was not statistically significantly different compared with CFU changes in the control, the variation between agar plates was extremely high. This reduces the chances of gaining a significantly different value. Other possibilities to explain why CFU counts did not detect any potential effects of SMX are discussed in section 3.4.1.

Chapter 5: Presence and Development of Antimicrobial Resistance in Aquatic Microcosms

5. Presence and Development of Antimicrobial Resistance in Aquatic Microcosms

Experiments that were conducted in previous chapters had suggested that varying degrees of recovery had taken place within microcosms that had been spiked with CTC and, to a lesser extent, SMX. Several publications point to the fact that this may have been a result of antibiotic resistance forming as a result of selective pressure being exerted by CTC and SMX. There have also been reports of the presence of antibiotic resistance in treated sewage and natural river waters. As such, a degree of antibiotic resistance may hve already been present within microcosm systems at the start of the exposure. A review of antibiotic resistance and antibiotic resistence in the environment shall be presented before the presence and development of antibiotic resistance in laboratory microcosms is evaluated experimentally.

5.1. Specific Antibiotic Resistance

5.1.1. Tetracycline Resistance

Tetracycline resistance mechanisms are phenotypic traits whereby bacteria are able grow in otherwise inhibitory tetracycline concentrations. Tetracycline resistance is the result of bacteria possessing one or more copies of a range of genetic determinants, of which there are at least 38 *tet* genes and three *otr* genes (Roberts, 2005; Thompson *et al*., 2007). Each gene codes for a specific mechanism of resistance which can be categorised into distinct groups. The most abundant of these groups are the efflux proteins. Tetracycline efflux proteins actively transport tetracycline-divalent metal complexes across modified regions of the microbial cell membrane in exchange for protons or potassium ions (Krulwich *et al*., 2001).

Tetracycline efflux pumps belong to the Major Facilitator Superfamily (MFS) of efflux pump classes (Marger and Siaer, 1993). Interestingly, tetracycline efflux pumps also play a key role in non-antibiotic efflux such as alkali efflux (Padan and Krulwich, 2000; Alekshun and Levy, 2000). The genes *tet* A, A (41), B, C, D, E, G, H, J, Y, Z, 33, 35, 38, 39 and *otr* C are all genetic determinants for tetracycline efflux pumps.

Another major group of tetracycline resistance mechanisms are those that act as ribosomal protection proteins. These are coded by the tetracycline resistance genes *tet* M, O, S, W, Q, T, 32, 36, B (P) and *otr* (A). With the exception of *tet* (M) and *tet* (O) ribosomal protection proteins display a high degree of homology with elongation factors EF-Tu and EF-G. As such they are able to bind to bacterial ribosomes and cause a conformational change that prevents tetracycline binding (and therefore reduces its inhibitory effects). *Tet* (M) and *tet* (O) display ribosome-dependent GTP-ase (guanosine triphosphate-ase) activity. The energy liberated by the hydrolysis of GTP provides the energy required for conformational changes within the structure of the ribosome (Speer *et al*., 1992; Roberts, 2005).

Two more *tet* genes, *tet* (X) and *tet* (37) code for mechanisms that enzymatically inactivate tetracycline molecules (Lambert, 2005). *Tet* X for example codes for a flavoprotein that catalyses the monohydroxylation of the Tetracycline-Mg^{2+} binding domain. After several hours the tetracycline molecule degrades into several degradation products (Wright, 2005). The enzymatic activity of *tet* (37) is very similar to *tet* (X) in that a monohydroxylation reaction is catalysed which is NADPH-dependent (Diaz-Torres *et al*., 2003). Another *tet* gene, *tet* (U) has also been discovered, although the function of its product has yet to be deciphered (Ridenhour *et al*., 1996).

5.1.2. Sulfonamide Resistance

Sulfonamide resistance is genetically encoded by just three acquired *sul* genes, *sul I, sul II* and *sul III*. Each genetic determinant codes for a modified DHPS enzyme that has a higher affinity for PABA than the sulfonamide analogue. Each protein is similar to the original 263 amino acid form with only slight substitutions (Perreten and Boerlin, 2002). The phylogenetic origins of *sul* genes are unknown due to their clear distinction from all known chromosomal DHPS genes (Radstrom and Swedberg, 1988). The most recent sulfonamide resistance gene (*sul III*) was described in 2002 in Swiss pig farms. This gene accounts for 30% of environmental sulfonamide resistance alongside an unrelated mutation to the *folA* gene. Again, a single amino acid substitution is responsible (proline to serine at position 68). This mechanism is restricted to mutations in chromosomal DNA. *Sul* genes however have been detected on chromosomes and highly mobile elements called transposons (Huovinen *et al*., 1995).

5.1.3. Environmental Monitoring of Tetracycline and Sulfonamide Resistance Genes

Tet and *sul* genes have been discovered in all environmental matrices. The aquatic environment represents an important environmental gene-pool for *tet* and *sul* genes with several studies having detected specific tetracycline and sulfonamide resistance determinants. The results of these studies are summarised in table 5.1. This table highlights the variety of aquatic sources that *sul* and *tet* genes have been discovered in. These include a diverse array of natural matrices, such as natural waters and sediment and also a range of anthropogenic sources such as hospitals and STPs. Table 5.1 also shows that *sul* and *tet* genes have been detected in drinking water. Also included is information on whether specific genes are located on a plasmid or chromosome. Genes found on plasmids are generally very mobile and transferable. Chromosomal resistance genes are much less likely to be transferred, although it has been speculated that some genes may have been transferred via gene transduction, the process whereby genes are transferred between cells virally (Thompson *et al*., 2007). The transfer of genetic elements shall be discussed later in this chapter.

Table 5.1: Reported occurrence of *tet* and *sul* resistance genes in the aquatic environment.

Gene	Biological Source	Environmental Source	Plasmid or Chromosomal?	Reference(s)
tetA	*Aeromonas, Alcaligenes, Arthrobacter, Comamonas, Escherichia, Listeria, Pseudomonas, Salmonella and Vibrio*; Plasmids pB10, pTB11 and pRSB101	AS, DW, EW, NW, SD, SW, US	Plasmid	Szczepanowski *et al.*, 2004; Agersø and Sandvang 2005; Srinivasan *et al*., 2005; Tennstedt *et al.*, 2005; Poppe *et al*., 2006; Rodríguez *et al*., 2006; Cernat *et al*. 2007; Dang *et al*., 2007; Macauley *et al.*, 2007; Hu *et al*., 2008
tetA(41)	*Serratia*	NW	Chromosomal	Thompson *et al.*, 2007
tetB	*Afipia, Alcaligenes, Arthrobacter, Burkholderia, Escherichia, Pseudomonas, Serratia, Staphylococcus and Vibrio*	AS, DW, EW, NW, SW, US	Chromosomal or plasmid	Agersø and Sandvang 2005; Cernat *et al*., 2007; Dang *et al*., 2007; Jacobs and Chenia 2007; Kim *et al*., 2007; Kobashi *et al.*, 2007, Macauley *et al.*, 2007
tetC	*Aeromonas, Alcaligenes, Arthrobacter, Brevibacterium* and *Pseudomonas*	AS, EW, SW, US	Plasmid	Agersø and Sandvang, 2005; Akinbowale *et al*., 2007a; Macauley *et al*., 2007
tetD	*Aeromonas, Escherichia*; microbial community	AS, DW, EW, SW, US	Plasmid	Schmidt *et al*., 2001; Auerbach *et al*., 2007a; Cernat *et al*., 2007

Table 5.1 (cont).

Gene	Biological Source	Environmental Source	Plasmid or Chromosomal?	Reference(s)
tetE	*Aeromonas, Pseudoalteromonas* and *Vibrio*	AS, EW, SD, SW, US	Chromosomal and plasmid	Schmidt *et al*., 2001; Dang *et al*., 2006; Agersø and Petersen 2007
tetG	*Pseudomonas*; microbial community	AS, EW, SW, US	Plasmid	Auerbach *et al*., 2007; Macauley *et al*., 2007; Jacobs and Chenia 2007; Macauley *et al*., 2007
tetJ	*Pseudomonas*	SW	Plasmid	Macauley *et al*., 2007
tetY	*Acidiovorax, Acinetobacter, Comamonas,* and *Proteus*	SW	Plasmid	Macauley *et al*., 2007
tetZ	*Actinomycetales, Afipia, Brevibacterium, Burkholderia, Dietzia, Leucobacte*r and *Microbacterium*	SW	Plasmid	Tauch, 2000; Kobashi *et al*., 2007; Macauley *et al*., 2007
tet33	*Alcaligenes, Arthrobacter* and *Pseudomonas*	SW	Plasmid	Agersø and Sandvang, 2005

Table 5.1 (cont).

Gene	Biological Source	Environmental Source	Plasmid or Chromosomal?	Reference(s)
tet39	*Acinetobacter*	SD, SW	Plasmid	Agersø and Petersen 2007
tetA(41)	*Serratia*	NW	Chromosomal	Thompson *et al*., 2007
otrB	*Streptomycete*	AS, NW, SW		Nikolakopoulou *et al*., 2005
tetB(P)	Microbial community	SD, SW	Plasmid	Chee-Sanford *et al*., 2001; Pei *et al*., 2006
tetM	*Aeromonas, Bacillus, Escherichia, Lactococcus, Pseudoalteromonas and Vibrio*	AS, EW, NW, SD, SW, US	Chromosomal and plasmid	Mackie *et al*., 2006; Akinbowale *et al*., 2007b; Auerbach *et al*., 2007; Dang *et al*., 2007; Kim *et al*., 2007; Nonaka *et al*., 2007; Hu *et al*., 2008; Rahman *et al*., 2008; Suzuki *et al*., 2008
tetO	*Paenibacillus, Pseudoalteromonas, Shewanella, Sporosarcina and Vibrio*; microbial community	AS, EW, NW, SD, SW, US	Chromosomal and plasmid	Chee-Sanford *et al*., 2001; Smith *et al*., 2004; Mackie *et al*., 2006; Pei *et al*., 2006; Auerbach *et al*., 2007; Nonaka *et al*., 2007
tetQ	Microbial community	AS, EW, NW, SW, US	Chromosomal	Smith *et al*., 2004; Auerbach *et al*., 2007; Mackie *et al*., 2006

Table 5.1 (cont).

Gene	Biological Source	Environmental Source	Plasmid or Chromosomal?	Reference(s)
tetS	Lactococcus and Vibrio; microbial community	AS, EW, SD, SW, US	Chromosomal or plasmid	Chee-Sanford *et al*., 2001; Kim *et al*., 2004; Auerbach *et al*., 2007; Suzuki *et al*., 2008
tetT	Microbial community	SD, SW	Plasmid	Chee-Sanford *et al*., 2001; Pei *et al*., 2006
tetW	Microbial community	SD, NW, SW	Plasmid	Chee-Sanford *et al*., 2001; Mackie *et al*., 2006; Pei *et al*., 2006; Suzuki *et al*., 2008
otrA	*Streptomycete*; microbial community	AS, NW, SW	Plasmid	Chee-Sanford *et al*., 2001; Nikolakopoulou *et al*., 2005
sulI	*Aeromonas, Escherichia and Listeria*; Plasmids pB2, pB3, pB8, and pB10; Microbial community	AS, DW, NW, SD, SW	Plasmid	Heuer *et al*., 2004; Lin and Biyela 2005; Schlüter *et al*., 2005; Srinivasan *et al*., 2005; Akinbowale *et al*., 2007a; Cernat *et al*., 2007; Hu *et al*., 2008

Table 5.1 (cont).

Gene	Biological Source	Environmental Source	Plasmid or Chromosomal?	Reference(s)
sulII	*Acinetobacter, Escherichia, Salmonella, and Vibrio*; Microbial community	DW, NW, SD, SW	Chromosomal or plasmid	Pei *et al*., 2006; Agersø and Petersen 2007; Cernat *et al*., 2007; Hu *et al*., 2008; Mohapatra *et al*., 2008; Michael *et al*., 2012
sulIII	*Escherichia*; Microbial community	NW, SD	Plasmid	Pei *et al*., 2006; Hu *et al*., 2008

[a] SW: special wastewater from hospital, animal production and aquaculture areas; US: untreated sewage; AS: activated sludge of STP; EW: effluent water of STP: NW: natural water; SD: sediments: DW: drinking water

5.1.4. Multidrug Resistance (MDR) in the Environment

As well as a high reported occurrence of single tetracycline and sulfonamide resistance in the environment, there have also been a number of reported occurrences of environmental bacteria possessing more than one resistant gene, a process that shows features of positive epistasis (Trindade *et al*., 2009). This is a process whereby the effects of one gene are modified by the effects of another. In such cases members of the aquatic microbial community that have acquired multiple resistance genes are able to negate the inhibitory effect of a number of biocidal compounds that may be present in their habitat. Several authors have reported a surprisingly low fitness cost of obtaining antibiotic resistance (Ward *et al.,* 2009; Trindade *et al.,* 2009); it is therefore an evolutionarily sound strategy for environmental microbes to be resistant to several antibacterial toxicants.

The benefit of possessing a range of resistant genotypes is reflected in the wealth of literature that reports multidrug resistance in a range of environmental matrices. Methods for detection of MDR in the environment include phenotypic techniques (mainly agar disk–diffusion) and molecular methods, including PCR and electrophoresis gels and qPCR.

5.1.5. MDR in the Aquatic Environment

A range of phenotypic MDR studies have been conducted in the aquatic environment. As early as 1991, Magee and Quinn tested the MDR profiles of 183 heterotrophic aquatic isolates. They observed that 40% of isolates showed phenotypic resistance to 8 antibiotics and 35% of isolates tested were resistant to at least 2 of the 8 antibiotics tested. Ozgumus *et al.* (2009) also tested the susceptibility of a mixed aquatic microbial community (from 10 Turkish rivers) to a range of antibiotics. Of the 183 isolates tested, the most resistance was detected to ampicillin (58% resistance) and streptomycin (51.9%), with decreasing resistance shown to tetracycline (28.4%), trimethoprim (24%) and chloramphenicol (12.5%).

Other studies have targeted studying the MDR profiles of specific portions of a microbial community. Park *et al.* (2003) tested 1400 coliform isolates for resistance to sulfamethoxazole, aminoglyosides and β–lactams. They observed that 53.6% of isolates were resistant to one or more of their test compounds. Zhang *et al.* (2009) targeted *Acinetobacter sp.* and found that 28.6% of samples (from an STP) showed MDR using a phenotypic approach.

Jianying *et al.* (2008) observed the MDR profile of *E.coli* strains from a Beijing river in summer and winter. They showed that the MDR index was slightly higher in the winter (0.14) than in the summer (0.11). They found that a maximum of 48.7% of isolates showed resistance to one or more antibiotics, with sulfonamide, tetracycline and ampicillin resistance being the most frequent. Lima Bittencourt *et al.* (2007) studied the susceptibility of a range of *Enterbacteriaceae* isolates to

several antibiotics in a pristine riverine environment. They found that 61% of isolates tested were resistant to multiple antimicrobial compounds. They additionally observed that resistance to one or more antibiotics was higher in rainy (100%) than it was in dry seasons (89%).

There are also several reported occurrences of genotypic MDR in the aquatic environment. Jianying *et al.* (2008) complemented phenotypic MDR data with specific resistance gene detection. They found high levels of the ampicillin resistance gene *TEM*. 90% of tetracycline resistance and 96% of sulfonamide resistance could be explained by combinations of different *tet* (*tet (A), Tet (B)* and *Tet (M)*) and *sul* genes (*Sul I*, *Sul II* and *Sul III*). The different combinations of distinct resistance genes showed a wide diversity of MDR. Verner–Jeffreys *et al*. (2009) investigated MDR in ornamental fish carriage water. 47 of 94 isolates that were tested were resistant to more than 15 antibiotics. This could be explained by the detection of several genetic resistance determinants including *qnrS2, $bla_{TEM,}$ tet (A), Tet (E), Tet (D), qacE2, sul I* and an aminoglycoside transferase gene.

5.1.6. MDR in the Marine and Estuarine Environment

Several studies have quantified phenotypic MDR in the marine and estuarine environment. The majority of these studies have focused on MDR in *E. coli* strains. Florian-Fricke *et al*. (2008) observed that an *E. coli* SMS 3–5 that had been isolated from a tidal harbor system showed resistance to a range of antibiotic classes (fluoroquinolones, β–Lactams, aminoglycosides, quinolones, macrolides, sulfonamides and tetracyclines). The isolate was resistant to a number of specific compounds within each class, with MICs reaching as high as 10 000 µg/ml. Parveen *et al*. (1997) also demonstrated MDR profiles of marine *E .coli* strains. A wide range of MDR profiles were observed, the most prominent one being resistance conferred to CTC and sulfathiazole, followed by CTC, penicillin and sulfamethoxazole. 2% of *E. coli* isolates showed resistance to all antibiotics tested.

Laroche *et al.* (2009) also found multidrug resistance profiles in *E. coli* isolates of marine origin. The authors reported that the frequency of isolates being resistant to between 2 and 12 of antibiotics tested was between 60.5–80%. Chandran *et al*. (2008) also studied *E. coli* MDR isolates in an Indian tropical estuary. A relatively large proportion of cultured isolates (95%) were resistant to all 12 of the antibiotics that were tested. Fernandez–Delgado and Suarez (2009) also observed relatively high MDR profiles of *E. coli* that had been cultured from a warmer marine environment. They demonstrated that *E. coli* isolates were resistant to 9 out of 10 antibiotics tested. The also observed that 21 isolates were resistant to at least 5 antibiotics and one isolate (*Enterococcus durans*) was resistant to all 20 antibiotics tested.

Molecular characterisation of marine isolates has also shown MDR profiles. Dang *et al*. (2007) analysed mariculture isolates from China. Oxytetracycline resistance was commonly found along with chloramphenicol and ampicillin resistance. The genes *tet (A), tet (B), tet (M) and tet (D)* were

often found in conjunction with other genetic resistance determinants such as *cat II* and *floR.* McIntosh *et al.* (2008) detected resistant gene cassettes in the marine isolate *Aeromonas salmonicida*. One of these cassettes containined *floR, tetA, suIII* and *strA* / *strB* sequences and another coded for a *bla* (CMY-2) beta-lactamase in association with *sugE* and *blc* sequence.

5.1.7. MDR in the Soil Environment

Phenotypic MDR profiles of isolates that have been cultured from the soil environment have also been investigated. It is noteworthy that all of these studies have been conducted in areas where livestock were being reared or agricultural activity was taking place. Hayes *et al.* (2004) studied MDR profiles of isolates that had been cultured from a commercial poultry farm. MDR profiles were observed for all but one species of *Enterococcus.* One species (*E. faecium*) was resistant to 18 out of the 20 antibiotics that were tested. Santos *et al*. (2007) also examined phenotypic MDR profiles in soils that had been sampled from 12 North Carolina poultry farms. The study found that 5% of all isolates were resistant to all of the antibiotics that were tested. Other isolates were resistant to 9 (2%), 6 (5%), 7 (12%), 4 (10%), 3 (21%), 2 (24%) and 1 antibiotic (10%).

Peron *et al*. (2008) looked at the phenotypic MDR profile of swine production isolates. 362 *Salmonella* strains were tested for resistance to 12 antibiotics. They observed that more than 25% of isolates displayed MDR, with the most common MDR isolate being *Salmonella typhimurium,* a serotype with links to public health concerns. Singh *et al.* (2007) studied the presence of MDR *Salmonella* strains in two Indian vegetable farms. They observed that 82.9% of the isolates that were screened displayed MDR. Of these, 25% were resistant to all 10 of the antibiotics tested. Byrne-Bailey *et al.* (2009) investigated cross–resistance of soil isolates that were resistant to sulfonamides to other antibacterial compounds. It was observed that the 12 isolates were resistant to 3 of the 12 antibiotics tested, including nalidixic acid, tetracycline, neomycin and trimethoprim.

One study has provided evidence in favor of multiple resistance genes being present in the soil environment. Burgos *et al.* (2005) tested the resistance profiles of a range of soil–dwelling microbial species in the presence and absence of salicylate, a known promoter of the *mar* (multiple antibiotic resistance) operon. They observed a widespread increase in MIC values of all four of the antibiotics that had been tested in the presence of salicylate, in some cases up to 30 times the control values. These results indicated the expression of a wide array of resistance genes that the *mar* operon carries. Frequently these were resistance determinants of the antibiotics that had been tested (chloramphenicol, nalidixic acid, penicillin and tetracycline).

5.1.8. Multidrug Efflux in the Environment

A common strategy of Gram negative environmental microbes in becoming resistant to antibiotics (along with other toxic waste products and xenobiotics) is to express a multidrug efflux pump phenotype (MEPP). Gram negative efflux pumps exhibit a tripartite structure comprising a

transporter, an adapter and exit duct proteins. A typical Gram negative pump comprises an AcrB / MexB–Acr/ Mex A–Tol C/OprM structure (Symmons *et al.*, 2009). MEPPs allow environmental microbes to expel a huge number of unrelated toxins and drugs out of their cytoplasm (Koronakis *et al.,* 2004). Despite their ability to export anthropogenic xenobiotics out of their intracellular space, bacteria are likely to have evolved MEPPs to export natural chemicals out of their cell such as plant root exudates (Bais *et al*., 2006). It has been hypothesised that MEPPs may ultimately have played a key role in initiating microbe–plant interactions which would not have been possible if plant defenses were effective against microorganisms (Espinosa-Urgel *et al*., 2002). There are several main classes of MEPPs. These are the RND (Resistance Nodulation–Cell Division) family, the MFS (Major Facilitator Superfamily), ABC (ATP-Binding Cassette) superfamily, the SMR (Small Multidrug Resistance) family and the MATE (Multi Antimicrobial Extrusion) family (Martinez *et al*., 2009).

5.1.9. Reported Incidences of MEPPs in the Environment

In addition to specific resistance mechanisms, MEPPs have been detected in the environment. Species such as *Stenotrophomonas maltophilia* for example are known to be extremely tolerant to a range of antimicrobial compounds and much of this is thought to be due to the presence of MEPPs such as smeDEF efflux mechanisms (Hernandez *et al*., 2009). Florian-Frike *et al.* (2008) conducted recombinant studies to see if MDR phenotype profiles could be explained by MEPPs in the aquatic MDR isolate *E. coli* SMS 3–5. Their results implicated the role of AcrAB-TolC, together with MarA as being responsible for conferring resistance to tetracycline, chloramphenicol, ampicillin, nalidixic acid, and rifampin. In the same study it was also suggested that expression of the *fsr* gene (the product of which is an efflux pump) conferred resistance to both ofloxacin and ciprofloxacin.

Long *et al*. (2008) also studied MEPPs in *E .coli* as well as *Neisseria gonorrhoeae.* The study was able to demonstrate that the proteins NorM (from *N. gonorrhoeae*) and YdhE (from *E .coli*) greatly increased resistance to several compounds including antimicrobials (fluoroquinolones, ethidium bromide, rhodamine 6G, acriflavine, crystal violet, berberine, doxorubicin, novobiocin, enoxacin, and tetraphenylphosphonium chloride).

Groh *et al.* (2006) investigated the presence of MEPPS in the aquatic sediment–dwelling isolate *Shewanella oneidensis*. Knockout studies revealed the importance of the MexF MEPP in conferring resistance to chloramphenicol and tetracycline, suggesting the role of MexF in multidrug efflux in this isolate. Further studies also revealed the importance of a mutated TetR regulatory protein (a protein that is involved in tetracycline resistance) that interacts with the MexAB operon to confer multidrug resistance in *Shewanella oneidensis.* In addition, Szezepanowski *et al.* (2004) found MEPPs in bacterial plasmids in an STP that conferred genotypes to two MEPPs, an ABC–binding cassette efflux pump and an efflux membrane fusion protein (EMF) associated with the RND

family of MEPPs. It is also notable that that these genes were plasmid-based, suggesting that they may be highly transferrable.

5.1.10. Transfer of Genetic Elements between Environmental Microbes

So far in this review multidrug resistance has been discussed with regard to environmental microorganisms possessing multiple genotypes that have lead to the expression of multiple resistant phenotypes. A major mechanism by which bacteria can possess a range of resistance profiles is by the transfer of genetic elements from other bacteria, regardless of genera or species. Gene transfer can occur via three processes, transduction (DNA is taken up by cells into their genome from phage DNA), conjugative transfer (which relies upon mobile genetic elements) and transformation (when DNA is taken up by competent cells) (Droge *et al*., 1999). A wealth of literature has described both single and multiple gene transfer. Such studies are frequently combined with the detection of mobile genetic elements called intergrons or transposons which are responsible for carrying transferred genetic material. Such mobile elements may carry single resistance or so called multiple gene cassettes. Interestingly though, mobile genetic elements seem to have origins from before the "antibiotic era", suggesting that bacteria are using evolutionarily–conserved mechanisms to cope with a relatively modern environmental stressor of anthropogenic origin (Stokes *et al*., 2006).

5.1.11. Direct Experimental Evidence for Gene Transfer between Environmental Bacteria

Several studies have attempted to show the occurrence of gene transfer experimentally by conducting microbial "mating" experiments. Mating experiments have revealed that resistance gene transfer can occur in both an intragenic (transfer between same species) and intergenic (transfer between different species) manner (Zahid *et al*., 2008). Silva *et al*. (2006) for example were able to successfully show the transfer of multidrug resistance phenotypically between donor coliform isolates and a recipient *E. coli* K-12 strain by observing an increase in MIC values post–mating. Nagachinta and Chen (2008) also used a phenotypic approach in mating experiments. After mating an MDR donor (a shiga toxin producing strain of *E. coli*) with a multidrug sensitive *E. coli* K-12 strain, they observed that the sensitive K-12 transconjugant had developed resistance to sulfathiazole, streptomycin and oxytetracycline.

In another study that had been utilizing *E. coli* as a recipient (Agerso and Sandvang, 2005), various MDR soil strains acted as donors. This study used molecular techniques to quantify gene transfer. They observed co–transfer of various *tet* resistance determinants and class 1 intergrons, suggesting that tetracycline resistance was transferred via a gene region that was present on a mobile genetic element. Mukhergee *et al*. (2005) also utilized molecular techniques to study MDR transfer. They

found that three plasmids associated with MDR (D1QN–9, D2QN–14 and MR-1) were transferable between aquatic MDR isolates and an *E. coli* DH-5α recipient. Byrne–Bailey *et al.* (2009) conducted mating experiments between 12 MDR soil isolates (as donors) using both *E. coli* and *P. putida* as recipients. The transfer of *sul I, sul II* and *sul III* were investigated. They observed 64% successful transfer of *sul I* between soil isolates and recipients and a 36% transfer success to both recipients of *sul II. Sul III* was shown to be non-transferrable. These data would suggest that the *sul III* gene is not transferrable between bacteria that are present in the soil environment.

5.1.12. Reported Transfer of Single Drug Resistance Determinants in the Environment

A number of studies have conducted molecular genetic studies that have demonstrated the transfer of genetic determinants that confer resistance to a single compound. Knapp *et al.* (2008) demonstrated that a range of tetracycline resistance genes were transferrable in aquatic mesocosm studies. They observed an indirect association between the frequency of tetracycline resistance genes and Tn916/1545 genes, genes that code for transferable genetic structures called transposons. The results of this study had therefore suggested that certain *tet* genes can be transferred between bacteria in the aquatic environment.

Peterson *et al.* (2000) investigated the presence of a transferrable trimethoprin gene cassette in the aquatic isolate *Acenetobacter*. They were able to detect a *dhfr1* gene (linked to trimethoprin resistance) which was associated with an open frame sequence (orf) *orfC* which had previously been linked with a class 1 intergron of a clinical *enterobacterial* isolate.

Byrne–Bailey *et al*. (2009) investigated the transfer of various *sul* genes (*sul I, sul II and sul III*) via class I intergrons in soil studies. They found that 8% of isolates carried the *sul I* gene alongside the gene that encodes for class 1 intergrons (*int1*), compared with 17.9% of *sul II* containing isolates. Interestingly, very few isolates possessed the *int2* gene (which codes for class 2 intergrons), implying that class 1 integrons may be more significant resistance gene carrying elements.

In another study, McIntosh *et al*. (2008) showed that a gene cassette containing an aminoglycoside –3-transferase (conferring aminoglycoside resistace) was present on a class 1 intergron in *Aeromonas* strains that had been isolated from the marine environment. They found that the intergron/gene cassette was present on a conjugative incA/C plasmid. It has also been demonstrated that resistance to other antimicrobial compounds can be transferred. Gillings *et al.* (2009) for example were able to associate quaternary ammonium compounds (QAC) with transferrable elements in freshwater biofilm bacteria. They observed that the *qac* gene (which codes QAC resistance) could be detected on class 1 intergrons in a similar manner to which antibiotics can.

5.1.13. Reported Transfer of Multiple Drug Resistance Determinants in the Environment

In addition to the transfer of genetic elements that confer resistance to a single antibiotic (or other compound or element) in the environment, there have been several reports of multiple gene transfer or transfer of MEPPs. This has mainly been studied in the aquatic environment and has been observed in several countries. Laroche *et al.* (2009) studied MDR transfer in the River Seine in France. They detected *int1* and *int2* in 11% of their isolates. When these regions were further investigated two dominant gene cassettes were discovered, *aadA* and *dfr*. These gene cassettes are known to confer resistance to a number of antibiotics, including some of those that the isolates in the study were resistant to, such as CTC, kanamycin and amoxicillin. Ozgumus *et al.* (2008) examined the potential for MDR isolates to transfer multiple resistance genes in Northern Turkish rivers. In addition to reporting nalidixic acid strains that harboured quinolone resistance, the study also found class 1 and class 2 intergrons on 15 and 4% of coliform isolates respectively. Sequence analysis of the gene cassettes revealed the presence of *dfrA1, dfr2d, dfrA7, dfrA16, dfrA17, aadA1, aadA5, bla* (*oxA-30*), and *sat1*. These resistance determinants were all transferrable.

Park *et al*. (2003) also detected the presence of class I intergrons in aquatic isolates (24%), this time in South Korea. They also observed multiple resistance determinants on mobile gene cassettes. These included *dfrA5, dfrA7, dfrA12, dfrA17, aaA2, aaA5*, and *aad (3').* Mukherjee and Chakraborty (2006) studied multidrug gene transfer in Indian rivers. As well as finding class 1 intergrons on 40% of isolates tested, they were also able to show a statistically significant correlation between MAR and presence of class 1 intergrons. The study also found the genes *dfrA1, dfrA5, dfrA7, dfrA17* and *dfrA12.* They additionally detected variant gene sequences conferring resistance to amikacin, tobramycin, streptomycin and spectinomycin.

The transfer of MDR has also been studied in the marine environment. McIntosh *et al*. (2008) studied MDR gene transfer elements in the marine species *Aeromonas salmonicida*. They examined the structure of class 1 intergrons that were present in MDR isolates. One of these gene cassettes coded for an inA/C plasmid that carried the resistance determinants *floR, tetA, suIII* and *strA/strB.* Jacobs and Chenia (2007) investigated MDR transferrable genes in South African aquaculture systems. They found that 37.8% of isolates carried multiple resistance plasmids. Upon sequencing they discovered that class 1 intergrons (and related structures) carried different combinations of the genes *ant (3'') Ia, aac (6) Ia, dhfr1, oxa2a* and/or *pse1*. Interestingly, no variable resistance gene cassettes could be found on class 2 intergrons providing more evidence that antibiotic resistance gene transfer is mediated mainly by class 1 intergrons in the environment.

5.1.14. Development of Antibiotic Resistance in the Environment

5.1.14.1. External Sources of Resistance Genes in the Environment

Several studies have investigated the potential for external sources of resistance genes to become part of environmental resistance gene reservoirs. Most studies have focused their attention on studying the fate of resistance genes from highly polluted environments into relatively less polluted ones. Lu *et al.* (2010) for example looked at the clonal relatedness between MDR bacteria from different origins (animals, workers and environment) in a range of farms. They observed a high degree of homology between samples. The results of this study suggested that resistance genes had been exchanged between animals, the environment and farm workers, although the exact pathways were not investigated. Heuer and Smalla (2007) produced data that supported some of these findings. Their study investigated the effects of manuring on the abundance of *sul* genes in soil samples. They found that *sul1* and *sulII* genes could be transferred from manure to soil, suggesting the role of animal intestinal bacteria in contributing to the soil resistome (a collection of all of the antibiotic resistance genes that are present in an environment) (Wright, 2007).

Byrne–Bailey *et al.* (2009) also provided evidence that manure application onto soils can increase the *sul* gene reservoir in the environment. Their work tracked *sul* gene levels in soils that had been amended and that had not been amended with pig slurry. They observed that the *sulIII* genotype was only present in soil samples that had been treated with pig slurry. In addition, it was reported that soil columns that had received a post-application of slurry contained a higher number of *sul* genes. These two results would suggest that *sul* genes in pig slurry can enter the terrestrial environment. *Sul* genes could also be detected in soil leachate, suggesting a possible reservoir of *sul* genes that may be further transported into different matrices, such as groundwater.

Schmitt *et al*. (2006) also investigated the effect of manuring soil on the number of *sul* gene copies that were present in microcosm and field studies. They also detected possible increases in *tet* gene copy numbers. In manure amended microcosms a large increase in *tet* and *sul* gene copies were observed. It was further demonstrated that the genes *tet (Y), tet (S), tet (C), tet (Q),* and *tet (H)* were only present in soil as a result of manuring. Field studies were slightly less conclusive, as the sites that were investigated had a considerable number of *tet* and *sul* gene copies that were already present. These results did indicate however that the soil environment contains a considerable resistance gene reservoir.

Knapp *et al.* (2010) investigated the fate of erythromycin and β–lactamase genes from swine lagoon waste into the aquatic environment using mesocosms. They observed that resistance genes disappeared from the water column rapidly. After 2 days they were able to detect a variety of genes in aquatic biofilms, although these genes disappeared over time. Interestingly, the transfer of specific genes was altered by different light and darkness regimes, suggesting that the transfer of at

least some resistance genes had depended upon specific abiotic factors. Mackie *et al.* (2006) conducted field studies to investigate the impact of manure management practices on the number of *tet* gene copies in nearby groundwater. They found a greater detection frequency of *tet* genes in groundwater underlying or slightly down–stream of the facility than in wells that were further away. The authors concluded that *tet* genes from farming represent a persistent environmental contaminant.

STPs as a source of antibiotic resistance genes into the environment have also been investigated. Szczepanowski *et al.* (2004) investigated the possibility of bacteria in STPs transferring genetic material into the aquatic environment. They analysed a range of plasmids that were harbouring antibiotic resistant genotypes. They observed that one of these plasmids, pRSB1 01 contained a number of resistance genes that were contained on a class I intergron associated with a highly mobile Tn–402 transposon-like sequence. This region of DNA also contained a number of genetic resistance determinants that potentially conferred resistance to β–lactams, trimethoprim, macrolides, tetracyclines and sulfonamides. A mobile region coding for two types of tripartite MEPPs was also detected, suggesting the possibility that these could be transferred also.

Silva *et al.* (2006) showed that STP effluent contained elevated levels of antibiotic resistant coliform bacteria. Such bacteria therefore act as a source of resistance genes into the aquatic environment. Zhang *et al.* (2009) also investigated the release of resistant isolates into the aquatic environment from STPs. The study tracked the presence of multidrug resistant *Acinetobacter* strains throughout the sewage treatment process. They observed an increase in multidrug resistant isolates that were present in effluent compared with raw influent samples. They also observed that isolates downstream of an STP showed greater multidrug resistance than isolates sampled from upstream. The results of these studies therefore show the potential of STPs to contribute to the natural resistome of aquatic habitats.

As mentioned in chapter 1, sewage sludge is often applied to land as a fertilizer. Selvaratnam and Kunberger (2004) studied this as a source of resistance genes into surface water via run–off from sludge–amended fields. They observed that *AmpR* (conferring ampicillin resistance) frequency could be positively correlated with *AmpR* input from agricultural run–off.

The transfer of resistance genes from land to seas has also been observed. Chandrasekaran *et al.* (1998) were able to successfully mate the soil isolate *P. flourescens* with a halophilic and halo–tolerant marine species. They observed that mating was successful even when *P fluorescens* was in a viable but non–culturable state and in a variety of nutrient levels. The potential for terrestrial influence of the marine resistome was thus exhibited.

5.1.14.2. Antibiotics as a Selective Pressure for the Formation of Antibiotic Resistance in the Environment

For some time it has been hypothesized that antibiotic residues in the environment may select for antibiotic resistance. The concept is based upon Darwin's theory of Natural Selection (Darwin, 1859) and has long been used as an example of the concept. With regard to antibiotic resistance it has been theorized that the members of the microbial community that can tolerate antibiotic exposure will survive and replicate; the microbial community will then contain more antibiotic-resistant genetic material. It has also been theorized that this scenario will be greatly helped by the ability of the microbial community to mutate at a rapid rate due to their short generation time (20–30 minutes). This has been observed with mutations to the *folA* gene that can confer sulfonamide resistance. As discussed in the previous section, the ability of environmental microbes to exchange genetic material also acts to increase the total microbial resistome in the environment.

In a recent study, Knapp *et al.* (2010) examined the presence of resistance genes in archived Dutch soils (from 1940–1968). This represented a gradient of antibiotic discovery and usage, ranging from low in the 1940s to high in 2008. The results indicated that the antibiotic resistance profile of the soils had increased over time. The abundance of tetracycline resistance genes showed a particularly sharp rise, with a > 15 times increase between 1970 and 2008. The authors commented on the fact that the abundance of resistance genes had risen despite improvements in waste management. These results show a global temporal trend of antibiotic use resulting in an increase in antibiotic resistance.

5.1.14.3. Selection of Single–Drug Resistance

The extent to which tetracyclines act as selecting agents for resistant genotypes in the environment has been the subject of a large body of literature. Kim *et al.* (2007) used activated sludge that had been spiked with tetracycline concentrations of 1–250 µg/L. As the tetracycline concentration increased, an increase in the number of resistant cells was observed. It was also observed that resistant isolates showed higher growth rates at higher tetracycline concentrations.

Rhysz and Alverez (2004) conducted aquifer studies to study the effects of tetracycline on the formation of resistance in run–off from agricultural land. A tetracycline concentration of 50 mg/L reduced the number of heterotrophic bacteria in the system while also increasing the number of tetracycline resistant bacteria. Interestingly, the study also observed the effects of removing tetracycline from the system (depuration period). It was observed that the system had completely recovered in terms of the bacterial community structure returning to that of the control as shown by 16S RNA profiles becoming more similar during the depuration period.

Kanpp *et al.* (2008) also used a simulated environment (mesocosm studies) when looking at oxytetracycline as a selector for resistance. They observed that an oxytetracycline concentration of

20 µg/L resulted in an increase in the ratio of *tet* genes: total 16S RNA. In addition, an increased abundance of *tet (M)* was positively correlated to all tetracycline concentrations, suggesting that this gene is particularly sensitive to the selective pressure exerted by tetracyclines. The results of a study by Yu *et al*. (2009) agree with these findings. In this investigation it was found that an oxytetracycline concentration of 5 mg/L resulted in elevated tetracycline resistance levels in the indicator species *Enterococcus faecalis* in an aquatic test system. The study also concluded that selection for resistance was highest when tetracycline was added with animal feed.

The ability of tetracyclines to act as a selecting agent for antibiotic resistance has also been investigated in the marine environment. Using marine microcosms, Kerry *et al.* (1996) observed a > 90% frequency in resistant CFU counts at an oxyteracycline concentration of 25 µg/g compared with a < 7% frequency at an oxytetracycline concentration of 6.25 µg/g or less. When the effect of adding oxytetracycline after 3 days of microcosm incubation was tested, oxytetracycline lost its selective pressure, perhaps suggesting that marine resistance may form more readily in a microbial community that is growing quicker and is able to establish itself within an environment.

Several studies have also investigated sulfonamides as selective agents for resistance in the terrestrial environment. Brandt *et al.* (2009) examined the effect of sulfadiazine on pollution induced community tolerance profiles. They found that sulfadiazine and nutrient amendment showed a tendency to increase the tolerance of the microbial community. The authors commented that these results showed that resistance to sulfonamides could proliferate in certain soil "hotspots." Heuer and Smalla (2007) investigated the effect of sulfadiazine that had beenamendment to soil. They showed that sulfadiazine, along with manure amendment, had an effect on *sulI* and *sulII* abundance in soils over time, suggesting that a synergistic effect occurred. Heuer *et al.* (2008) further investigated the effect of sulfadiazole on the selection of sulfonamide resistance, using a modeling approach. They concluded that a selective effect of sulfathiazole was observed on *sulII* selection at concentrations as low as 0.15 mg/Kg.

A Chinese study recently investigated the effect of several sulfonamides (sulfamethoxazole, sulfadiazine and sulfachloropyridazine) on the selection of sulfonamide resistance selection in the aquatic environment (Yi *et al*., 2010). They found a positive correlation between total sulfonamide concentration and the total abundance of resistance genes in aquatic sediment. Interestingly, the authors detected 120–2000 more *sul* gene copies in sediment then in the water column, implicating aquatic sediments as an important *sul* gene reservoir in the environment (Yi *et al*., 2010).

The influence of other antibiotics as selective agents of resistance formation has been investigated to a much lesser extent. Cermak *et al.* (2008) observed an increased selection of *lmrB* homologues in soils that had been treated with lincomycin compared with untreated soils. Lu *et al*. (2004) investigated the effect of vancomycin–exposed soils on the long term selection of the *vanHAX* genotype in animal commensal bacteria. The authors concluded that exposure to vancomycin had

selected for vancomycin resistance in animal commensal bacteria based on gene cluster analysis. They further reported that humans faced exposure to animal bacteria that had become vancomycin–resistant. Yu *et al.* (2009) studied the effect of ciprofloxacin on the formation of resistance in the aquatic environment. They observed that 2 mg/L of ciprofloxacin administered in animal feed resulted in the selection of resistant bacteria.

5.1.14.4. Co–Selection of Antibiotic Resistance: Development of Multidrug Resistance

As well as antibiotics acting as a selective agent for single drug resistance in the environment, a number of studies have examined co–selection of a number of resistance profiles. Peterson *et al.* (2002) tracked resistance levels to a number of antibiotics over time in aquatic systems that been amended with farming animal waste. Although the exact antibiotic composition in the waste were unknown they were likely to contain mainly tetracyclines based upon normal farming procedures. The authors observed co–selection of chloramphenicol, ciprofloxacin, erythromycin, oxytetracycline, sulfamethizine and trimethoprim over the study period.

Wright *et al.* (2008) investigated the effect of heavily polluted water (containing antibiotic and metals) on the abundance of class 1 intergrons in microcosm experiments. The results of this study therefore suggested that polluted microcosms had a greater potential for gene transfer and possibly the formation of multidrug resistance.

Heuer *et al.* (2007) investigated the effect of exposing soils to sulfadiazole on the composition of class 1 intergrons. They observed that sulfadiazine had a synergistic effect with manure on increasing the number *int1* gene copies in soil. Sulfadiazine was thus shown to play at least some role in increasing the number of mobile elements that could (in theory) lead to a rise in MDR in manure soils.

From the available literature, it is evident that the exposure of microbial communities to general contaminants (that may not be antibiotic compounds) may also result in the proliferation of antibiotic resistance. A major reason for this is likely to be the movement of several genetic elements on mobile elements such as gene cassettes on integrons and transposons (eg: Ozgumus *et al.*, 2008). The presence of multidrug efflux pumps and the possibility for these to be transferred on mobile genetic elements also increases the likelihood that co-selection of resistance will occur in the presence of a general environmental stressor (such as heavy metal pollution).

5.1.15. Fears over Antibiotic Resistance in the Environment: The Broader Issues

There has been widespread media coverage concerning antibiotic resistance in clinical settings, such as the spread of MRSA (methicillin-resistant *Staphylococcus aureus*) and the rise of so called

“superbugs.” For some time now the propensity for these microorganisms to exchange genetic material and mutate has been known. Scientists therefore understand that clinical isolates can (and do) rapidly adapt to conventional antibiotic chemotherapy. The next question the scientific community are asking themselves is whether or not antibiotic resistance in the environment can have an impact on human health. Or more specifically, can resistance that started in the environment be transferred to bacteria that are human pathogens? On the same note, can environmental resistance be transferred to pathogens of animals that are economically important?

There has already been some research that provides an insight into some of these issues. Kruse and Sorum (1994) investigated resistance gene transfer (a multidrug resistant R plasmid) between bacteria that been isolated from different microhabitats. They found that multidrug resistance was transferrable between the human pathogen *Vibrio cholera* and a previously susceptible fish pathogen, *Aeromonas salmonicida.* The fish pathogen could also act as a recipient to pathogenic bovine *E. coli*. In addition, MDR bovine pathogenic *E. coli* could also transfer multidrug resistance to a previously susceptible human pathogenic *E. coli*. Transfer of resistance from animal (pocine and fish) pathogens to human pathogens was also demonstrated during this study. Lu *et al.* (2004) described a study in which they saw vancomycin resistance form in animal feed. The gene cluster involved was then passed to animal commensal bacteria and eventually to bacteria that humans regularly came into contact with. It had therefore been demonstrated that vancomycin resistance determinants (and therefore vancomycin resistance) had been transferred between different gene pools. Opegaard *et al.* (2001) found genetic homology between R plasmids from cattle and humans associated with farm work. The authors speculated that the spread of the MDR R plasmid was due to antibiotic residues on in the farm environment acting as a selective pressure for resistance formation.

Mukherjee and Chakraborty (2005) studied multidrug resistant gene transfer in Indian rivers. They isolated a *Margonella sp.* that harbored a multidrug resistant gene cassette that shared homology to a multidrug resistant gene cassette that was associated with the human pathogenic bacteria *V. cholerae*. The results of this study indirectly suggested that exchange of genetic material between pathogenic and non–pathogenic bacteria is possible.

Gillings *et al.* (2009) found more direct evidence that suggested gene transfer had taken place between environmental bacteria and a clinical isolate. They found identical gene cassettes (encoding for QAC resistance) associated with aquatic proteobacteria and clinical human pathogens. Rhodes *et al.* (2000) investigated resistance gene transfer between an aquaculture location and bacteria found in a nearby hospital. They observed significant genetic similarities between *Aeromonas sp.* and *E. coli*, suggesting that the aquaculture and human environmental compartments can act as one resistance reservoir.

5.1.16. The Biolog PM11C and PM12B Assays as a Phenotypic Reporter of Multidrug Resistance

Biolog PM11C and PM12B arrays are comprised of a 96 well microplate format. Each well contains a coating of a specific antibiotic at set a concentration; each antibiotic is present at 4 different concentrations. Low cellular suspensions of a particular isolate are prepared then inoculated onto PM plates. The inoculating solution is also prepared with a redox dye which is reduced and changes colour when microbial growth occurs. This colour change can be measured spectrally at 595 nm. The system therefore acts a "phenotypic reporter" of microbial growth in the presence of antibiotics. Figure 5.1 and 5.2 show the configuration of antibiotic compounds that are present on PM11C and PM12B plates respectively.

A1 Amikacin 1	A2 Amikacin 2	A3 Amikacin 3	A4 Amikacin 4	A5 Chlortetracycline 1	A6 Chlortetracycline 2	A7 Chlortetracycline 3	A8 Chlortetracycline 4	A9 Lincomycin 1	A10 Lincomycin 2	A11 Lincomycin 3	A12 Lincomycin 4
B1 Amoxicillin 1	B2 Amoxicillin 2	B3 Amoxicillin 3	B4 Amoxicillin 4	B5 Cloxacillin 1	B6 Cloxacillin 2	B7 Cloxacillin 3	B8 Cloxacillin 4	B9 Lomefloxacin 1	B10 Lomefloxacin 2	B11 Lomefloxacin 3	B12 Lomefloxacin 4
C1 Bleomycin 1	C2 Bleomycin 2	C3 Bleomycin 3	C4 Bleomycin 4	C5 Colistin 1	C6 Colistin 2	C7 Colistin 3	C8 Colistin 4	C9 Minocycline 1	C10 Minocycline 2	C11 Minocycline 3	C12 Minocycline 4
D1 Capreomycin 1	D2 Capreomycin 2	D3 Capreomycin 3	D4 Capreomycin 4	D5 Demeclocycline 1	D6 Demeclocycline 2	D7 Demeclocycline 3	D8 Demeclocycline 4	D9 Nafcillin 1	D10 Nafcillin 2	D11 Nafcillin 3	D12 Nafcillin 4
E1 Cefazolin 1	E2 Cefazolin 2	E3 Cefazolin 3	E4 Cefazolin 4	E5 Enoxacin 1	E6 Enoxacin 2	E7 Enoxacin 3	E8 Enoxacin 4	E9 Nalidixic acid 1	E10 Nalidixic acid 2	E11 Nalidixic acid 3	E12 Nalidixic acid 4
F1 Chloramphenicol 1	F2 Chloramphenicol 2	F3 Chloramphenicol 3	F4 Chloramphenicol 4	F5 Erythromycin 1	F6 Erythromycin 2	F7 Erythromycin 3	F8 Erythromycin 4	F9 Neomycin 1	F10 Neomycin 2	F11 Neomycin 3	F12 Neomycin 4
G1 Ceftriaxone 1	G2 Ceftriaxone 2	G3 Ceftriaxone 3	G4 Ceftriaxone 4	G5 Gentamicin 1	G6 Gentamicin 2	G7 Gentamicin 3	G8 Gentamicin 4	G9 Potassium tellurite 1	G10 Potassium tellurite 2	G11 Potassium tellurite 3	G12 Potassium tellurite 4
H1 Cephalothin 1	H2 Cephalothin 2	H3 Cephalothin 3	H4 Cephalothin 4	H5 Kanamycin 1	H6 Kanamycin 2	H7 Kanamycin 3	H8 Kanamycin 4	H9 Ofloxacin 1	H10 Ofloxacin 2	H11 Ofloxacin 3	H12 Ofloxacin 4

Figure 5.1: Schematic configuration of different antibiotics on a Biolog PM11C plate (Biolog, 2007).

A1 Penicillin G 1	A2 Penicillin G 2	A3 Penicillin G 3	A4 Penicillin G 4	A5 Tetracycline 1	A6 Tetracycline 2	A7 Tetracycline 3	A8 Tetracycline 4	A9 Carbenicillin 1	A10 Carbenicillin 2	A11 Carbenicillin 3	A12 Carbenicillin 4
B1 Oxacillin 1	B2 Oxacillin 2	B3 Oxacillin 3	B4 Oxacillin 4	B5 Penimepicycline 1	B6 Penimepicycline 2	B7 Penimepicycline 3	B8 Penimepicycline 4	B9 Polymyxin B 1	B10 Polymyxin B 2	B11 Polymyxin B 3	B12 Polymyxin B 4
C1 Paromomycin 1	C2 Paromomycin 2	C3 Paromomycin 3	C4 Paromomycin 4	C5 Vancomycin 1	C6 Vancomycin 2	C7 Vancomycin 3	C8 Vancomycin 4	C9 D,L-Serine hydroxamate 1	C10 D,L-Serine hydroxamate 2	C11 D,L-Serine hydroxamate 3	C12 D,L-Serine hydroxamate 4
D1 Sisomicin 1	D2 Sisomicin 2	D3 Sisomicin 3	D4 Sisomicin 4	D5 Sulfamethazine 1	D6 Sulfamethazine 2	D7 Sulfamethazine 3	D8 Sulfamethazine 4	D9 Novobiocin 1	D10 Novobiocin 2	D11 Novobiocin 3	D12 Novobiocin 4
E1 2,4-Diamino-6,7-diisopropyl-pteridine 1	E2 2,4-Diamino-6,7-diisopropyl-pteridine 2	E3 2,4-Diamino-6,7-diisopropyl-pteridine 3	E4 2,4-Diamino-6,7-diisopropyl-pteridine 4	E5 Sulfadiazine 1	E6 Sulfadiazine 2	E7 Sulfadiazine 3	E8 Sulfadiazine 4	E9 Benzethonium chloride 1	E10 Benzethonium chloride 2	E11 Benzethonium chloride 3	E12 Benzethonium chloride 4
F1 Tobramycin 1	F2 Tobramycin 2	F3 Tobramycin 3	F4 Tobramycin 4	F5 Sulfathiazole 1	F6 Sulfathiazole 2	F7 Sulfathiazole 3	F8 Sulfathiazole 4	F9 5-Fluoroorotic acid 1	F10 5-Fluoroorotic acid 2	F11 5-Fluoroorotic acid 3	F12 5-Fluoroorotic acid 4
G1 Spectinomycin 1	G2 Spectinomycin 2	G3 Spectinomycin 3	G4 Spectinomycin 4	G5 Sulfa-methoxazole 1	G6 Sulfa-methoxazole 2	G7 Sulfa-methoxazole 3	G8 Sulfa-methoxazole 4	G9 L-Aspartic-β-hydroxamate 1	G10 L-Aspartic-β-hydroxamate 2	G11 L-Aspartic-β-hydroxamate 3	G12 L-Aspartic-β-hydroxamate 4
H1 Spiramycin 1	H2 Spiramycin 2	H3 Spiramycin 3	H4 Spiramycin 4	H5 Rifampicin 1	H6 Rifampicin 2	H7 Rifampicin 3	H8 Rifampicin 4	H9 Dodecyltrimethyl ammonium bromide 1	H10 Dodecyltrimethyl ammonium bromide 2	H11 Dodecyltrimethyl ammonium bromide 3	H12 Dodecyltrimethyl ammonium bromide 4

Figure 5.2: Schematic configuration of different antibiotics on a Biolog PM11B plate (Biolog, 2007).

5.1.17. Aims and Objectives

1) To investigate the formation of CTC and SMX resistance as a result of the selective pressure exerted by the exposure of aquatic microbial communities to CTC and SMX and to examine the hypothesis that this influenced the functional recoveries that were observed in Chapters 3 and 4.

2) To investigate whether exposure to CTC and SMX selects for a wider resistance profile.

3) To investigate the extent to which antibiotic resistance is already present in test systems and to what extent antibiotic resistance forms in the absence of antibiotics as a selective pressure.

5.2. Materials and Methods

5.2.1. Reagents

All reagents and chemicals were purchased and prepared according to section 2.5.1. Biolog PM11C and PM12B plates and all associated equipment were purchased from Biolog (Hayward, CA).

5.2.2. Preparation of R2A/CTC and Sulfamethoxazole Agar Plates

R2A agar (11.5g) was mixed with 1 liter of deionised water. The R2A agar/deionised water suspension was then slowly heated while being stirred on a magnetic infrared hotplate. When the mixture was completely melted it was autoclaved at 121°C for 15 minutes. Following cooling (~ 15 minutes), CTC or sulfamethoxazole stock solutions were pipetted into the melted R2A agar to achieve a final [CTC] / [SMX] of 1 mg/L. The spiked melted R2A agar was then poured into sterile Petri dishes leaving an air space of ~0.25cm.

5.2.3. Preparation of Biolog BUG B Media

Biolog BUG B media (15 g) was mixed with 1 liter of deionised water. The agar / water suspension was then slowly heated and stirred on a magnetic infrared hotplate. When the mixture was completely melted it was autoclaved at 121°C for 15 minutes. Following cooling the melted agar was poured into sterile petri dishes in a class II Hera cabinet.

5.2.4. Preparation of Biolog PM Media

Biolog IF-10a inoculating fluid (a proprietary nutrient broth used for inoculating Biolog PM Plates) was prepared by mixing 125 ml 1.2 x Biolog IF-10a fluid with 37.5 ml deionised H_20. Biolog IF-10a inoculating fluid was prepared by mixing 125 ml of 1.2 x IF-10a fluid with 36 ml deionised H_20 and 1.5 ml Biolog Dye Mix A (an aqueous tetrazolium dye). All media was prepared in a Hera class II flow cabinet to minimize contamination.

5.2.5. CFU Counts of CTC and Sulfamethoxazle–Resistant Bacteria

Serial dilutions (0, 10^{-1}, 10^{-2} and 10^{-3}) of microcosm liquid were prepared using sterile water. Triplicate R2A -CTC / sulfamethoxazole agar plates were spread-plated with each 0.1 ml of each dilution factor using a sterile plate spreader. Plates were then incubated at 20°C for 72 hours. After incubation, the numbers of individual CFUs on each plate were enumerated by eye on each agar plate. Only counts of 30-300 CFU were considered as being valid. This process was repeated on days 1 and 7 of the exposure period. CFU counts of each CTC and SMX resistant morphotype was also taken in plates spread from control microcosms following the same procedure.

5.2.6. Preparation of R2A Slopes

11.5g R2A agar was weighed on a calibrated balance and mixed with 1 litre of deionised water. The R2A agars / deionised water suspension was then slowly heated while being stirred on a magnetic infrared hotplate. When the mixture was completely melted it was autoclaved at 121°C for 15 minutes. Following a period of cooling, 5.5 ml melted R2A agar was poured into a sterile sample tube at an angle of 25º - 35º and allowed to set. Slopes were prepared and seeded in a Hera class II flow cabinet to minimise contamination of R2A slopes.

5.2.7. Morphotyping and Storage of Antibiotic Resistant Bacteria

Each R2A + CTC/SMX agar plate (plated on day 1 and 7 of each corresponding exposure and including inoculations of control microcosm liquid) that contained visible colony growth was screened for individual morphtypes. Each morphotype is considered as being a distinct isolate during the current investigation. Morphotypes were harvested from R2A plates that had been spiked with both treated and dosed microcosms liquid. Criteria for individual morphotypes was based on colony colour, size, shape, translucence and other features that made them different from other colonies such as distinct patterns (such as veined colony). Each morphotype description was noted. Each individual morphotype was then picked up with a flamed loop and streak–plated onto a new sterile R2A plate. If pure cultures were not obtained then the desired colony was re–streaked onto a new sterile R2A plate. When a pure culture was obtained a single colony was picked up with a sterile loop and a pre–poured slope was seeded. Slopes were stored at 20ºC for 72 hours to allow growth then transferred to a 4ºC cold–store to slow growth until future use of cultures.

5.2.8. Biolog PM11C and PM12B Array Procedure

A Biolog IF–10a / pure culture suspension was prepared by swirling a Biolog longswab covered in a pure morphotype culture in a sterile tube containing IF–10a. Cultures were grown on Biolog BUG B agar plates. A sample of the suspension (~1 ml) was then read at 600 nm with a spectrophotometer. Depending on the absorbance reading, the suspension was amended with either more pure culture or IF-10a until an absorbance of 0.85 was obtained. A 1:5 ratio of the final cell suspension was then prepared using fresh IF–10a. A further dilution of 1:200 was made in IF–10a + Biolog Dye Mix A solution to obtain the final diluted culture suspension. 100 µl of 1:200 pure culture / IF-10a + dye mix A suspension was used to seed Biolog PM11C or PM12B plates, depending on the antibiotic the individual morphotype was resistant to (CTC-resistsnt isolates were used to seed PM11C plates, sulfamethoxazole- resistant isolates were used to seed PM12B plates as corresponding plates contained the original antibiotic that these microbes were resistant to). This was seen as being a trade-off between relevance and cost as all isolated morphotypes could not feasibly be plated on both types of plate. Plates were then incubated at 20ºC for at least 94 hours. During this period, plates were read spectrally at 595 nm periodically. This procedure was repeated for each isolated morphotype (from day 1 and day microcosms) that could be isolated during the

exposure. Blank wells on a standard 96–well plate were also inoculated with 100 µl 1.2 x diluted IF–10a fluid to obtain blank readings. All procedures were performed in a Hera Class II flow cabinet to minimise contamination.

5.2.9. Resistant CFU Counts

Serial dilutions (0, 10^{-1}, 10^{-2} and 10^{-3}) of microcosm liquid (all doses and control) were prepared using sterile water. Triplicate R2A-SMX and R2A-CTC agar plates were spread-plated with each 0.1 ml of each dilution factor using a sterile plate spreader. Plates were then incubated at 20°C for 72 hours. After incubation, the numbers of individual CFUs on each plate were enumerated by eye on each agar plate. Only CFU counts of between 30 and 300 were accepted for further analysis.

5.2.10. Data Analysis

5.2.10.1. Total Resistant CFU Counts over Time: Formation of Culturable Resistance

Total resistant CFU count data were treated in an identical manner as described in section 3.2.8.

5.2.10.2. Morphotype Data

The total number of distinct resistant morphotypes was enumerated for each antibiotic resistance profile (CTC and SMX resistance) on days 1 and 7 of the exposure. These data were then graphed using Microsoft Excel. Morphotypes from control and dosed microcosms were included. The profile of each morphotype that was inoculated onto each R2A + CTC/SMX plate was then cross referenced using the descriptions of each unique morphotype. The presence of each specific morphotype at each dose group could therefore be ascertained qualitatively. The specific features of each morphotype could also be compared. This also allowed for a comparison of which antibiotic resistant morphotypes were present at the start of the exposure and which specific antibiotic resistant morphotypes had appeared in miocrocosms after day 1. In addition, a total CFU count of each specific morphotype that had been plated onto control microcosm inoculated R2A + CTC/SMX agar plates was conducted. Counts of between 30 and 300 CFU were considered valid. Log CFU/ml values for each specific morphotype were then graphed using Microsoft Excel. This analysis allowed the relative presence of each morphotype in control microcosms to be calculated.

If a distinct morphotype was not present in control microcosms at the start of the exposure and could not be cross referenced to a specific day 1 resistant morphtype then it was assumed that such a morphotype had developed antibiotic resistance during the exposure as the result of selective pressure of the corresponding antibiotic that microcosms had been dosed with (CTC or SMX). If a specific morphotype could only be detected in day 7control microcosms only but could not be cross referenced with a specific day 1 morphotype, then it was assumed that the resistance of this morphotype had formed due to a mechanism other than the selective pressure of dosed SMX or

CTC. Day 7 resistant morphotypes that could be detected in day 1 control microcosms and could also be cross referenced to a specific day 1 morphotype were considered to be resistant morphotypes that were added to a specific microcosm via river water of treated sewage. Finally, if a specific morphotype could be detected in day 1 microcosms but could not be cross-referenced with a day 7 morphotype, then it was assumed that these morphotypes had not thrived beyond the 7 day exposure (they were all dead and had possibly been degraded themselves by other bacteria).

5.2.10.3. Biolog PM Data: MDR Profiling of SMX/CTC-Resistant Morphotypes

A total growth value for each morphotype that could be grown on Biolog PM plates was calculated by subtracting the initial well values against the final well values using Microsoft Excel ($\Delta_{DAY\ 1} - _{DAY\ 7}$). If an absorbance value of > 0.1 was recorded (~ 2 x background noise) a positive growth outcome was noted. All values < 0.1 were noted as having no significant growth and therefore the isolate had been inhibited by the corresponding antibiotic. The number of isolates that could grow in *x* number of antibiotic–containing wells was then graphed for days 1 and 7 and the frequency distribution was graphed using Microsoft Excel. The number of incidences of resistance to each antibiotic was also graphed against antibiotic–type using Microsoft Excel.

5.3: Results

5.3.1: Total Resistant CFU Counts: Formation of Single Drug Resistance over Time

The data shown in figure 5.3 highlight the temporal change in the total number (log cells/ml) of culturable CTC-resistant bacteria (figure 5.3a) and SMX-resistant bacteria (figure 5.3b) that were present in microcosms that had been spiked with 3 antibiotic doses. It can be seen that resistant CFU counts showed a large rise over time at each dose group, including in controls. These data indicate that the density of resistant bacterial cells increased regardless of antibiotic dose. In CTC-treated microcosms, the resistant CFU count showed an increase with increasing CTC concentration. Each resistant CFU count was significantly higher than the control CFU count value ($P < 0.01$). These data indicate that a statistically significant rise in resistance formation had occurred as a result of exposing microbial communities to all CTC doses. There was however no statistically significant rise in resistant CFU counts at any SMX dose group. These data suggest that exposing microbial communities to these SMX concentrations has no significant effect on the formation of SMX resistance.

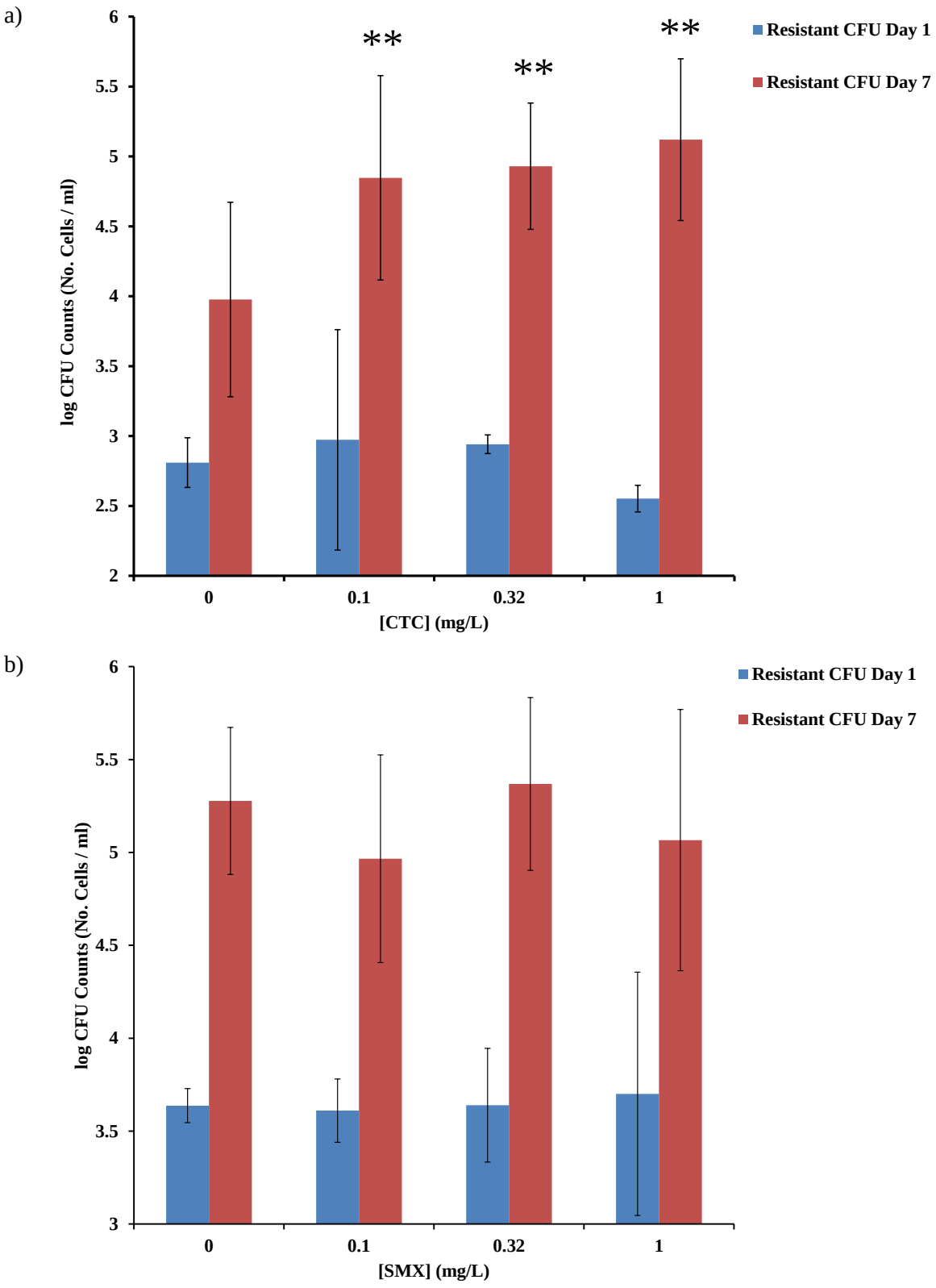


Figure 5.3: Total resistant CFU counts of a) CTC-resistant bacteria and b) SMX-resistant bacteria that could be cultured from microcosms from each dose group on day 1 and day 7. Statistical significance is represented by asterisk notation (*, $P \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$).

5.3.2. Total CFU Counts of CTC and SMX-Resistant Isolates: Day 1 versus Day 7

The data in figure 5.3 show the combined (control plus dosed) total number of distinct morphotypes that could be harvested from each microcosm on day 1 and day 7 of the exposure. Note that these data are an initial indication of how many distinct morphotypes could be isolated from combined microcosms before further cross-referencing had been conducted. This initial analysis revealed that 52.6 % more CTC-resistant morphotype could be isolated on day 7 compared with day 1. It was additionally revealed that 57.1 % more SMX-resistant morphotypes could be isolated on day 7 compared with day 1. It can be concluded therefore that a temporal rise in CTC and SMX-resistant morphotypes occurred over the 7 day exposure in combined microcosms.

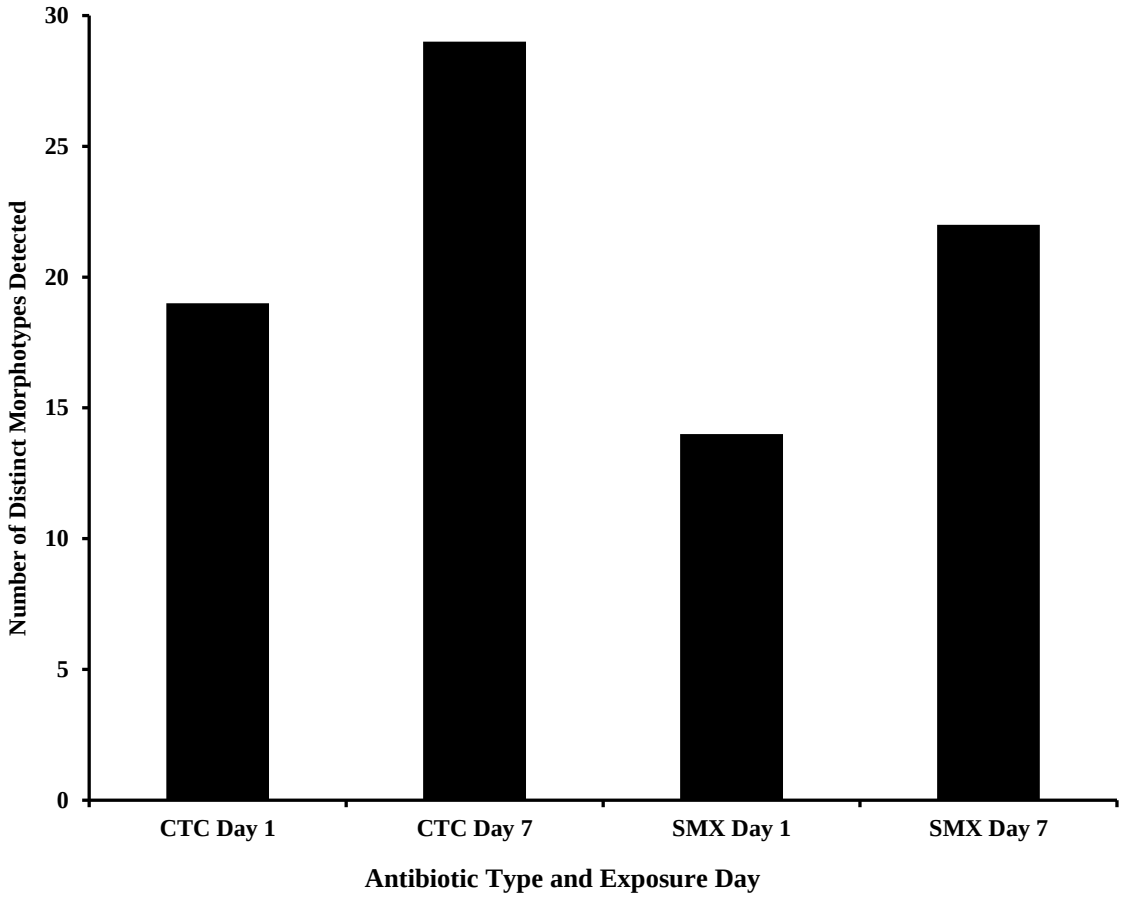


Figure 5.4: Total number of distinct morphotypes that could be harvested from combined microcosms on day 1 (CTC Day 1 and SMX Day 1) and day 7 (CTC Day 7 and SMX Day 7) of the exposure.

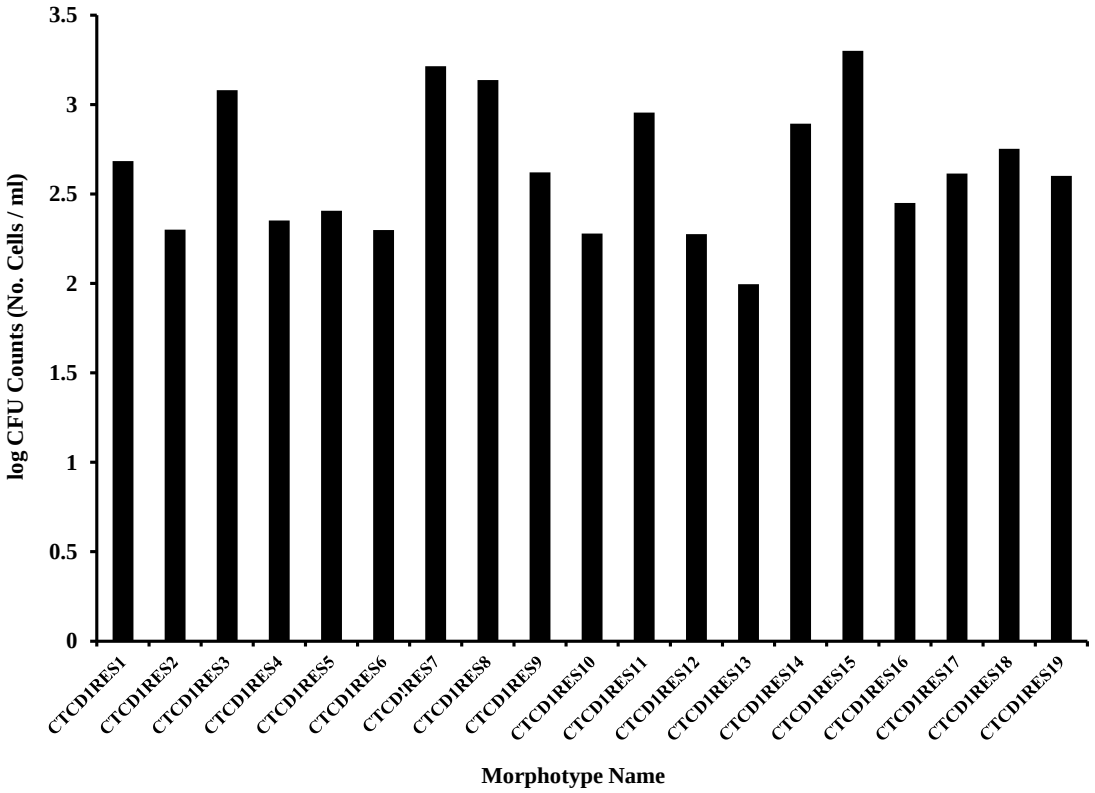
5.3.3. CFU Counts and Morphotyping of Resistant Bacteria in Control Microcosms on day 1 and 7

The data in figure 5.5 show CTC-resistant CFU counts in control microcosms on a) day 1 and b) day 7. The data shown by figure 5.6 show SMX-resistant CFU counts in control microcosms on a) day and b) day 7. The information in table 5.2 shows which other microcosms each CTC-resistant morphotype could be isolated from. It additionally shows which day 1 and day 7 CTC-resistant morphotypes could be cross referenced with each other based on their distinct characteristics. Detailed CTC-resistant morphotype characteristics can be found in appendix 1. The information in table 5.3 shows which other microcosms each SMX-resistant morphotype could be isolated from. It additionally shows which day 1 and day 7 SMX-resistant morphotypes could be cross-referenced with each other based on their distinct characteristics. Detailed SMX-resistant morphotype characteristics can be found in appendix 1.

It can be seen form the data in figure 5.5a and the information in table 5.2 that every distinct CTC-resistant morphotype that was isolated from day 1 control microcosms could be cross-referenced with a corresponding day 7 CTC-resistant morphotype, with two exceptions (*CTCD1RES4* and *CTCD1RES10*). It can therefore be suggested that the majority of day 1 CTC-resistant morphotypes could be accounted for on day 7. This was also the case for SMX-resistant morphtypes that were isolated from day 1 control microcosms (figure 5.6a and table 5.3). Each of these morphotypes could be cross-referenced with a similar day 7 morphotype, with the exception of two distinct isolates (*SMXD1RES2* and *SMXD1RES6)*. It is likely that all day 1 CTC and SMX-resistant morphotypes that were found in control microcosms (and day 7 isolates that could be cross referenced with these morphotypes) were added to each microcosm (control and dosed) via river water, treated sewage or both due to the fact that they were expressing CTC/SMX resistance at the start of the exposure in unexposed microcosms. It also likely that *CTCD1RES4*, *CTCD1RES10*, *SMXD1RES2* and *SMXD1RES6* were also present at the start of the exposure, but disappeared from microcosms at some point during the seven day exposure. Data shown by figures 5.5b and 5.6b also confirm that each CTC and SMX-resistant day 7 morphotype that was successfully cross-referenced with a corresponding day 1 morphotype could be accounted for in terms of varying cell densities (as shown by CFU data).

Further data shown by figures 5.5b and 5.6b demonstrate that a number of day 7 CTC and SMX-resistant morphotypes could not be accounted for in day 1control microcosms, as demonstrated by a zero CFU count. These data suggest that such morphotypes were not added to microcosms via river water or treated sewage at the start of each exposure. It is likely that these isolates developed antibiotic resistance during the exposure period. The next section (5.3.4) shall present more data regarding these morphotypes.

a)



b)

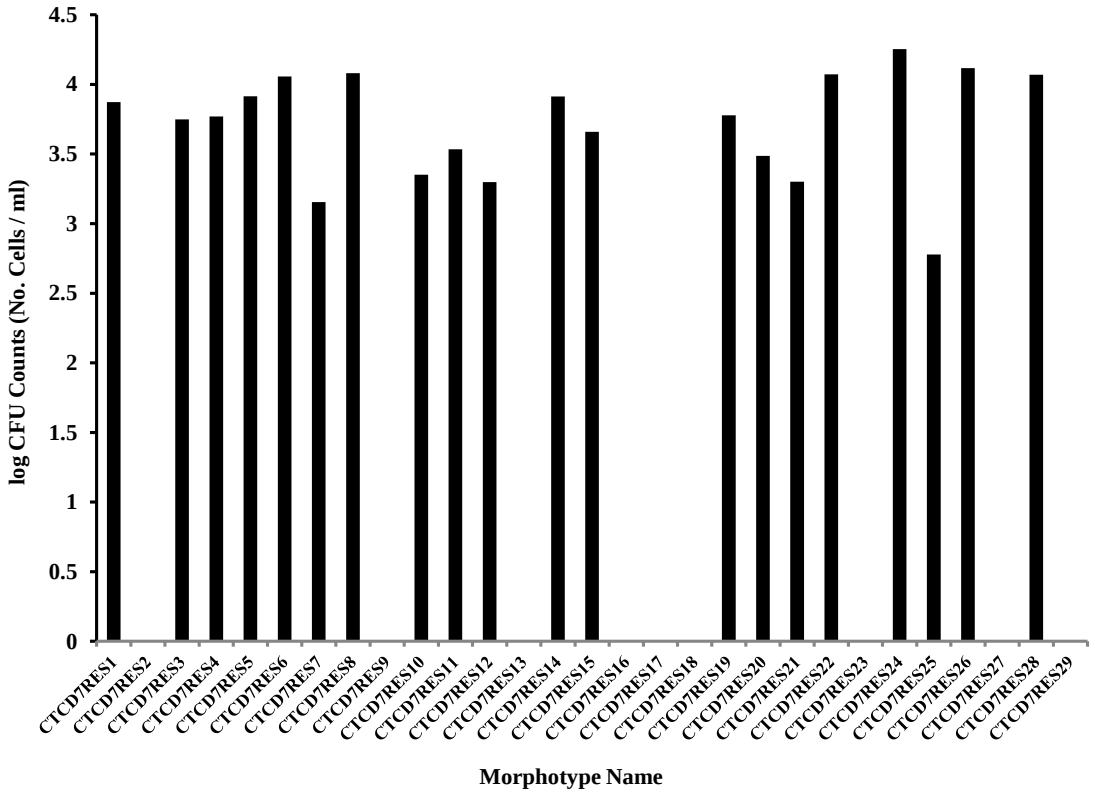
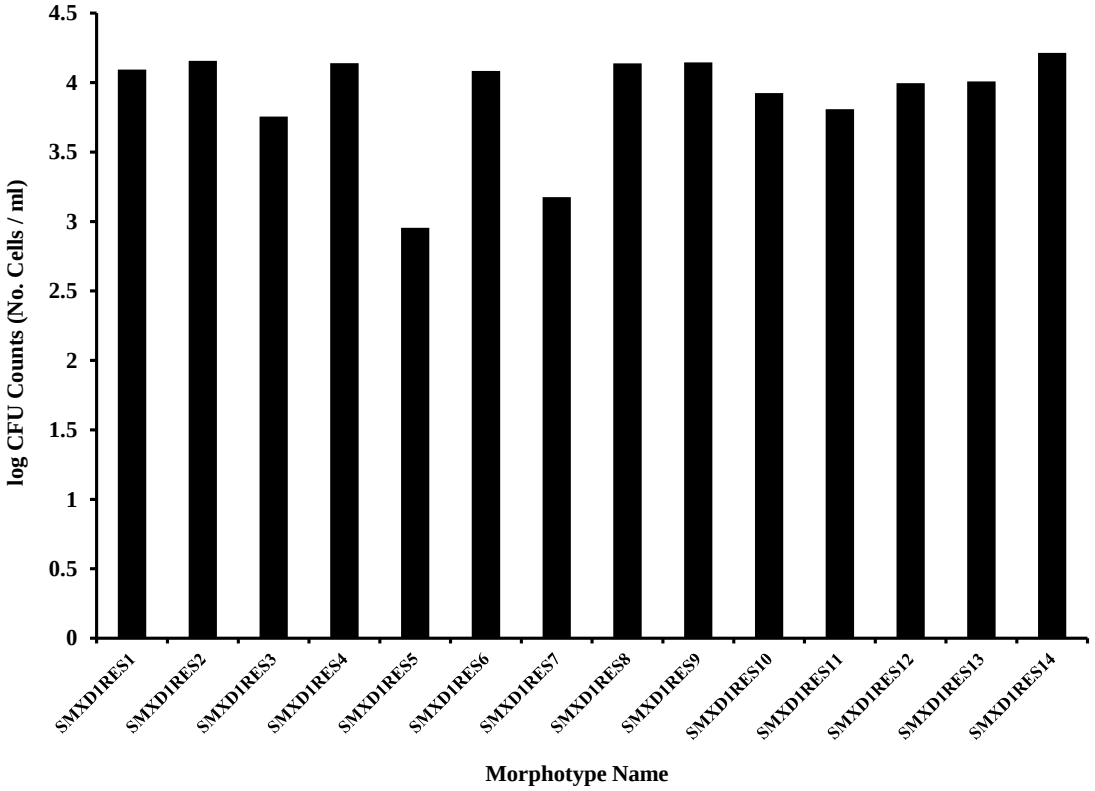


Figure 5.5: Resistant CFU counts for CTC-resistant bacteria that were present in control microcosms on a) day 1 and b) day 7.

a)



b)

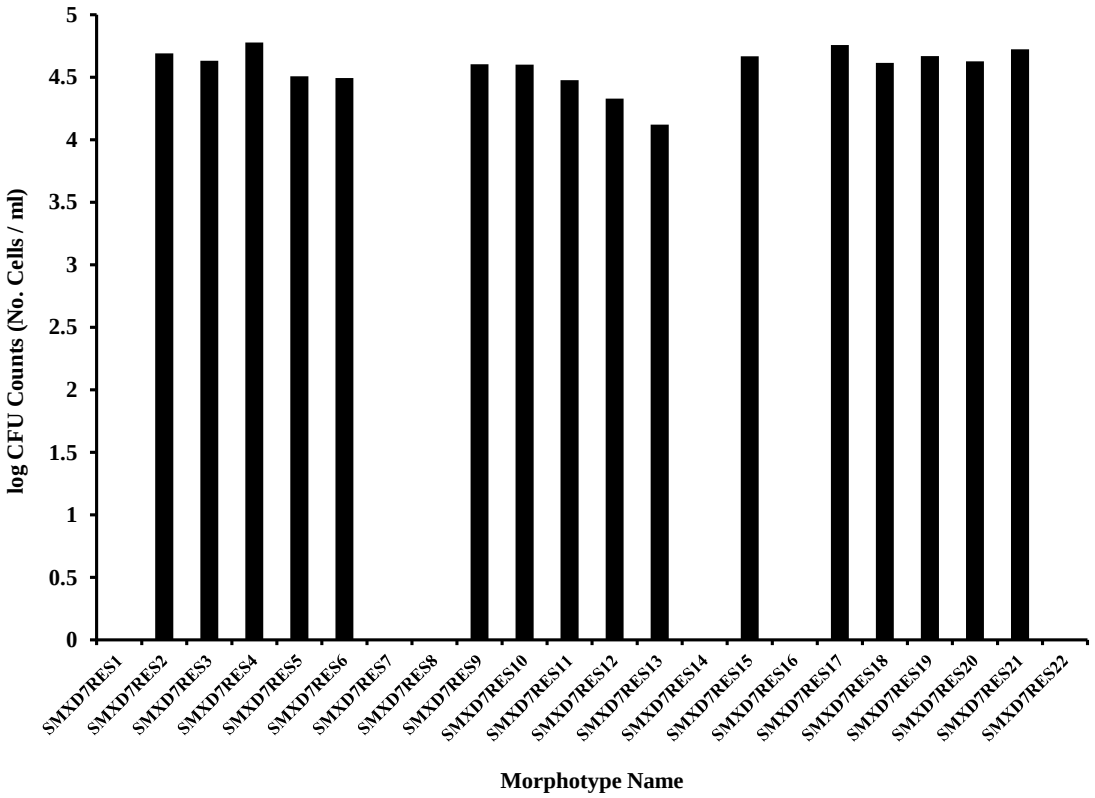


Figure 5.6: Resistant CFU counts for SMX-resistant bacteria that were present in control microcosms on a) day 1 and b) day 7.

Table 5.2: Matching CTC-resistant morphotypes that could be detected on day 1 and day 7 following cross referencing of their morphotype characteristics. The dose groups in which they could be detected in are also listed.

	Successful or Unsuccessful Detection (+/-)					Successful or Unsuccessful Detection (+/-)			
Day 1 Morphotype	**0 mg/L CTC**	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**	**Corresponding Similar Day 7 Morphotype**	**0 mg/L CTC**	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**
CTCD1RES1	+	+	+	-	*CTCD7RES14*	+	+	+	+
CTCD1RES2	+	+	+	+	*CTCD7RES28*	+	-	-	+
CTCD1RES3	+	+	+	+	*CTCD7RES7*	+	+	+	+
CTCD1RES5	+	+	+	+	*CTCD7RES26*	+	+	+	+
CTCD1RES6	+	+	+	+	*CTCD7RES3*	+	+	+	-
CTCD1RES7	+	+	-	+	*CTCD7RES4*	+	+	-	-
CTCD1RES8	+	+	+	+	*CTCD7RES6*	+	+	+	+
CTCD1RES9	+	+	+	+	*CTCD7RES20*	+	+	+	+
CTCD1RES11	+	+	+	+	*CTCD7RES22*	+	+	+	+
CTCD1RES12	+	+	+	+	*CTCD7RES10*	+	-	-	+
CTCD1RES13	+	+	+	+	*CTCD7RES12*	+	+	+	-
CTCD1RES14	+	+	+	+	*CTCD7RES19*	+	+	+	+
CTCD1RES15	+	+	+	-	*CTCD7RES8*	+	+	+	-
CTCD1RES16	+	+	+	+	*CTCD7RES15*	+	+	+	+
CTCD1RES17	+	+	+	+	*CTCD7RES21*	+	-	-	+
CTCD1RES18	+	+	+	+	*CTCD7RES24*	+	+	+	+
CTCD1RES19	+	+	+	+	*CTCD7RES11*	+	+	+	+

Table 5.3: Matching SMX-resistant morphotypes that could be detected on day 1 and day 7 following cross referencing of their morphotype characteristics. The dose groups in which they could be detected in are also listed.

	Successful or Unsuccessful Detection (+/-)					Successful or Unsuccessful Detection (+/-)			
Day 1 Morphotype	**0 mg/L SMX**	**0.1 mg/L SMX**	**0.32 mg/L SMX**	**1 mg/L SMX**	**Corresponding Similar Day 7 Morphotype**	**0 mg/L SMX**	**0.1 mg/L SMX**	**0.32 mg/L SMX**	**1 mg/L SMX**
SMXD1RES1	+	+	+	+	*SMXD7RES21*	+	+	-	+
SMXD1RES3	+	+	+	+	*SMXD7RES11*	+	+	+	+
SMXD1RES4	+	+	+	+	*SMXD7RES2*	+	+	+	+
SMXD1RES5	+	+	+	-	*SMXD7RES10*	+	-	+	+
SMXD1RES7	+	+	+	+	*SMXD7RES20*	+	+	+	-
SMXD1RES8	+	+	-	+	*SMXD7RES18*	+	+	-	+
SMXD1RES9	+	+	+	+	*SMXD7RES6*	+	+	+	+
SMXD1RES10	+	+	+	-	*SMXD7RES13*	+	+	+	-
SMXD1RES11	+	+	+	+	*SMXD7RES19*	+	+	+	+
SMXD1RES12	+	+	+	+	*SMSD7RES17*	+	-	+	+
SMXD1RES13	+	+	+	+	*SMXD7RES5*	+	+	+	-
SMXD1RES14	+	+	+	+	*SMXD7RES9*	+	+	+	+

5.3.4. Analysis of Day 7 Isolates that could not be Cross-Referenced with a Day 1 Control Morphotype

The information in tables 5.4 and 5.5 indicate the microcosms from which resistant morphotypes that were not detected in day 1 control microcosms, or cross-referenced with these morphotypes could be detected in. Table 5.4 shows that one morphotype could only be detected in a day 7 control microcosm, two could be detected in both control and treated microcosms and nine could be detected only in microcosms that had been exposed to CTC. Table 5.5 also shows that only one unique day 7 SMX-resistant morphotype was present in exclusively day 7 control microcosms. In addition, four unique day 7 SMX-resistant morphotypes were found in both control and treated microcosms, while another five SMX-resistant morphotypes were found only in microcosms that had been exposed to SMX. By looking at tables 5.4 and 5.5 there is no obvious link between the presence of a resistant morphotype and antibiotic concentration. Indeed, antibiotic resistant morphotypes were detected at every antibiotic dose.

Table 5.4: Microcosms in which unique day 7 CTC-resistant morphotypes were detected in with an indication of whether resistance developed in control or treated microcosms.

	Successful or Unsuccessful Detection (+/-)				
Morphotype	**0 mg/L CTC**	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**	**Formation of Resistance in Treated (Tr) or Control (C) Microcosms**
CTCD7RES1	+	-	-	-	C
CTCD7RES2	-	-	+	+	Tr
CTCD7RES5	+	+	-	-	C + Tr
CTCD7RES9	-	+	+	-	Tr
CTCD7RES13	-	+	+	+	Tr
CTCD7RES16	-	+	+	+	Tr
CTCD7RES17	-	+	-	-	Tr
CTCD7RES18	-	+	+	-	Tr
CTCD7RES23	-	-	+	+	Tr
CTCD7RES25	+	+	+	-	C + Tr
CTCD7RES27	-	+	-	-	Tr
CTCD7RES29	-	+	+	+	Tr

Table 5.5: Microcosms in which unique day 7 SMX-resistant morphotypes were detected in with an indication of whether resistance developed in control or treated microcosms.

	Successful or Unsuccessful Detection (+/-)				
Morphotype	**0 mg/L SMX**	**0.1 mg/L SMX**	**0.32 mg/L SMX**	**1 mg/L SMX**	**Formation of Resistance in Treated (Tr) or Control (C) Microcosms**
SMXD7RES1	+	+	+	+	C + Tr
SMXD7RES2	+	+	+	+	C + Tr
SMXD7RES3	+	-	+	+	C
SMXD7RES7	-	+	-	-	Tr
SMXD7RES8	-	+	+	+	Tr
SMXD7RES12	+	+	-	+	C + Tr
SMXD7RES14	-	+	+	-	Tr
SMXD7RES15	+	+	+	+	C + Tr
SMXD7RES16	-	-	+	-	Tr
SMXD7RES22	-	+	+	+	Tr

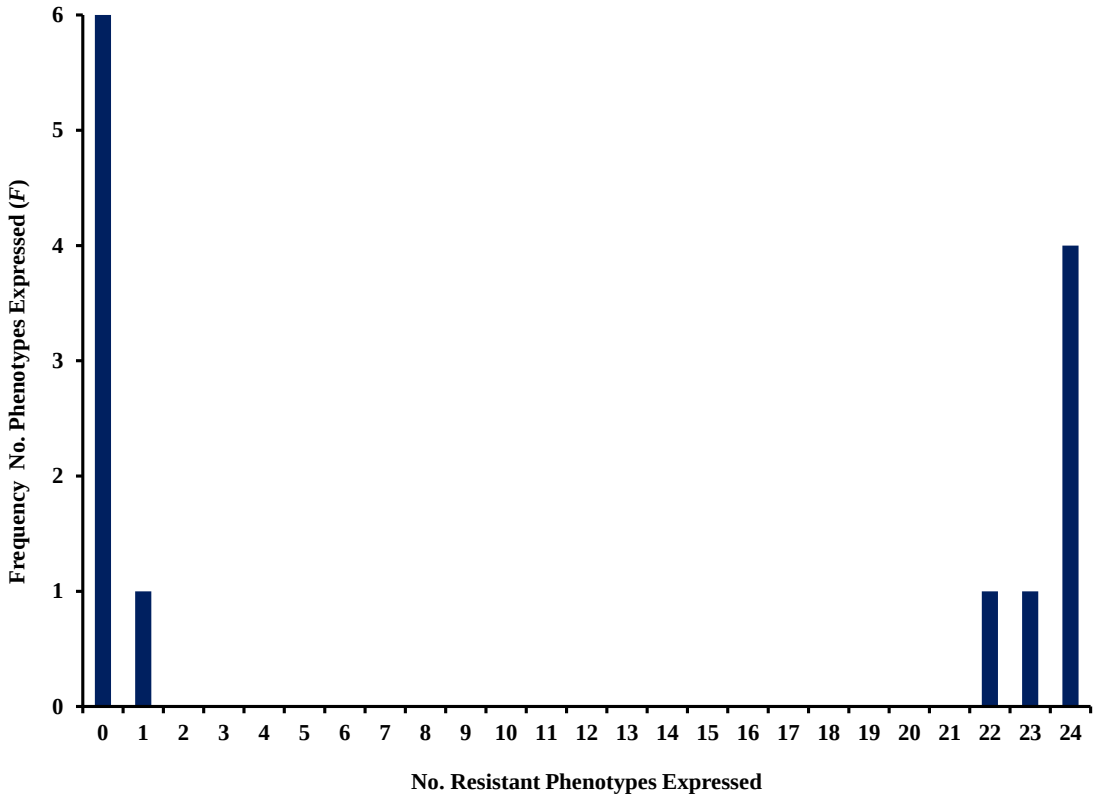
5.3.5. Temporal Expression of MDR by the Total Microbial Community (Control and Exposed)

5.3.5.1. Frequency of MDR Phenotypes on Day and Day 7 of CTC and SMX Exposures

Previous experiments have shown that morphotypes that are resistant to either CTC or SMX were either present at the start and throughout the exposure or have been detected at the end of the exposure. The data in figure 5.7 show the frequency different antibiotic resistant phenotypes were expressed by CTC-resistant morphotypes (isolated from all microcosms, control and treated) on a) day 1 and b) day 7 of the exposure. As antibiotic resistance was observed at all CTC and SMX concentrations, control morphotypes were also included in this study. On day 1 of the exposure, isolates expressed resistance to either a low number of antibiotics (or none at all) or a high number of antibiotics. Indeed on day 1 six morphotypes did not show resistance to any of the antibiotics that were tested, whereas six morphotypes were found to be resistant to between 21 and 24 antibiotics. On day 7 the resistance distribution profile of all morphotypes tested showed a marked shift. Six morphotypes were shown to express between 2 and 21 resistant phenotypes and only two morphotypes expressed a resistant phenotype to no antibiotics at all. Conversely, nine instances of morphotypes being resistant to between 21 and 24 antibiotics were recorded.

The data in figure 5.8 show the frequency different antibiotic resistant phenotypes were expressed by SMX-resistant morphotypes (isolated from all microcosms, control and treated) on a) day 1 and b) day 7 of the exposure. On day 1 of the exposure, the number of resistance incidences was shown to be relatively more dispersed compared with the resistance frequency distribution on day 1 of the CTC exposure. At the lower end of the frequency scale, a total of 4 incidences of morphotypes expressing between zero and four resistant phenotypes was recorded. Conversely, there were eight incidences of isolates expressing between 18 and 24 resistant phenotypes. On day 7 there was a massive shift in the distribution of resistant phenotype occurrences. In fact, all day 7 SMX-resistant morphotypes expressed resistance to all 24 antibiotics.

a)



b)

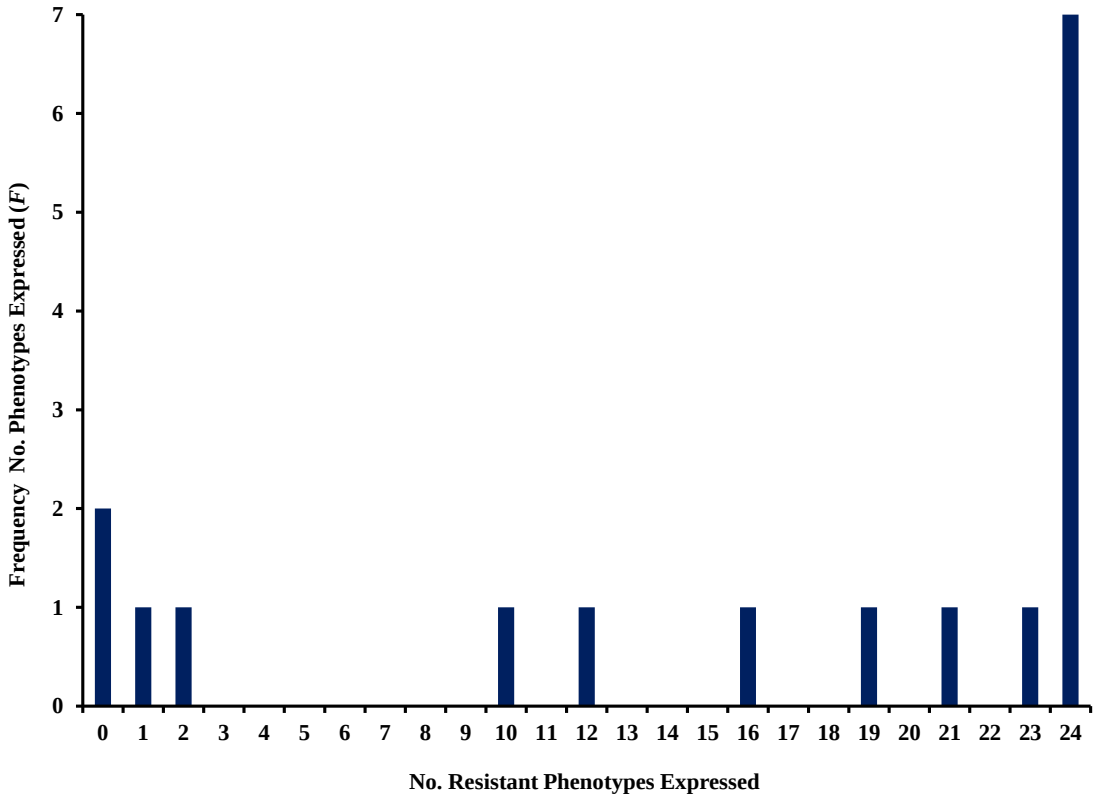


Figure 5.7: Number of resistant phenotypes that were expressed to number of specific antibiotics by CTC-resistant morphotypes on day 1 (a) and 7 (b) of the CTC exposure period.

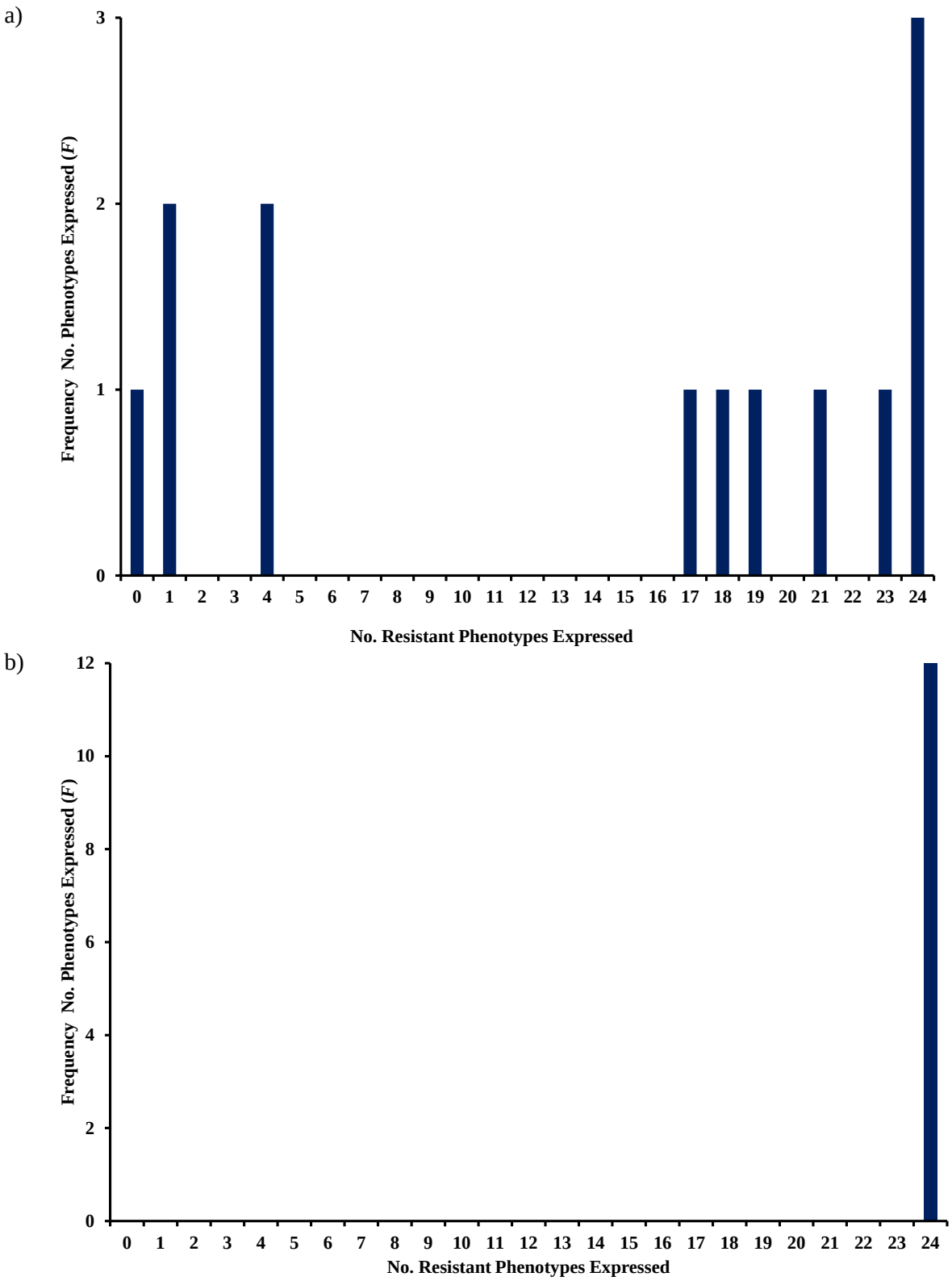


Figure 5.8: Number of resistant phenotypes that were expressed to number of specific antibiotics by SMX-resistant morphotypes on day 1 (a) and 7 (b) of the SMX exposure period.

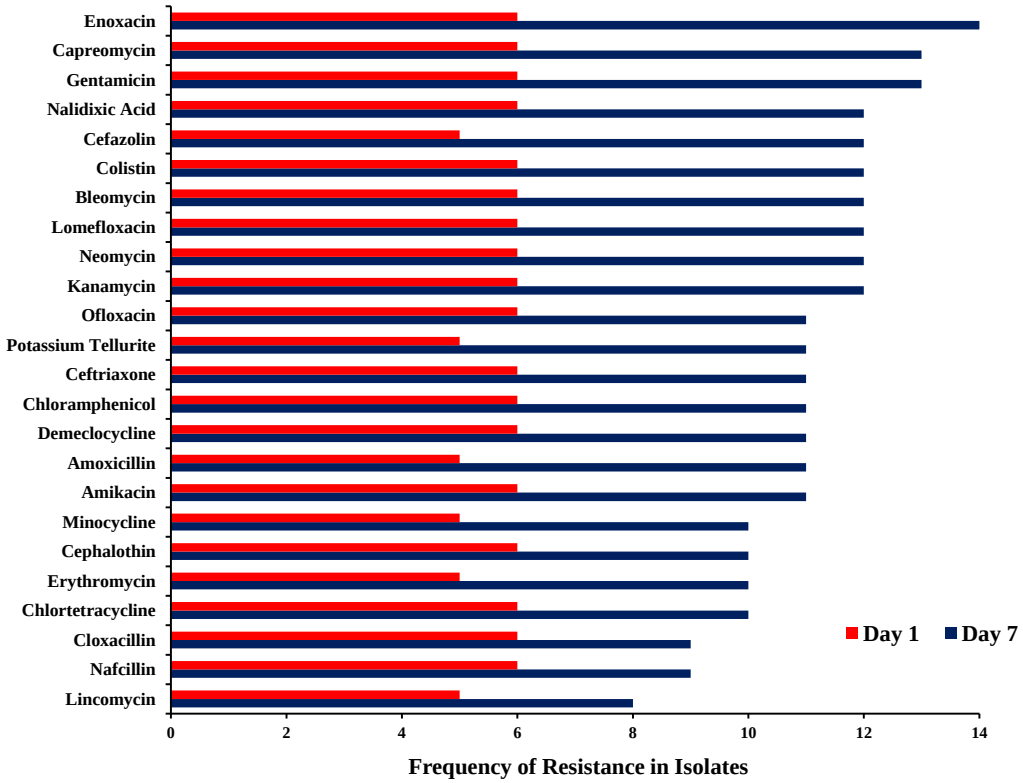
5.3.5.2. Temporal Change of MDR Profile of the Total Microbial Community

Section 5.3.5.1 demonstrated changes in the number of specific antibiotic resistance phenotypes that CTC and SMX-resistant bacteria expressed over time. The data in figure 5.9a show the number of morphotypes that were resistant to each antibiotic on PM11C plates on day 1 and day 7 of the exposure. The data shown by figure 5.9b show the number of morphotypes that were resistant to each antibiotic on PM12B plates on day 1 and day 7 of the exposure.

On day 1 between five and six morphotypes were resistant to each antibiotic. There was little difference in the number of morphotypes that were resistant to each compound however; the number of incidences was always five or six. The data representing day 7 incidences of resistance to each antibiotic showed that the number of morphotypes that expressed resistance to every compound had increased. The greatest increases in resistance could be recorded for enoxacin, capreomycin and gentimycin. The lowest increases in resistance could be observed in the case of cloxacillin, nafcillin and lincomycin. The overall trend therefore showed that the exposure of the microbial community to CTC generally resulted in an increase in resistance to a number of different antibiotics, in most cases from a different class altogether to the test compound.

The data representing day 1 resistance profiles for SMX-resistant morphotypes (figure 5.9b) indicate that resistance was expressed to between four and eleven individual antibiotics. The most resistance on day 1 was expressed to enoxacin, capreomycin and gentimycin. On day 7, there was a marked increase in the number of morphotypes that expressed resistance to each of the antibiotics tested. In fact, the data show that by day 7 of the exposure all morphotypes were resistant to every compound tested. In general, the data illustrate that exposure of the microbial community to SMX resulted in all of the SMX-resistant morphotypes expressing resistance to an increased number of compounds present on Biolog PM12B plates.

a)



b)

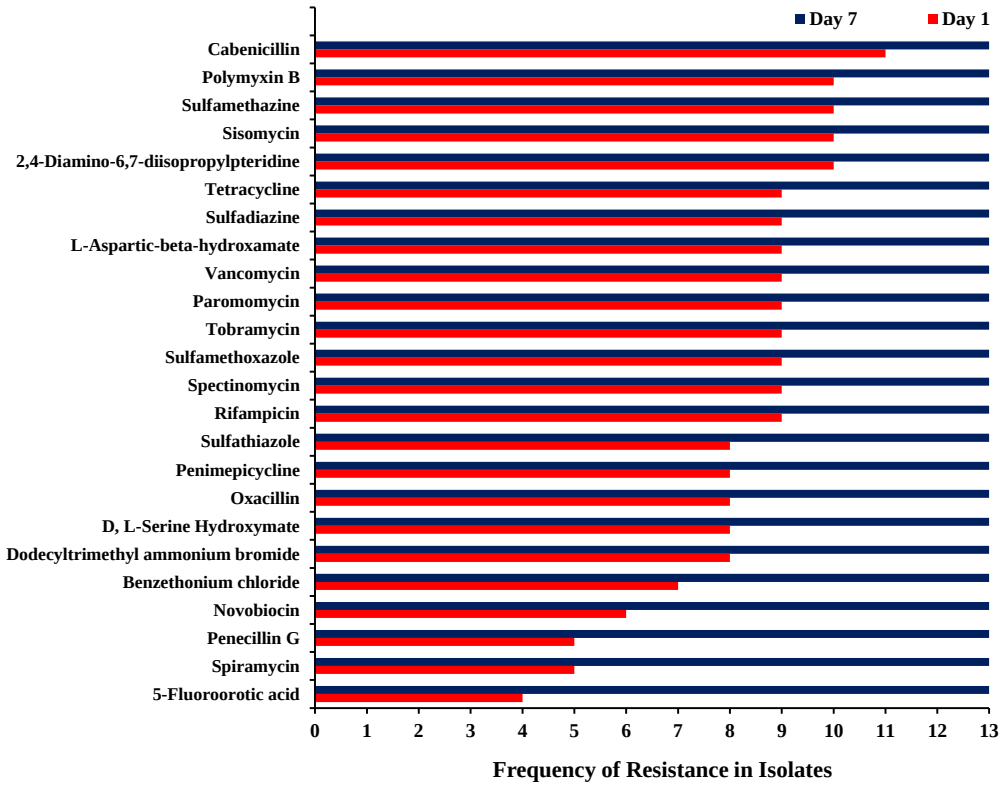


Figure 5.9: Number of isolates that expressed specific resistance to particular antibiotics as a result of exposure to a) CTC and b) SMX on day 1 and day 7 of the exposure.

5.3.6. Co-Selection of MDR in CTC and SMX-Resistance Microbes by CTC and SMX Exposure

Previous sections in this chapter have demonstrated that a general increase in the incidence of MDR can be detected over time. It has also been demonstrated that nine CTC-resistant morphotypes (*CTCD7RES2*, *CTCD7RES9*, *CTCD7RES13*, *CTCD7RES16*, *CTCD7RES17*, *CTCD7RES18*, *CTCD7RES23*, *CTCD7RES27* and *CTCD7RES29*) and six SMX-resistant morphotypes (*SMXD7RES1*, *SMXD7RES2*, *SMXD7RES3*, *SMXD7RES7*, *SMXD7RES8*, *SMXD7RES12*, *SMXD7RES14*, *SMXD7RES15*, *SMXD7RES16* and *SMXD7RES22*) developed single drug resistance (to CTC and SMX) only in microcosms that had been exposed to antibiotics. The information in table 5.6 and 5.7 shows the MDR resistance profile of those isolates that could be grown in Biolog media for CTC and SMX-resistant morphotypes respectively. It should be noted that several isolates could not be transferred from R2A to Biolog PM Medium. These were *CTCD7RES9*, *CTCD7RES17*, *CTCD7RES23*, *CTCD7RES27*, *SMXD7RES2*, *SMXD7RES2*, *SMXD7RES3*, *SMXD7RES7*, *SMXD7RES12*, *SMXD7RES14*, *SMXD7RES15* and *SMXD7RES16*. It can be seen that each morphotype that developed single drug resistance exclusively in the presence of CTC and SMX also expressed a much wider resistance profile. CTC-resistant morphotypes expressed resistance to between 14 and 24 distinct antimicrobial compounds. SMX-resistant morphotypes all expressed resistance to 24 antimicrobial compounds. It can be concluded therefore that morphotypes that have developed single drug resistance under the selective pressure of CTC and SMX are likely to also express a wider MDR profile. It is possible that these additional resistance phenotypes may be co-selected.

Table 5.6: MDR profile of CTC-resistant morphotypes that formed single drug resistance only in CTC-treated microcosms.

Morphotype Name	Total MDR Profile	Total AB Resistance Count
CTCD7RES2	amoxicillin, cefazolin, erythromycin, potassium tellurite, minocycline, neomycin, enoxacin, amikacin, CTC, cloxacillin, lomefloxacin, bleomycin, colistin, capreomycin, demeclocycline, nafcillin, nalidixic acid, chloramphenicol, ceftrixone, gentimicin, cephalothin, kanamycin, ofloxacin	23
CTCD7RES13	lincomycin, amoxicillin, cefazolin, erythromycin, potassium tellurite, minocycline, neomycin, enoxacin, amikacin, CTC, cloxacillin, lomefloxacin, bleomycin, colistin, capreomycin, demeclocycline, nafcillin, nalidixic acid, ceftrixone, gentimicin, cephalothin, kanamycin, ofloxacin	23
CTCD7RES16	lincomycin, amoxicillin, cefazolin, erythromycin, potassium tellurite, minocycline, neomycin, enoxacin, amikacin, CTC, cloxacillin, lomefloxacin, bleomycin, colistin, capreomycin, demeclocycline, nafcillin, nalidixic acid, chloramphenicol, ceftrixone, gentimicin, cephalothin, kanamycin, ofloxacin	24
CTCD7RES18	amoxicillin, cefazolin, erythromycin, neomycin, enoxacin, amikacin, CTC, cloxacillin, lomefloxacin, capreomycin, chloramphenicol, gentimicin, kanamycin, ofloxacin	14
CTCD7RES29	lincomycin, amoxicillin, cefazolin, erythromycin, potassium tellurite, minocycline, neomycin, enoxacin, amikacin, CTC, cloxacillin, lomefloxacin, bleomycin, colistin, capreomycin, demeclocycline, nafcillin, nalidixic acid, chloramphenicol, ceftrixone, gentimicin, cephalothin, kanamycin, ofloxacin	24

Table 5.7: MDR profile of SMX-resistant morphotypes that formed single drug resistance only in SMX-treated microcosms.

Morphotype Name	Total MDR Profile	Total AB Resistance Count
SMXD7RES1	penecillin G, tetracyline, cabenicillin, oxacillin, penimepicycline, polymyxin B, paromomycin, vancomycin, D, L-serine hydroxymate, sisomycin, sulfamethazine, novobiocin, 2,4-diamino-6,7-diisopropylpteridine, sulfadiazine, benzethonium chloride, tobrmycin, sulfathiazole, 5-fluoroorotic acid, spectinomycin, sulfamethoxazole, L-aspartic-beta-hydroxamate, spiramycin, rifampicin, dodecyltrimethyl ammonium bromide	24
SMXD7RES8	penecillin G, tetracyline, cabenicillin, oxacillin, penimepicycline, polymyxin B, paromomycin, vancomycin, D, L-serine hydroxymate, sisomycin, sulfamethazine, novobiocin, 2,4-diamino-6,7-diisopropylpteridine, sulfadiazine, benzethonium chloride, tobrmycin, sulfathiazole, 5-fluoroorotic acid, spectinomycin, sulfamethoxazole, L-aspartic-beta-hydroxamate, spiramycin, rifampicin, dodecyltrimethyl ammonium bromide	24
SMXD7RES22	penecillin G, tetracyline, cabenicillin, oxacillin, penimepicycline, polymyxin B, paromomycin, vancomycin, D, L-serine hydroxymate, sisomycin, sulfamethazine, novobiocin, 2,4-diamino-6,7-diisopropylpteridine, sulfadiazine, benzethonium chloride, tobrmycin, sulfathiazole, 5-fluoroorotic acid, spectinomycin, sulfamethoxazole, L-aspartic-beta-hydroxamate, spiramycin, rifampicin, dodecyltrimethyl ammonium bromide	24

5.4. Discussion

A series of experiments were conducted to determine the extent to which SMX and CTC selected for single and multidrug resistance in the aquatic environment. The influence of resistance which was likely to have been added to microcosms (via treated sewage and river water) was also evaluated. A range of culture based approaches were utilised, including selective plating and multidrug susceptibility tests. Initial experiments revealed that CTC but not SMX exposure caused a significant increase in the number of resistant bacterial cells over time. All CTC doses caused this effect. A general increase in CTC and SMX-resistant morphotypes was observed over time in microcosms that had been exposed to both antibiotics. It was also observed that several antibiotic-resistant morphotypes were present in day 1 control and dosed microcosms in both exposures (CTC and SMX), as well as being present in day 1 dosed microcosms. Some of these isolates were also present at the end of the exposure (day 7). It was also observed that several SMX and CTC-resistant morphtypes emerged over time in both control and treated microcosms, in control only microcosms and exclusively in dosed microcosms. In general, the entire microbial community showed an increase in MDR over the exposure period. Morphotypes that had formed single drug resistance over time also mirrored the trend of the total microbial community by expressing a wider MDR profile on day 7 of the exposure.

5.4.1. Culture of CTC / SMX - Resistant Morphotypes from Aquatic Microcosms

Resistant isolates could be selectively cultured from microcosm contents that had been plated on R2A agar + 1 mg/L CTC or SMX. However, when stored isolates were grown on Biolog BUG B agar the agar could not sustain the growth of all isolates. In addition to this, there were some Biolog PM11C and PM12B plates that did not show any growth at all when inoculated. The result of this was that a reduced resistance profile of culturable bacteria. The most likely cause of this is that some isolates were nutrient (media) shocked having been transferred from R2A agar to glycerol deep freeze then to Biolog BUB B agar. The cells were also subjected to a different growth medium in the form of Biolog IF-10a inoculating fluid. It is highly likely that all Biolog media was too nutrient–rich for aquatic isolates as it is a general medium and is not specifically designed for oligotrophic bacteria. Future experiments should investigate this. Azevedo *et al*. (2004) observed the shocking of aquatic *H. pylori* by increasing the nutrient content of growth medium. Reasoner and Geldreich (1985) observed that a wider array of aquatic microorganisms were able to grow on R2A media compared with a standard plate count media. Straskravoba (1983) demonstrated that starved aquatic organisms (simulating natural conditions) that were transferred to a more nutrient rich media showed a short term loss in viability and a long term loss of biochemical function. Accordingly, a repeat of the study should be conducted using R2A agar only and R2A broth should

be used as inoculating fluid. Both of these measures would potentially reduce any media–specific growth factors.

In the present study it was however possible to isolate a number of morphotypes that expressed resistance to CTC and SMX. It was possible to culture more morphotypes on day 7 compared with day 1 of the study. It was shown that a portion of the microbial community that was present in aquatic microcosms were already resistant to CTC and SMX. The microcosm contained both river water from a "pristine" location and 5% treated sewage, so these are the two possible sources of resistant bacteria. Other studies have detected the presence of antibiotic resistance in pristine aquatic environments (Lima-Bittencourt *et al.*, 2007). In addition to this, there has been a wealth of literature reporting the presence of resistant isolates in sewage effluent. Many of these studies have detected *tet* and *sul* genes (Zhang *et al*., 2009). It is highly likely therefore that a significant pool of antibiotic resistance was added to every microcosms system at the start of each exposure.

The addition of resistance genes to an aquatic system via an STP has also been reported in the aquatic environment, acting to increase the natural resistance gene reservoir (Szczepanowski *et al.*, 2004; Silva *et al.*, 2006; Zhang *et al.*, 2009). The aquatic microcosms that were developed in the current study therefore provide a realistic simulation of natural aquatic habitats. The 1:20 dilution of surface water: treated sewage used in aquatic microcosms is likely to vary between different rivers and different aquatic systems within different countries however. Accordingly, the contents of the microcosm system could be changed. This would allow site and geographically–specific simulations. The resistome of aquatic systems can also be influenced by other additional factors, such as manure amendment (Mackie *et al*., 2006) and surface run–off from agricultural areas (Selvaratnam and Kunberger, 2004). Again, the contents of the microcosm system presented in the current study could be altered accordingly to simulate a range of variables.

Wide arrays of resistance mechanisms are likely to be responsible for conferring tetracycline and sulfonamide resistance in aquatic microcosms. In the case of tetracycline resistance, genes have been detected from each of the mechanistic groups of tetracycline resistance (efflux, ribosomal protection and enzymatic modification) in sewage effluent and surface water. All three *sul* genes (*sul I, sulII and sulIII*) have also been detected in the same matrices (Zhang *et al*., 2009). The resistance genotypes that were expressed in the present study are therefore likely to be comprised of a wide and diverse array of genetic resistance determinants.

5.4.2 Development of CTC and SMX Resistance over Time in Aquatic Microcosms

The fact that more resistant morphotypes could be isolated on day 7 compared with day 1 indicates that the portion of the culturable microbial community had developed tetracycline or sulfonamide resistance during the exposure period. This was further shown by the emergence of SMX and CTC-resistant morphotypes exclusively in microcosms that were dosed with CTC and SMX. Resistant

CFU counts also showed that each CTC concentration caused a significantly higher resistant-cell density compared with control values on day 7 of the exposure. A similar result was not repeated during the SMX exposure however, with no significant increase in day 7 resistant CFU counts being observed compared with control values. In both exposures a large increase in CTC and SMX-resistant CFU counts was observed on day 7 compared with day 1 at all CTC and SMX concentrations (including controls).

Other studies have also observed an increase in CFU counts as a result of antibiotic exposure. Kim *et al.* (2007) observed an increase in cell density and production rate of tetracycline–resistant activated sludge bacteria as a result of exposing the microbial community to 250 µg/L tetracycline. Kanpp *et al.* (2008) found that a lower oxytetracycline concentration of 20 µg/L caused an increase in the ratio of *tet* genes: total 16S RNA, inferring that tetracycline resistance had developed over time in a series of aquatic microcosm experiments. Other investigations have observed the formation of tetracycline resistance in systems that were exposed to higher tetracycline concentrations. Yu *et al.* (2009) showed that an oxytetracycline concentration of 5 mg/L resulted in an increased resistant cell density of *Enterococcus faecalis.* Rhysz and Alverez (2004) found that 50 mg/L tetracycline (in agricultural run–off) resulted in an increased concentration of resistant bacteria in the aquatic environment. Kerry *et al.* (1996) investigated tetracycline resistance formation in the marine environment. Their findings indicated that oxytetracycline concentrations of 6.25 and 25 µg/g resulted in an increase of resistant CFU frequencies (7% and 90% respectively). The literature therefore agrees with the findings of the present study, with some higher and lower inputs of selective pressure (tetracycline concentration) having resulted in the formation of tetracycline resistance.

Other studies have investigated the formation of sulfonamide resistance in the environment. Brandt *et al.* (2009) observed that soil microbial communities had become tolerant to a sulfadiazine concentration of 1 µg/g after 15 weeks of exposure to the compound based on PICT experiments. Heuer *et al.* (2008) were able to show a direct correlation between the formation of sulfonamide resistance (indicated by a temporal increase in *sulII* gene abundance) following sulfadiazole application to soil over time. Modeling approaches suggested that a sulfadiazole concentration of 0.15 mg/kg acted as a sufficient selective pressure for resistance to form. Heuer and Smalla (2007) concluded that sulfadiazine (10 and 100 mg/kg) had a synergistic effect with manuring of soils on the formation of sulfonamide resistance over time. A recent aquatic study concluded that sulfonamide resistance in aquatic sediments (measured as *sul* gene abundance) could be positively correlated with higher total sulfonamide concentrations down a 72 km stretch of river (Yi *et al*., 2010). The results of these studies generally agree with the findings of the present investigation in terms of resistant morphotypes emerging in SMX-exposed microcosms; the concentrations observed to have selected for sulfonamide resistance are generally in the same magnitude also. CFU counts could detect a significant rise in resistance compared with control values on day 7 of the exposure however. It is possible that a greater portion of SMX-resistant bacteria (than CTC-

resistant bacteria) are not culturable. This could be confirmed by a molecular technique such whole community sequencing or PCR-DGGE.

In general though, the findings of Knapp *et al.* (2010) that antibiotic resistant gene abundance (especially *tet* genes) has increased in the environment over the last few decades of heavy antibiotic usage is perhaps the greatest insight into how antibiotics residues in the environment have increasingly selected for resistance in the environment.

Interestingly, distinct resistant morphotypes also emerged in control microcosms over the 7 day period. A total rise in CTC and SMX-resistant CFU counts was also observed between day 1 and day 7. These findings could perhaps be explained by the increasing presence of mechanisms that are responsible for the general efflux of toxic/waste materials from microbial cells (Koronakis *et al.,* 2004). General efflux mechanisms such RND and MATE family efflux systems can expel a massive range of unwanted but unrelated compounds from a bacterial cell. Hernandez *et al*. (2009) for example reported the presence of the general efflux mechanism smeDEF in the aquatic isolate *Stenotrophomonas maltophilia.*

In general however it is possible to explain the increase in resistance in tetracycline and sulfonamide resistance in terms of natural selection, in the sense that only those members of the microbial community that could genetically code for resistance mechanisms could survive antibiotic treatment. Some of the microbial community may have acquired resistance by horizontal gene transfer. The transfer of tetracycline and sulfonamide resistance has been shown experimentally by mating studies (Nagachinta and Chen, 2008; Byrne–Bailey *et al.*, 2009; Agerso and Sandvang, 2005). Moreover, transfer of tetracycline and sulfonamide resistance has also been shown to occur in specific environmental matrices. Knapp *et al.* (2008) for example detected a link between *tet* gene transfer and the presence of transposons in aquatic studies. Byrne–Bailey *et al.* (2009) found a possible link between *sulI* gene transfer and class 1 integrons. It is therefore possible that certain *sul* and *tet* genes were transferred from resistant members of the microbial community to non–resistant members. The total resistance within a microcosm would thus be increased (which could be detected by selective plating).

5.4.3. Presence of Multidrug Resistance on Day 1 of Aquatic Microcosm Experiments

The results of the present study have indicated that a degree of multidrug resistant isolates were present in both microcosms (exposed to CTC and SMX) on day 1 of the exposure period (6 MDR profiles in CTC microcosms and 8 MDR profiles in SMX microcosms). From the available literature it is clear that multidrug resistant bacteria have been isolated from various environmental matrices including the soil (Peron *et al.*, 2008; Byrne-Bailey *et al.,* 2009; Singh *et al.,* 2007; Santos *et al.,* 2007; Burgos *et al.,* 2005; Hayes *et al.,* 2004) and the marine and estuarine environment

(Fernandez–Delgado and Suarez, 2009; Laroche *et al.,* 2009; Fricke *et al.,* 2008; Chandran *et al.*, 2008; Dang *et al.*, 2007; Parveen *et al.*, 1997).

There have also been several reported occurrences of multidrug resistance in the aquatic environment. This has been reported as far back as 1991, when Magee and Quinn isolated 73 aquatic isolates that were resistant to 8 antibiotics. Since then several more investigations have detected MDR in the aquatic environment (Ozgumus *et al.,* 2009; Zhang *et al.,* 2009; Verner–Jeffreys *et al.,* 2009; Jianying *et al.,* 2008; Lima-Bittencourt, 2007; Park *et al.,* 2003). When the individual resistance profiles were analysed, there did not appear to be any particular pattern however. It is likely therefore that MDR profiles in the environment are dependent upon sample site and geographical location. Indeed MDR profiles have been shown to depend on other factors, such as season (Jianying *et al.,* 2008). The results of the current study are therefore consistent with other studies that have detected MDR in the environment.

It is also possible that MEPPs were present in aquatic microcosms. These mechanisms have also been detected in various environments including surface water (Hernandez *et al*., 2009; Frike *et al.*, 2008; Long *et al.*, 2008), aquatic sediment (Groh *et al.*, 2006) and also in STPs (Szczepanowski *et al.,* 2004). Such resistance mechanisms are not compound–specific and as such are capable of expunging a huge number of toxicants out of a bacteria cell (Koronakis *et al.,* 2004). Interestingly, one study reported the presence of multiple resistance genotypes and MEPPs (Florian–Fricke *et al*, 2008). The possibility of multidrug resistance being present on day 1 in aquatic microcosms as a result of multiple general mechanisms and general efflux mechanisms is therefore presented.

5.4.4. Development of Multidrug Resistance in Aquatic Microcosms

The results of the current study have indicated that multidrug resistance forms over time in the aquatic environment. Some of this MDR formation was not due to exposing the microbial community to CTC or SMX it would appear. However, the presence of several MDR isolates were not detected in control microcosms, suggesting that the selective pressure exerted by CTC and SMX selected for the formation of MDR also. With the exception of one isolate that was screened for MDR, resistant morphotypes expressed between 22 and 24 different resistant phenotypes. These data would suggest that antibiotic resistance can be co-selected in aquatic systems.

There have been other reported incidences of co–selection of antibiotic resistance in the environment. Peterson *et al.* (2002) observed co–selection of chloramphenicol, ciprofloxacin, erythromycin, oxytetracycline, sulfamethizine and trimethoprim as a result of exposing an aquatic system to farm animal waste (likely to contain tetracyclines).

Other studies have concentrated on detecting an increase in mobile genetic elements (that are highly likely to carry multiple transferrable genotypes) as a result of the exposure of microbial communities to antibiotics and metals (Wright *et al.,* 2008) and sulfonamides in particular (Heuer

et al., 2007). The literature therefore provides some corraboration although the current study potentially provides novel evidence of tetracycline and sulfonamide–mediated co–selection of antibiotic agents.

The available literature also presents a number of reported occurrences of multiple gene transfer in the environment. It is possible that these processes provide the explanation for why MDR characteristics were co–selected by exposure to CTC and SMX in the aquatic microcosms studied in the current investigation.

The transfer of multiple resistance genes is a possible explanation to explain why there was an increase in multiple resistance phenotypes over the 7 days of exposure to both compounds. Many studies have shown that multiple genetic resistance determinants can be transferred between environmental microbes. A number of these studies have additionally identified the role of mobile genetic elements such as class 1 integrons and transposons in the transfer of multiple resistance genes.

Ozgumus *et al.* (2009) reported that ampicillin, tetracycline, trimethoprim, streptomycin, and nalidixic acid resistance traits were all transferrable between isolates that had been cultured from Turkish rivers. PM11C studies in the current investigation showed that CTC resistance can potentially be co–transferred with nalidixic acid resistance. Both studies have therefore demonstrated that tetracycline and nalidixic acid resistance mechanisms can be co–selected. The same study also detected class 1 and class II integrons that were carried by many of the isolates tested. This provides some evidence that tetracycline and nalidixic acid genes may be co–transferrable of these mobile genetic elements.

Laroche *et al.* (2009) investigated the presence of multidrug resistance profiles in aquatic isolates in relation to the presence of class I and class II integrons. They observed that the presence of both tetracycline and sulfonamide resistance were often accompanied by several other resistance profiles. One isolate for example showed resistance to amoxicillin, kanamycin, CTC, tetracycline and nalidixic acid and sulfamethoxazole. In the present study CTC resistance was co–selected with amoxicillin, nalidixic acid and kanamycin resistance. In addition, sulfamethoxazole resistance was co–selected with tetracycline resistance. Tetracycline and sulfonamide resistance could also be co selected with cephlalosporin resistance and tetracycline resistance could also be transferred with chloramphenicol resistance; these selection patterns were also observed in the present study. This investigation therefore shows some similar results to the present study. The *int2* gene was also present suggesting the possibility that resistance genes could be horizontally transferred. It is noteworthy however that sequence analysis of the variable intgeron regions could not explain the multitude of resistance profiles.

Zahid *et al.* (2008) investigated the co–transfer of tetracycline resistance (the gene was not specified and only referred to as "tet^R") in a series of mating experiments involving aquatic

bacteria. They found that tet^R could be co–transferred with sulfamethoxazole, trimethoprim, erythromycin and streptomycin. The present study has shown that tetracycline and erthythromycin resistance were co-transferrable also. It was additionally shown that sulfamethoxazole and tetracycline resistance were co–selected. The two data sets therefore demonstrated some agreement in their findings.

In another study, Park *et al.* (2003) found that sulfamethoxazole resistance had been co–selected alongside resistance to aminoglycosides and β–lactam resistance. In the present study sulfamethoxazole resistance was co–selected with aminoclycoside resistance. One example of this was the co–selection of tobramycin resistance. Co–selection of β–lactam resistance was also witnessed in the present study, in the case of penicillin G resistance co–selection for example. The findings of this study therefore provide more corraborating evidence with the present investigation.

Mukherjee and Chakraborty (2006) also detected co–selection of antibiotic resistance. Their investigation revealed the presence of a variable gene cassette (found on class I integrons) that contained sequences for several resistance genes, including sequences that coded for β–lactam and aminoglycoside resisance determinants. Although the study did not show co–selection of resistance along with tetracycline or sulfonamide resistance studied in the present investigation, it still provided more evidence of the occurrence of co–selection of antibiotic resistance in the environment.

It also possible that MDR formed in aquatic microcosms due to the transfer of genes that code for MEPPs. To the best of my knowledge this has not yet been studied in the environment or in mating studies. If genes coding for MEPPs were shown to be transferrable this would provide more evidence to explain why MDR was formed in the present study. Alternatively, it is also possible that MEPPs were present in one or more resistant isolates all along but were only expressed when antimicrobial compounds were added to their habitat (ie: gene expression was being induced). Another scenario could be that a portion of the aquatic microbial community was expressing MEPPs in response to the build-up of toxic waste products within the microcosm system. In this scenario, antibiotic molecules could potentially have been expelled from microbial cells as general toxicants. Bacteria that would have produced these systems may not have been affected by selective pressure as they would have been able to avoid antimicrobial toxicity. It is conceivable that such bacteria would also have been able to thrive equally as well in control microcosm and spiked microcosms. In this scenario, antibiotic exposure may have been of reduced significance.

Several studies have reported the apparently low fitness cost of developing MDR (Ward *et al.*, 2009; Trindade *et al.,* 2009). It is therefore in a microbe's best interest (in an evolutionary sense) to become resistant to as many antibiotics as possible considering the low risks involved. The current investigation detected the presence of morphotypes that had developed resistance in control only systems (over the seven day exposure). As antibiotics were not added to these microcosms one must seek an alternative selective pressure which selected resistance. It is however possible that a

cocktail of antimicrobial compounds already have existed within the river water and treated sewage that comprised the majority of microcosm liquid (see table 1.3). It is possible that these compounds may have been selecting and co-selecting for the expression of specific and general resistance mechanisms. In the scenario that the build-up of toxic waste (such as ammonia) was causing an increased expression of general efflux mechanisms (which will expel toxic waste from bacterial cells) it is also possible that antibiotics will be expunged inadvertently. If toxic waste products are present within microcosms then it possible that toxic waste products are acting as a selective pressure for general efflux genes being horizontally transferred. It has been previously demonstrated that toxic compounds can co-select for mechanisms that also protect microbes from other, unrelated toxic compounds (eg: Burgos *et al*., 2005). Also, given the presence of bacteria that were resistant to more than one antibiotic at the start of the exposure, it is clear that a resistant gene pool was available from which a range of resistance genotypes (possibly general and specific) could be transferred to previously susceptible bacteria. It is also worth mentioning that class 1 integron genes have been detected (in sediment-dwelling bacteria) which lacked the type of transposons (Tn402-like) that would normally be associated with the spread of antibiotic resistance. These integrons also lacked any antibiotic resistance genes. This study therefore demonstrates that genetic determinants were being transferred bfore the "antibiotic era" and that transfer of genetic antibiotic reistance determinants may occur in the absence of a positive selective pressure such the presense of antibiotic resistance (Stokes *et al*., 2006).

It remains unclear as to whether resistance developed in control microcosms due to a particular selective pressure (such as exposure to ammonia for example) or occurred passively due to the microbial developing antibiotic resistance at a low fitness cost. The only way to study this would be to follow temporal resistance formation in truly pristine test systems. One option would be to utilize synthetic media (such as synthetic pond water or mineral medium) and study the formation/donation of resistance by pure cultures with known resistance profiles.

Chapter 6: General Discussion

6. General Discussion

Antibiotics are specifically targeted to kill pathogenic bacteria in both human and veterinary medicine. As a result of the necessary usage of antibiotics they may enter the aquatic environment *via* a number exposure routes such as STP effluent and surface run-off from agricultural areas. As a result, antibiotics have been detected in several aquatic environmental matrices, such as surface water (e.g.: Kolpin *et al*., 2002), sediment (e.g.: Lalumera *et al*., 2004) and groundwater (e.g.: Karthikeyan and Bleam, 2003). Several studies have demonstrated that antibiotics exert a targeted effect on the soil microbial community, such as inhibiting microbial respiration (e.g.: Thiele-Bruhn and Beck, 2005).

Despite this, there is a lack of data on how antibiotics may affect aquatic microbial communities. This is reflected in the current environmental risk assessment of antibiotics, which relies on tests that are ecologically irrelevant for the aquatic environment as well as being conducted in too brief a time period.

To address these issues, a series of experiments were conducted that initially focused on developing a more ecologically relevant test system that could detect the effects of two antibiotics from classes that have been frequently detected in the environment due to their heavy global usage (sulfamethoxazole and CTC). A microcosm system was developed that contained 90% river water as a basic test medium, 5% treated sewage to increase biomass and provide environmental relevance and 5% OECD synthetic sewage as a nutrient source. As such, the test system provided a "bridge between theory and nature" (Fraser, 1999). In addition to this, a multisubstrate utilisation assay (Biolog GN2) was optimized for use in aquatic microbial ecotoxicological studies and some issues that had been raised in the literature were resolved (Preston-Mafham *et al.*, 2002; Garland, 1996; Haack *et al.*, 1995). These were concerning relating to inoculum density, detection of substrate utilization rates in relation to lag times, nutrient carryover, taking multiple time-point readings and adding equal volumes of inoculum to each well. The current investigation demonstrated methods that should ultimately take away these concerns. The combined microcosm-GN2 system was shown to be repeatable in the absence of a toxicant and could be validated against the OECD reference toxicant 3, 5-DCP. In addition, the system could detect the effects of and recovery to 3, 5-DCP.

The present study has therefore developed an approach that is arguably more environmentally relevant than the current standard test battery. As pointed out in section 2.4, laboratory test systems will never fully recreate a natural aquatic environment in terms of its full complexity; there are far too many variables to take into consideration. Having said this, conducting the current investigation under field conditions would have been logistically and financially infeasible. The current test system has also been shown to be capable of detecting the effects of environmental toxicants on

aquatic microbial communities. Further, the system also allows the removal of contents for further analysis with minimum disruption. As such, A "one system fits all" was developed.

A test system was now in place that could test the effects of antimicrobial compounds on microbial function in a simulated aquatic environment. As the Biolog GN2 assay can assess a range of carbon and nitrogen sources belonging to 5 ecologically relevant "guilds" (Preston-Mafham, 2002) the system can detect carbon and nitrogen turnover in the aquatic environment. The system can therefore detect many more ecologically relevant end-points than the current range of microbial toxicity tests. The current test system can track the effects of pharmaceuticals on aquatic microbial communities, not sludge communities (as the ASRIT does) or single algal or marine species (as the Microtox or other single species testing do). In addition, the system is generally equallyas sensitive than these tests, with a LOEC of 0.1 mg/L having been detected. Table 6.1 summarises the toxicity of CTC and SMX that have been previously reported, alongside the results of other published standard test information. Toxicity values obtained in the present study are generally comparable. However, this generally holds true for Microtox only when the test duration is increased (Backhaus *et al*., 1997).

This test system was then used to test the effects of two antibiotics on microbial function. Acute effects were observed on total substrate utilisation for both compounds in terms of lag phase, max rate and multivariate analysis. This was in accordance with other studies that had tested the effects of antibiotics on total substrate utilisation in Biolog plates in other environmental matrices (Kong *et al*., 2006; Maul *et al.*, 2006; Schmitt *et al.*, 2005). The mode of action of each antibiotic could explain these effects; tetracyclines inhibit protein synthesis and sulfonamides prevent the biosynthesis of folic acid. Both of these compounds would therefore be expected to prevent microbial growth and therefore reduce the extent to which bacteria can utilize substrates.

Jankhe and Craven (1995) report the ability of aquatic microorganisms to utilise a broad range of molecules contained within DOC (dissolved organic carbon). Blomqvist *et al*. (2001) demonstrated the importance of DOC in terms of its conversion into microbial biomass while Tranvik and Hofle (1987) commented on the efficiency with which microbes achieve this. Landi *et al.* (1993) have already reported a significant decrease in soil respiration rates as a result of exposure to streptomycin. The results of the present study therefore raise concerns over the ability of aquatic bacteria to utilize a wide range of substrates in their environment.

Table 6.1: Reported toxicity by microbial standard tests.

Test	Result	Reference
Microtox	oxytetracycline: EC_{50} = 64.5 mg/L; sulfamethoxazole: EC_{50} = 23.3 mg/L; oflaxacin: 25% growth inhibition at 100 mg/L	Isidori *et al*., 2005
Microtox	oxytetracycline, erythromycin, ofloxacin and oxolinic acid: EC_{50} = 66-550 mg/L	Christensen *et al*., 2006
Microtox	sulfamethoxazole: EC_{50} (15 and 30 mins) = 74.2 and 78.1 mg/L; sulfachloropyridizine: EC_{50} (15 and 30 mins) = 53.7 and 26.4 mg/L	Kim *et al*., 2007
Microtox	flumequine: EC_{50} = 12.12-15.24 mg/L; oxytetracycline: EC_{50} = 121.01 - 139.31 mg/L	Lalumera *et al*., 2004
Microtox	nalidixic acid: EC_{50} (24 hour) = 0.21 mg/L; streptomycin sulphate: EC_{50} (24 hour) = 20.6 mg/L; chloramphenicol: 0.074 mg/L	Froehner *et al*., 2000
Microtox	nalidixic acid: EC_{50} (24 hour) = 0.184 mg/L; tetracycline: EC_{50} (24 hour) = 0.024 mg/L	Backhaus *et al*., 1997
Microtox	chloramphenicol, tetracycline, nalidixic acid, norfloxacin and norfloxacin: EC_{50} (24 hour) < 1 mg/L	Backhaus and Grimme, 1999
Pseudomonas putida Growth Inhibition Test	ciprofloxacin: EC_{50} = 80 µg/L; ofloxacin: EC_{50} = 10 µg/L	Kummerer *et al*., 2000
Pseudomonas putida Growth Inhibition Test	tetracycline: MIC_{50} = 2 mg/L; CTC: MIC_{50} = 0.5 mg/L; oxytetracycline: MIC_{50} = 1 mg/L	Halling Sorensen *et al*., 2002
Microcystis aeruginosa Growth Inhibition Test	levofloxacin: EC_{50} = 7.9 µg/L; ciprofloxacin: EC_{50} = 17 µg/L; ofloxacin: EC_{50} = 21 µg/L; enrofloxacin: EC_{50} = 49 µg/L; flumequine: EC_{50} = 1960 µg/L	Robinson *et al*., 2005
Microcystis aeruginosa Growth Inhibition Test	mecillinam: EC_{50} = 0.06 mg/L; ciprofloxacin: EC_{50} = 0.005 mg/L	Sorensen *et al*., 2000

Both antibiotics also exerted significant effects on substrate guild utilisation. For both antibiotics acute effects could be detected for amine and amide, amino acid, carbohydrate, carboxylic acid and polymer utilisation. Each of these end-points indicated the inhibition of key ecological processes within the aquatic environment. These can be generally categorized as effects on the nitrogen cycle (amines and amides and amino acids), the carbon cycle (carbohydrate and carboxylic acids) and on degradatative processes (polymer utilisation).

The nitrogen cycle is responsible for recycling inorganic nitrogen from nitrogenous compounds such as amines and amino acids. Any disruption of this process could lead to a reduction in key processes such as the ammonification, deamination and nitrification. Other studies have also gathered data that points to the potential disruption of nitrogen cycling by antibiotics (Brandt *et al.*, 2009; Verma *et al.*, 2007; Kong *et al.*, 2006; Halling Sorensen *et al.*, 2000; Halling Sorensen, 2000; Klaver and Mathews, 1994; Wheeler and Kirchman, 1986; Sevag and Green, 1944). This area therefore warrants further investigation. This should start with the testing of as many antibiotics as possible as a range of compounds from different classes have shown potential toxicity towards nitrogen cycling. The effects of various antibiotics on microorganisms that perform key distinct roles within the nitrogen cycle should also be investigated using molecular techniques such DGGE and TRFLP for community analysis and q-RT-PCR for specific gene analysis. Interestingly, there is a suite of nitrogen metabolizing end-points within a specially designed Biolog plate for investigating the utilisation of nitrogenous compounds (PM3B), nitrogen peptide compounds (PM6, PM7 and PM8) and also an array for testing the utilisation of nitrogen sources at different pHs (PM10) (Biolog 2010). A combination of these approaches could potentially be employed within the context of microcosm studies similar to the one used in the current investigation.

Carbon turnover (which can be linked to processes that occur within the carbon cycle) is also a key ecological process. The importance of aquatic bacteria in the utilization of DOC constituents has been previously reported (Blomqvist *et al*., 2001; Tranvik and Hofle, 1987). Specifically, carbohydrates are an important energy source for aquatic microorganisms. Carboxylic acids are an important constituent of humic material in the environment (Kawahigashi and Sumida, 2006). The importance of carboxylic acid utilisation to the functioning of microbial function has also been reported (eg: Naumann, 1918). The current study saw that both antibiotics inhibited the utilisation of both carbohydrates and carboxylic acids. Interestingly, the mode of action of each antibiotic can be linked to this inhibitory effect in terms of retardation of protein synthesis and the inhibition of purine base biosynthesis (Tritton, 1977; Gale *et al.*, 1981; Chopra, 1985; Rasmussen *et al.*, 1991; Altman, 1946).

Other studies have also found that a range of antibiotics tend to interfere with carbon turnover by microorganisms (Kong *et al.*, 2006; Maul *et al.*, 2006; Zielezny *et al.*, 2006; Boleas *et al*., 2005; Thiele-Bruhn and Beck, 2005; . Vaclavik *et al.*, 2004; Sevag *et al.*, 1945; Clinton and Loeuringer, 1942; Dorfman and Koser, 1942; Sevag and Shelburne, 1942). The results of these studies, as well

as those presented in the current investigation therefore raise concerns over the tendency of antibiotics to interfere with carbon turnover in the environment. Consequently, the issue merits further investigation. This could take the form of testing as wide a range of antibiotics as possible, in particular new antibiotics such as fourth generation cephelosporins and tigecycline (a new tetracycline antibiotic). In addition to this, detailed metabolomics technology could tease out specific biochemical alterations in the microbial metabolome. Specific gene analysis of important enzymes involved in the utilisation of carbohydrates could also be conducted.

Polymer utilisation was another specific end point that was affected by both antibiotics. The current study has suggested that this could have consequences for degradative processes that occur in the aquatic environment. Microorganisms play a key role in degrading natural polymers in the environment, such as lignin for example (Kirk and Farrell, 1987), but have also been shown to be able to degrade a huge array of polymeric compounds some of which were once thought to be recalcitrant to degradation (Shimano, 2001; Howard, 2002; Szostac-Kotowa, 2004; Shah *et al*., 2008). Microrganisms ultimately assimilate polymers as cellular biomass and energy (Lucas *et al*., 2008). In addition, environmental microorganisms play a key role in the degradation of a wide array of xenobiotic compounds (Allocati *et al.,* 2009).

Other studies have also detected the inhibition of polymer utilisation by antibiotics (Kong *et al.*, 2006). Other studies have also demonstrated that antibiotics inhibit the degradation of a number of xenobiotic compounds such as synthetic hormones (Chun *et al.,* 2005), pesticides (Allen and Walker 1987) and pharmaceuticals (Montiero and Boxall, 2009). Montiero and Boxall also point out that pharmaceuticals are rarely present by themselves in the environment. Not only is this a concern in terms a possible additive antibacterial effect (Christensen *et al.,* 2006) but also raises concerns that a potentially huge array of degradative processes are being inhibited by antibiotics in the environment. Future work is therefore warranted. The effect of antibiotics on the degradation of both naturally occurring and xenobiotic polymeric compounds should be investigated. This is a daunting thought considering how many possible polymers and xenobiotic compounds are present in the environment. High-throughput methods would therefore have to be developed to save time and labour intensity. In addition to this, molecular techniques such as q-RT-PCR should be utilized to investigate the specific genes that code for degradative enzymes (both specific and general) and how the expression of these are affected by antibiotics. Another approach could be to biochemically assay specific degradative systems. For example the current investigation suggested that sulfonamides may interfere with the glutathione-s-transferase degradative mechanism. This also leads to the conclusion that more care should be taken to properly evaluate not just the mode of action of an antibiotic but also take into account the knock-on effects of what this will cause. This could lead to the development of more rigid hypotheses and targeted test design. The current investigation for example has highlighted the knock on effects of folate deficiency as result of sulfonamide intoxication, such as inhibiting crucial metabolic cofactors (Altman, 1946). This can often involve the review of complex biochemical pathways.

As well as detecting a range of specific acute effects of CTC and SMX, the current investigation also showed that effects can show later on in study period. It was interesting to speculate why this may have happened. In the case of CTC, it was hypothesized that there may have been a toxic effect of a CTC metabolite or transformation product, as was observed by Halling-Sorensen in 2002. This certainly merits further investigation. This could possibly take the form of specific metabolite analysis with mass spectrometry to identify the presence of any toxic metabolites and toxicity testing of the actual metabolites. However, this would not be easy in the case of CTC considering the 64 possible tautomer conformations, five protonation sites and four dissociation constants (Duarte *et al*., 1998; Jin *et al.*, 2007). Degradation studies have suggested that the half-lives of tetracyclines are between 1 and 4 days (Verma *et al.*, 2007; Sanderson *et al*., 2005). However these studies took place in the presence of light. Tetracyclines are known to photodegraded (Thiele-Bruhn and Peters, 2007; Bautitz and Nogueira, 2007; Sanderson *et al*., 2005). When studies in the absence of light have been investigated, half-lives of between 13 and 18 days have been reported (Verma *et al*, 2007). During the present study samples were taken and stored for specific analysis. Given the variation in degradation rate that have been reported, it would be wise to perform specific analysis in future exposures. Due to time and financial limitations during the present project, it was not possible to conduct the analytical procedures that were required.

In the case of SMX, it was hypothesized that microbial communities that were intermittently inhibited later in the study may have been an artifact of transient communities displaying a variable ability to regulate their internal pH (Tappe *et al.*, 2008). Due to the likely buffering effect of the phosphate buffer present in OECD synthetic sewage, an increase in sulfonamide activity as the result of a 1 unit drop in pH (1997 Zarfl *et al.*, 2008; Madigan *et al.*, 2003; Mengelers *et al*., 1997 Roland *et al.*, 1979) was tentatively ruled out. It is likely however that SMX did not degrade in the aquatic microcosm, as has been suggested by closed bottle tests (Alexy *et al*., 2004; Al-Amad *et al.*, 1999) and studies in the aquatic environment (Bartsch, 2009; Radke *et al.*, 2009; Benotti and Brownawell, 2008; Conkle *et al.*, 2008; Tamtam *et al.*, 2008; Bendz *et al.*, 2005). The compound was therefore likely to persist and exert an effect upon susceptible microbial communities over the 7 day period investigated in the present study.

In the aquatic environment therefore, both CTC and SMX are likely to exert an inhibitory effect on susceptible aquatic microbial communities and affect several effects on ecologically relevant end-points. Studies have shown that both compounds are present in the environment and were transported there via a number of exposure routes, as has been discussed extensively throughout the current investigation. More studies are therefore warranted to further investigate the effects of antibiotics on microbial function in the aquatic environment as suggested during this section.

Another key observation during the current investigation was that a degree of recovery occurred in microcosm exposed to both CTC and SMX. The recovery was much more pronounced in

microcosms exposed to CTC than with SMX. A degree of recovery was however observed at the highest SMX concentration. This may suggest that an SMX concentration of between 0.32 and 1 mg/L is required to select for sulfonamide resistance. A more widespread recovery was observed in microcosms spiked with CTC. This could be observed in microcosms spiked with 0.1 mg/L CTC, suggesting that a lower CTC concentration might select for antibiotic resistance. It is noteworthy that the effect of SMX on the functional diversity of total substrate utilisation could still be observed on day 7 of the exposure. No effect of CTC on functional diversity was observed after day 1. These data therefore highlight the differential recoveries of microbial communities to both compounds that were tested.

To test the hypothesis that CTC and SMX selected for antibiotic resistance a series of culture-based resistance experiments were conducted. It was initially shown that a greater number of distinct CTC and SMX-resistant bacteria could be cultured on day seven compared with day one. In addition, more resistant morphotypes could be cultured from microcosms that had been exposed to CTC than SMX. Total CFU counts of resistant bacteria were shown to significantly increase in all microcosms, including controls that had not been spiked with either CTC or SMX. Moreover, day 7 CTC resistant CFU counts indicated that exposure to all CTC concentrations resulted in a significantly higher increase in resistance formation compared to controls. SMX exposed microcosms did not result in a significantly higher development of SMX resistance however.

CFU counts of resistant bacteria from control microcosms and cross referencing thee with day 7 SMX and CTC-resistant morphotypes revealed that several resistant isolates had been added to microcosms via river water and treated sewage ("background' resistance). In addition, some resistant morphotypes had formed in exclusively control microcosms, some in control and treated microcosms and some had only formed resistance as the result of exposure to CTC and SMX. Multidrug resistance profiles were established for those CTC and SMX-resistant isolates that could be sub-cultured. This revealed that a wider resistance profile was formed on day seven compared with day one. For the resistant morphotypes that formed due to selective pressure, multidrug resistance profiles could be established for five CTC-resistant and three SMX-resistant isolates. It was found that these resistant morphotypes generally displayed a much wider resistance profile. The results therefore suggested that both single and multidrug resistance could form in both the presence and absence of CTC and SMX. The reduction in resistant bacteria that could be cultured was attributed to media (nutrient) shock.

The presence of "background" resistance in microcosms could be attributed to the resistant bacteria that were already present in river water and treated sewage within microcosms. Several studies have shown that STP effluent and river water contain antibiotic-resistant bacteria (Xiang Zhang *et al.*, 2009; Zhang *et al.*, 2009; Szczepanowski *et al.*, 2004; Silva *et al.*, 2006). Other studies have shown that exposure of microbial communities to tetracyclines can result in the formation of single drug resistance (Yu *et al.*, 2009; Kanpp *et al.*, 2008; Kim *et al.*, 2007; Rhysz and Alverez, 2004;

Kerry *et al.,* 2006). Exposure to sulfonamides has also been shown to result in selection of antibiotic resistance (Yi *et al*., 2010; Brandt *et al.,* 2009; Heuer *et al.,* 2008; Heuer and Smalla, 2007). Knapp *et al.* (2010) have reported an increase in antibiotic resistance (especially tetracycline resistance) with increasing usage over longer time periods.

The current investigation also suggests that the formation of both single and multidrug resistance may have been due to the presence of multidrug efflux systems that were also expelling toxic waste products that were likely to have been produce in all microcosms, including controls (Koronakis *et al.,* 2004). Aquatic bacteria have previously been shown to possess such mechanism (Hernandez *et al.,* 2009; Hernandez *et al*., 2009; Frike *et al.,* 2008; Long *et al.,* 2008; Groh *et al.,* 2006). Multidrug resistance mechanisms have also been detected in STPs (Szczepanowski *et al.,* 2004). It is possible therefore such genetic resistance determinants were added to microcosms via treated sewage.

The presence of multidrug resistance has been reported in aquatic systems (Ozgumus *et al.,* 2009; Zhang *et al.,* 2009; Verner–Jeffreys *et al.,* 2009; Jianying *et al.,* 2008; Lima-Bittencourt, 2007; Park *et al.,* 2003; Magee and Quinn, 1991). Day one results showed that a certain amount of multidrug resistance was also present in the aquatic microcosms studied in the present investigation. It is likely therefore that this contributed significantly to the multidrug resistance profile of the microbial community in the aquatic microcosm investigated during the present study.

The mechanism behind the formation of both single and multidrug resistance is likely to be the transfer of genetic material between environmental bacteria. This has been shown to occur by several mating studies that have observed the exchange of resistance genes (Nagachinta and Chen, 2008; Byrne–Bailey *et al.,* 2009; Agerso and Sandvang, 2005). Moreover, this has been shown to occur in an intergenic and intragenic manner (Zahid *et al*., 2008).

Chapters 3 and 4 revealed very different recovery profiles. It was hypothesized that a recovery may have occurred (at least partially) due to the formation of antibiotic resistance. In the case of the functional recovery shown by microbial communities during CTC exposure experiments, analysis of resistant CFU formation demonstrated that a significant temporal rise in resistant CFU counts occurred at all CTC doses. These data support the hypothesis that a recovery was observed due to development of antibiotic resistance. This is not to say that other factors, such as degradation of CTC, were also taking place. Interestingly, several *tet* genes code for tetracycline degradation/transformation mechanisms. Both degradation rates and formation of CTC resistance may therefore be linked. Future studies should employ specific analysis for *tet* genes and CTC concentration also.

In the case of SMX exposure, although a certain degree of recovery could be observed, a wide range of functional effects could still be observed on day 7 of the exposure, even at the lowest SMX dose (0.1 mg/L). In addition, a significantly greater rise in the temporal formation of SMX

resistance as the result of exposure to SMX could not be detected by culture-based methods. A rise in resistance was however detected at all SMX doses including controls. It must be concluded though that SMX did not seem to act as much as a selective pressure for resistance as CTC did. It is possible therefore that the less pronounced recovery that was observed in SMX exposures could have been caused by lack of significant formation of temporal resistance as the result of exposure to SMX.

To conclude, single resistance and co-selection of multidrug resistance were shown to have occurred as the result of "background" and selective processes. This would suggest that antibiotic resistance in the aquatic environment can be a naturally occurring process or can be influenced by the anthropogenic discharge of antimicrobial compounds. The current investigation would like to apply a cautionary message however. It is highly likely that control microcosms were not entirely free of antibiotic residues due to the occurrence of CTC, SMX and other antibiotics in both natural river systems and treated sewage. To investigate the formation of single and multidrug resistance formation in a truly pristine environment (whether this takes the form of filed or simulated environment study) may not be easy. This deserves a high degree of consideration in the view of the current study. Saying that however, it would perhaps not be surprising that single or multiple drug resistance did form in the absence of antibiotics due to the apparently low fitness cost of bacteria exchanging genetic information MDR (Ward *et al.,* 2009; Trindade *et al.,* 2009).

What were not investigated during the current investigation were the concentrations of CTC and SMX that were required to exert a selective pressure on the formation of single and multidrug resistance. Time constraints did not allow more extensive CFU counts of each resistant morphotype that were present in each microcosm on each day of the study. However, samples were kept aside for molecular analysis; this should reveal changes in the microbial community and complement phenotypic data well. Further molecular analysis (q-RT-PCR) would allow the detection of specific genes and when they were expressed during the exposure period. Moreover, metagenomic analysis of stored samples would give an even more detailed picture of genetic events during the study.

The results regarding antibiotic resistance studies during the present investigation raise some concerns over human and animal health. Antibiotic resistance has been reported to spread from the environment to bacteria that both humans and animals come into contact with (Mukherjee and Chakraborty, 2005; Opegaard *et al.,* 2001; Kruse and Sorum 1994). Moreover, other studies have suggested that antibiotic resistance may be transferable between the environment and humans/animals (Gilling *et al.,* 2009; Kruse and Sorum 1994). This seems worthy of further investigation. One method of achieving this would be to spike aquatic microcosms with human or animal pathogens that are susceptible to a range of antibiotics and detect changes in their resistance profile over a time period. Resistance could be detected by either molecular or phenotypic methods.

6.1. General Conclusions of Thesis

1) A test system was developed that was validated with an OECD reference toxicant and could detect functional and structural changes in microbial communities.

2) CTC had an inhibitory effect on various aspects of ecologically relevant structural and functional aspects of microbial communities in aquatic microcosms at a lower dose of 0.1 mg/L on the first two days of the exposure period.

3) SMX also had an inhibitory effect on various aspects of ecologically relevant structural and functional aspects of microbial communities in aquatic microcosms at a lower dose of 0.1 mg/L on the first two days of the exposure period.

4) The microbial community showed a variable degree of recovery to both antibiotics at certain concentrations, whereas at other concentrations less or no recovery was exhibited. More recovery was observed by microbial communities that were exposed to CTC.

5) Single and multidrug resistance was formed in the presence and absence of CTC and SMX, indicating that resistance can form due to the selective pressure of antibiotics or due to naturally occurring processes. A significantly greater rise in CTC resistance was observed at all CTC doses but not as the result of exposure of microbial communities to SMX.

6) A functional recovery to the effects of CTC could have have occurred due to the formation of CTC resistance over time, as suggested by the significant increase in CTC-resistant CFU counts over time. It is less clear as to whether the same may have happened during SMX exposures due to the lack of significant SMX-resistant CFU counts over time.

7) In general, CTC and SMX show signs that they may pose a risk to the environment and possibly the wider health of humans and animals.

Appendix

Appendix 1a: Morphotyping of CTC-resistant isolates and their presence in microcosms - Day 1.

			Presence of Distinct Morphotype at Different CTC Concs			
Similar To / Formed[2]	**Morphotype ID**	**Morphotype Description**	**0 mg/L CTC**	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**
CTCD7RES14	***CTCD1RES1***	White colonies. 1-2 mm diameter. Irregular edges. Non-translucent. Non-swarming	+	+	+	-
CTCD7RES28	***CTCD1RES2***	Orange colonies. 10-12 mm diameter. Translucent. Swarming.	+	+	+	+
CTCD7RES7	***CTCD1RES3***	Purple colonies. 4-6 mm diameter. Pulvonated. Translucent. Non-swarming.	+	+	+	+
UNIQUE	***CTCD1RES4***	White colonies. 5-7 mm diameter. Non-translucent. Non-swarming.	+	+	+	-
CTCD7RES26	***CTCD1RES5***	White colonies. 1-2 mm diameter. Translucent. Non-swarming.	+	+	+	+
CTCD7RES3	***CTCD1RES6***	Orange colonies. 5-6 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
CTCD7RES4	***CTCD1RES7***	White colonies. 20 mm diameter. Non-translucent. Swarming.	+	+	-	+
CTCD7RES6	***CTCD1RES8***	Yellow colonies. 5-7 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
CTCD7RES20	***CTCD1RES9***	White colonies. 25-27 mm diameter. Non-translucent. Swarmed to form dense plaque.	+	+	+	+
UNIQUE	***CTCD1RES10***	Purple colonies. 2-3 mm diameter. Non-translucent. Swarming.	+	+	+	+
CTCD7RES22	***CTCD1RES11***	White colonies. 10-12 mm diameter. Translucent. Non-swarming.	+	+	+	+
CTCD7RES10	***CTCD1RES12***	Bright pink colony. 3-4 mm diameter. Opaque. Non-translucent. Non-swarming.	+	+	+	+
CTCD7RES12	***CTCD1RES13***	Yellow colonies. 2-3 mm diameter. Translucent. Non-swarming.	+	+	+	+
CTCD7RES19	***CTCD1RES14***	White colonies. 10-12 mm diameter. Non-translucent. Swarming.	+	+	+	+
CTCD7RES8	***CTCD1RES15***	Orange colonies. 10-12 mm diameter. Non-translucent. Non-swarming.	+	+	+	-
CTCD7RES15	***CTCD1RES16***	Light pink colonies. 15-18 mm diameter. Non-translucent. Swarmed to form dense plaque.	+	+	+	+

Appendix 1a (cont).

Similar to/Formed[2]	Morphotype ID	Morphotype Description	Presence of Distinct Morphotype at Different CTC Concs			
			0 mg/L CTC	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**
CTCD7RES21	***CTCD1RES17***	Orange colony with veins. 14-16 mm diameter. Translucent. Swarming.	+	+	+	+
CTCD7RES24	***CTCD1RES18***	White raised colony. 8-10 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
CTCD7RES11	***CTCD1RES19***	White veined colony. 18-20 mm diameter. Translucent. Swarming.	+	+	+	+

[2] C = Formed in control microcosms only; Tr = Formed in treated microcosm only; C + Tr = Formed in treated and control microcosms

Appendix 1b: Morphotyping of CTCresistant isolates and their presence in microcosms - Day 7.

			Presence of Distinct Morphotype at Different CTC Concs			
Similar To / Formed[2]	**Morphotype ID**	**Morphotype Description**	**0 mg/L CTC**	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**
C	***CTCD7RES1***	White colonies. 50-55 mm diameter. Non-translucent. Non-swarming.	+	-	-	-
Tr	***CTCD7RES2***	Raised pink colonies. 3-5 mm diameter. Translucent. Non-swarming.	-	-	+	+
CTCD1RES6	***CTCD7RES3***	Orange colonies. 4-6 mm diameter. Non-translucent. Non-swarming.	+	+	+	-
CTCD1RES7	***CTCD7RES4***	White colonies. 18-20 mm diameter. Non-translucent. Swarming.	+	+	-	-
C + Tr	***CTCD7RES5***	Light blue colonies. 2-3 mm diameter. Non-translucent. Non-swarming. Relativey rare isolate.	+	+	-	-
CTCD1RES8	***CTCD7RES6***	Yellow colonies. 5-7 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
CTCD1RES3	***CTCD7RES7***	Purple colonies. 4-6 mm diameter. Pulvonated. Translucent. Non-swarming.	+	+	+	+
CTCD1RES15	***CTCD7RES8***	Orange colonies. 10-12 mm diameter. Non-translucent. Non-swarming.	+	+	+	-
Tr	***CTCD7RES9***	White, veined colonies. 10-12 mm diameter. Translucent. Non-swarming. Grows near to other colonies	-	+	+	-
CTCD1RES12	***CTCD7RES10***	Bright pink colony. 3-5 mm diameter. Non-translucent. Opaque. Non-swarming.	+	-	-	+
CTCD1RES19	***CTCD7RES11***	White veined colony. 19-21 mm diameter. Translucent. Swarming.	+	+	+	+
CTCD1RES13	***CTCD7RES12***	Yellow colonies. 2-3 mm diameter. Translucent. Non-swarming.	+	+	+	-
Tr	***CTCD7RES13***	Raised white colonies. 22-24 mm diameter. Non-translucent. Swarming; forms dense plaque.	-	+	+	+
CTCD1RES1	***CTCD7RES14***	White colonies. 1-2 mm diameter. Irregular edges. Non-translucent. Non-swarming	+	+	+	+
CTCD1RES16	***CTCD7RES15***	Light pink colonies. 16-19 mm diameter. Non-translucent. Swarmed to form dense plaque.	+	+	+	+

Appendix 1b (cont).

Similar To / Formed[2]	Morphotype ID	Morphotype Description	Presence of Distinct Morphotype at Different CTC Concs			
			0 mg/L CTC	**0.1 mg/L CTC**	**0.32 mg/L CTC**	**1 mg/L CTC**
Tr	***CTCD7RES16***	Raised off-white colonies. 4-5 mm diameter. Rough surface. Non-translucent. Non-swarming.	-	+	+	+
Tr	***CTCD7RES17***	White raised colonies. 1-2 mm diameter. Translucent. Non-swarming.	-	+	-	-
Tr	***CTCD7RES18***	Raised purple colonies. 9-11 mm diameter. Non-translucent. Non-swarming.	-	+	+	-
CTCD1RES14	***CTCD7RES19***	White colonies. 10-12 mm diameter. Non-translucent. Swarming.	+	+	+	+
CTCD1RES9	***CTCD7RES20***	White colonies. 26-29 mm diameter. Non-translucent. Swarmed to form dense plaque.	+	+	+	+
CTCD1RES17	***CTCD7RES21***	Orange colony with veins. 14-16 mm diameter. Translucent. Swarming.	+	-	-	+
CTCD1RES11	***CTCD7RES22***	White colonies. 12-14 mm diameter. Translucent. Non-swarming.	+	+	+	+
Tr	***CTCD7RES23***	Puplle colonies. 14-16 mm diameter. Shiny surface. Translucent. Non-swarming.	-	-	+	+
CTCD1RES18	***CTCD7RES24***	White raised colony. 8-10 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
C + Tr	***CTCD7RES25***	Yellow veined colonies. 10-13 mm diameter. Translucent. Swarming.	+	+	+	-
CTCD1RES5	***CTCD7RES26***	White colonies. 1-2 mm diameter. Translucent. Non-swarming.	+	+	+	+
Tr	***CTCD7RES27***	Off-white colonies. 40-42 mm in diameter. Filiform edges. Non-translucent. Non-swarming.	-	+	-	-
CTCD1RES2	***CTCD7RES28***	Orange colonies. 10-12 mm diameter. Translucent. Swarming.	+	-	-	+
Tr	***CTCD7RES29***	Orange raised veined colonies. 30-32 mm diameter. Translucent. Swarming. Mainly found by themselves on plates.	-	+	+	+

Appendix 1c: Morphotyping of SMX-resistant isolates and their presence in microcosms – day 1.

			Presence of Distinct Morphotype at Different SMX Concs			
Similar To / Formed[2]	**Morphotype ID**	**Morphotype Description**	**0 mg/L SMX**	**0.1 mg/L SMX**	**0.32 mg/L SMX**	**1 mg/L SMX**
SMXD7RES21	***SMXD1RES1***	White colonies. 1-2 mm diameter. Non-translucent. Non-swarming	+	+	+	+
UNIQUE	***SMXD1RES2***	Veined purple colonies. 23-25 mm diameter. Translucent. Non-swarming.	+	+	+	-
SMXD7RES11	***SMXD1RES3***	White colonies. 50-55 mm diameter. Non-translucent. Rough surface. Non-swarming.	+	+	+	+
SMXD7RES2	***SMXD1RES4***	Yellow colonies. 2-3 mm diameter. Opaque. Non-translucent. Non-swarming.	+	+	+	+
SMXD7RES10	***SMXD1RES5***	Light brown colonies. 3-4 mm diameter. Filiform margins.Translucent. Non-swarming.	+	+	+	-
UNIQUE	***SMXD1RES6***	Purple colonies. 3-4 mm diameter. Non-translucent. Swarming.	+	+	+	+
SMXD7RES20	***SMXD1RES7***	White veined colony. 18-20 mm diameter. Translucent. Swarming.	+	+	+	+
SMXD7RES18	***SMXD1RES8***	White raised colony. 8-10 mm diameter. Non-translucent. Non-swarming.	+	+	-	+
SMXD7RES6	***SMXD1RES9***	Bright pink colony. 1-3 mm diameter. Translucent. Non-swarming.	+	+	+	+
SMXD7RES13	***SMXD1RES10***	Orange colonies. 20-25 mm diameter. Translucent. Swarming.	+	+	+	+
SMXD7RES19	***SMXD1RES11***	Light blue colonies. 2-3 mm diameter. Convex. Non-translucent. Non-swarming. Relativey rare isolate.	+	+	+	+
SMSD7RES17	***SMXD1RES12***	Orange colonies. 5-6 mm diameter. Pulvonated. Non-translucent. Non-swarming.	+	+	+	+
SMXD7RES5	***SMXD1RES13***	Off-white colonies. 15-17 mm diameter. Translucent. Swarming.	+	+	+	+

Appendix 1d: Morphotyping of SMX-resistant isolates and their presence in microcosms - day 7 isolates.

			Presence of Distinct Morphotype at Different SMX Concs			
Similar To / Formed[2]	**Morphotype ID**	**Morphotype Description**	**0 mg/L SMX**	**0.1 mg/L SMX**	**0.32 mg/L SMX**	**1 mg/L SMX**
SMXD7RES9	***SMXD1RES14***	Raised white colonies. 1-2 mm diameter. Translucent. Non-swarming.	+	+	+	+
Tr	***SMXD7RES1***	White colonies. 30-33 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
C	***SMXD7RES2***	Off-white veined colonies. 3-4 mm diameter. Opaque. Filamentous edges. Non-swarming.	+	+	+	+
C + Tr	***SMXD7RES3***	Raised, veined orange colomnies. Non-translucent. Non-swarming.	+	-	+	+
SMXD1RES6	***SMXD7RES4***	Purple colonies. 2-3 mm diameter. Non-translucent. Swarming.	+	+	+	-
SMXD1RES13	***SMXD7RES5***	Off-white colonies. 15-17 mm diameter. Translucent. Swarming.	+	+	+	-
SMXD1RES9	***SMXD7RES6***	Bright pink colony. 1-3 mm diameter. Translucent. Non-swarming.	+	+	+	+
Tr	***SMXD7RES7***	Red colonies. 2-3 mm. Non-translucent. Non-swarming.	-	+	-	-
Tr	***SMXD7RES8***	Raised white colonies. 9-11 mm. Translucent. Non-swarming.	-	+	+	+
SMXD1RES14	***SMXD7RES9***	Raised white colonies. 1-2 mm diameter. Translucent. Non-swarming.	+	+	+	+
SMXD1RES5	***SMXD7RES10***	Light brown colonies. 3-4 mm diameter. Filiform margins.Translucent. Non-swarming.	+	-	+	+
SMXD1RES3	***SMXD7RES11***	White colonies. 52-56 mm diameter. Rough surface. Non-translucent. Non-swarming.	+	+	+	+
C + Tr	***SMXD7RES12***	Purple colonies. 4-6 mm diameter. Translucent. Non-swarming.	+	+	-	+
SMXD1RES10	***SMXD7RES13***	Orange colonies. 20-25 mm diameter. Translucent. Swarming.	+	+	-	-
Tr	***SMXD7RES14***	Off-white colonies. 10-15 mm diameter. Translucent. Non-swarming.	-	+	+	-
C + Tr	***SMXD7RES15***	Raised purple colonies. 10-12 mm diameter. Non-translucent. Non-swarming.	+	+	+	+
Tr	***SMXD7RES16***	Veined, off-white colonies. 5-6 mm diameter. Translicent. Non-swarming.	-	-	+	-

Appendix 1d (cont).

			Presence of Distinct Morphotype at Different SMX Concs			
Similar To/Formed[2]	**Morphotype ID**	**Morphotype Description**	**0 mg/L SMX**	**0.1 mg/L SMX**	**0.32 mg/L SMX**	**1 mg/L SMX**
SMXD1RES12	***SMXD7RES17***	Orange colonies. 5-6 mm diameter. Pulvonated. Non-translucent. Non-swarming.	+	-	+	+
SMXD1RES8	***SMXD7RES18***	White raised colony. 8-10 mm diameter. Non-translucent. Non-swarming.	+	+	-	+
SMXD1RES11	***SMXD7RES19***	Light blue colonies. 2-3 mm diameter. Convex. Non-translucent. Non-swarming. Relativey rare isolate.	+	+	+	+
SMXD1RES7	***SMXD7RES20***	White veined colony. 19-21 mm diameter. Translucent. Swarming.	+	+	+	-
SMXD1RES1	***SMXD7RES21***	White colonies. 1-2 mm diameter. Non-translucent. Non-swarming.	+	+	-	+
Tr	***SMXD7RES22***	Pink colonies. 20-22 mm diameter. Non-translucent. Swarms to form solid plaque.	-	+	+	+

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