The effects of antibiotics in waste water treatment plant effluent on the growth and metabolome of *Lemna minor* in constructed wetlands.

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Abstract:

Constructed Wetlands (CWs) are used for further treatment of Waste Water Treatment Plant (WWTP) effluents, to remove a range of harmful contaminants from the environment including antibiotics. Determining whether CW plants are experiencing stress, with their remediation potential being undermined is of great importance for contaminant removal globally. This thesis explores the effect of antibiotics in CWs on the physiology and metabolome of *Lemna minor*, a vital plant in CW ecology. In this study *Lemna minor* were exposed to the antibiotics metronidazole, erythromycin, erythromycin ethylsuccinate, flucloxacillin sodium, penicillin v, cefalexin, clarithromycin, ciprofloxacin, amoxicillin, and trimethoprim during a 9 day exposure study at environmentally relevant and elevated concentrations. Changes in growth rate, chlorophyll a content and the full plant metabolome with antibiotic exposures were investigated. No significant changes in growth rate (24 hour data set; $F(2) = 1.475$, $P = 0.244$, 5 day data set; $F(7.438) = 0.774$, $P = 0.619$, 9 day data set; $F(4.818) = 0.582$, $P = 0.708$) and chlorophyll a content ($F(8) = 0.689$, $P = 0.691$) were found with antibiotic exposure. A Principal Components Analysis (PCA) was performed on the metabolome of *Lemna minor* to identify compounds driving the biochemical response to the antibiotic exposures. Variation in the metabolome was observed following elevated antibiotic exposures of at least 5 days, and strongly with 9 days, but not with predicted environmental concentration level exposures. Potential identifications of important plant metabolites changing in abundance with antibiotic exposures included flavonoids, a neolignan and a gingerglycolipid. Changes in the abundances of these plant metabolites indicate plant defence responses with elevated antibiotic exposures. The results of this study suggest that *Lemna minor* in CWs is likely to not be experiencing physiological or metabolomic changes with predicted environmental exposures of the antibiotics, which is positive for CW contaminant remediation. However, an extended exposure study is needed to confirm no metabolomic changes occur with environmentally relevant exposures, which may ultimately undermine plant health. With elevated antibiotic exposures, full metabolomic analysis was found to be a more sensitive measure than growth rate and chlorophyll a content, which if incorporated into plant health assessments could potentially be used as an early warning sign of plant stress. As such metabolomics may prove to be a valuable tool in plant health assessments, revealing plant stress signs prior to them manifesting physiologically, providing crucial time to identify and reduce environmental pollution before potentially irreparable damage to plants. The results of this thesis may also be applied more broadly to the field of ecotoxicology, showing that metabolomics may be a useful analytical technique in ecotoxicology for a range of plant health assessments with a variety of pollutants, including but not limited to antibiotics.

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Declaration

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I declare that this thesis is a presentation of original work and I am the sole author. This work has not previously been presented for a degree or other qualification at this University or elsewhere. All sources are acknowledged as references.

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List of Abbreviations

AR	Antibiotic resistance
ARB	Antibiotic resistant bacteria
ARG	Antibiotic resistant gene
CW	Constructed wetland
CAS	Conventional activated sludge
HPLC-HRMS	High Performance Liquid Chromatography- High Resolution Mass Spectrometry
LC-MS	Liquid Chromatography-Mass Spectrometry
PEC	Predicted Environmental Concentration
PNEC-MIC	Predicted No Effect Concentration-Minimum Inhibitory Concentration
PCA	Principal Components Analysis
qPCR	quantitative Polymerase Chain Reaction
RQ	Risk Quotient
WWTP	Wastewater Treatment Plant

Chapter 1. Introduction

1.1. Research question, objectives and overview of the study

Research question: Does exposure to antibiotics in CWs treating WWTP effluent affect the physiology and metabolome of the plant *Lemna minor*?

Objectives:

1. To select a plant to be used in the exposure study, which meets the following criteria: a robust plant, with a short growth time manageable within the time constraints of the study, found in Constructed Wetlands, native to the UK, with a high remediation potential for a range of contaminants including antibiotics (Chapter 2; section 2.1).
2. To select the antibiotics to be used in the exposure study, using a risk-based prioritization method that considers the presence of antibiotics in Constructed Wetlands and the threat of antibiotic resistance (Chapter 2; section 2.3).
3. To determine the effect of antibiotic concentrations and time exposures on the growth rate (change in frond surface area) and changes in the frond diameter of *Lemna minor* (Chapter 4).
4. To determine the effect of antibiotic concentrations and time exposures on the frond chlorophyll a content of *Lemna minor* (Chapter 4).
5. To determine the effect of antibiotic concentrations and time exposures on the metabolome of *Lemna minor* (Chapter 5).

With an increasing misuse of antibiotics globally (Ávila et al., 2021; Huang et al., 2015), there are many important questions surrounding the removal of antibiotics from and their effect on the environment that require answers. Constructed Wetlands (CWs) are of particular interest to this study as CW plants often experience high antibiotic exposure due to the inputs of Waste Water Treatment Plant (WWTP) effluent (Gwenzi et al., 2020) and as they are vital in removing antibiotics and other pollutants from the environment (Abou-Kandil et al., 2021).

1.2. Bioremediation in Constructed Wetlands

1.2.1. Constructed Wetlands; Brief background and mechanisms

CWs are a well-established and promising example of using phytoremediation to reduce the contamination of surface waters for a range of pollutants, with high removal efficiencies (Abou-Kandil et al., 2021; Matamoros and Bayona, 2006). CWs are manmade and utilise natural processes to treat polluted waters (Helt et al., 2012). They use both biotic processes such as microbial degradation and plant uptake and abiotic processes such as photodegradation, oxidation and

hydrolysis, taking place in the vegetation, soil, and microbial assemblages (Almuktar et al., 2018; Helt et al., 2012; Hijosa-Valsero et al., 2016). CWs are broadly classified based on hydrology (surface flow vs subsurface flow), macrophyte types (free-floating, emergent, and submerged), and flow path (horizontal or vertical), which all affect performance efficiency and can be tailored to best suit the requirements of a certain location (Almuktar et al., 2018; Carvalho et al., 2014; García et al., 2020). Surface flow CWs are usually larger scale than subsurface flow and are more frequently used as a tertiary treatment following WWTP treatments, whereas subsurface flow systems may be included in WWTP facilities (García et al., 2020).

CWs offer a sustainable alternative to further treat polluted waters globally as they are cost effective with low maintenance and low energy requirements. Successful implementation can be seen around the world in a variety of different environments and situations (Abou-Kandil et al., 2021; Almuktar et al., 2018; Carvalho et al., 2014; Huang et al., 2015; Vymazal, 2009; Zhang et al., 2020). Great success can be seen, especially in tropical and sub-tropical regions (Varma et al., 2021). However, the use of CWs can be limited by the availability of land; CWs require 2 to 8 m^2/PE (person equivalent) compared to 0.06 m^2/PE for conventional treatment (White et al., 2006). This may result in CW treatment being more applicable for smaller populations away from large urban areas, where more space is available, however, this limitation can be overcome with enough willpower and resource availability; Orlando Florida pipes treated wastewater 20 km to a CW in a low population area (White et al, 2006). CWs can often serve many different purposes, functioning not just to treat water, but also as a form of flood control, carbon sequestration, and habitat creation (Almuktar et al., 2018; Carvalho et al., 2014; Vymazal 2013; Vymazal 2014).

1.2.2. Phytoremediation; Brief background and mechanisms

A large component of treating contaminated water in CWs revolves around phytoremediation; the use of plants (macrophytes) to remove contaminants (Almuktar et al., 2018; Carvalho et al., 2014). Phytoremediation is not limited to water, it can also be used in soils and even the atmosphere (Susarla et al., 2002). Macrophytes in wetland systems directly impact the water quality and are central in the removal processes of nutrients such as nitrogen and phosphorus (Shelef et al., 2013). Macrophytes are important in regulating the environmental conditions of a CW, and so enable the processes of other organisms such as microbes to function and remove contaminants as well by processes such as microbial degradation (Varma et al., 2021). Macrophytes provide a surface for biofilms (composed of organic matter, algae, and microorganisms) to form, which remove nitrogen through nitrification and denitrification (Hou et al., 2017; Li et al., 2014; Qin et al., 2019). Macrophytes also remove the contaminants from the wastewater directly by several pathways including evapotranspiration processes resulting in release to the atmosphere but also storage in the

plant, accumulating the contaminant in tissues which can then be removed from the environment by sequestration or incineration (Gerhardt et al., 2009; Vymazal, 2002). An example of this can be seen with the storage of heavy metals in vacuolar or granular compartments (Ha et al., 2011; Shelef et al., 2013), along with nutrients (Vymazal, 2007) and potentially antibiotics (Liu et al., 2013). However, plant uptake of contaminants varies with different environmental conditions such as climate but also the type of wastewater and its influx rate (Saeed and Sun, 2012; Wu et al., 2016).

A range of plant species are used around the world in CWs, their selection based on their effectiveness for the contaminants they are required to remediate and the environmental conditions they need to withstand (Carvalho et al., 2014). The selection of plants for a CW is of great importance and great attention is paid to selecting species tolerant to the specific environment they are being introduced to (Almuktar et al., 2018). The macrophytes must be tolerant to waterlogged, anoxic and hyper eutrophic conditions as these often occur from effluent entry and must have a high capability to take up certain contaminants and be able to withstand all those present (Almuktar et al., 2018; Wu et al., 2016). Environmental conditions vary with specific wetlands; plants may not only have to tolerate potentially toxic contaminant concentrations but also salinity, pH and O_2 concentrations that may not be optimal for some species growth and function (Almuktar et al., 2018). If such requirements are not met, the treatment efficiency of the CW may not be sufficient to effectively treat the effluent (Almuktar et al., 2018; Wu et al., 2016). When selecting macrophyte species, consideration of their potential to adapt to climate change is of increasing importance (Wu et al., 2016).

It is possible to broadly categorise CW plants into three groups: emergent, submerged and free floating, with plants in each group having unique problems and positives for their use in CWs (Almuktar et al., 2018; Carvalho et al., 2014; García et al., 2020; Saeed and Sun, 2012; Wu et al., 2016). Emergent plants are visible above the water's surface and are vital in stabilising CW substrate (Almuktar et al., 2018). Submerged also grow and stabilise substrate but remain underwater (Wu et al., 2016), whereas free floating contrast both as they are not fixed to soil substrate, instead floating on the surface of a water body (Almuktar et al., 2018). *Phragmites australis* is the most commonly used plant in CWs globally (Almuktar et al., 2018) and *Cyperus papyrus* is commonly used in Africa (Almuktar et al., 2018). Many of the key plant species used in CWs and their class can be seen listed in Table 1 below, with more comprehensive lists visible in review papers such as Almuktar et al. (2018), Saeed and Sun (2012) and Wu et al. (2016). There is significant research showing the effectiveness of macrophytes removing contaminants from water, for example *Eleocharis acicularis* has been found to accumulate heavy metals including lead, copper, cadmium and zinc, the latter it

stored in its root tissue up to 213 mg/kg dry weight (Ha et al., 2011). Removal efficiencies and uptake rates of antibiotics for several key plant species used in CWs can be seen below in Table 1.

The many complex interconnected mechanisms involved in antibiotic removal by macrophytes, associated plant-microbial interactions and other factors such as soil adsorption is still up for debate, as well as how varying environmental conditions such as climate and the presence of different pharmaceuticals alter removal effectiveness (Carvalho et al., 2014; Shelef et al., 2013). Effectiveness has been found to vary with the design of the CW altering the main removal mechanisms of antibiotics which include substrate adsorption, and plant uptake (Chen et al., 2019). For example, Chen et al. (2019) and Du et al. (2020) found that increasing artificial aeration influenced antibiotic CW removal efficiencies. Removal rates for antibiotics have found to be greater in subsurface flow CWs than surface flow, potentially due to increased contact with soils increasing substrate adsorption (Chen et al., 2016). Artificial aeration improves nitrogen and ammonium removal efficiencies due to increases in oxygen water content and mixing increasing microbial degradation, substrate adsorption and plant uptake (Chen et al., 2019). While there are indications in data that such mechanisms increased with artificial effluent that are responsible for reducing nutrient loads are likely to be involved in reducing antibiotics confirming this has not yet occurred (Chen et al., 2019).

Table 1: Commonly used plant species in CWs with their classification. Antibiotic removal efficiencies for highly studied plants can also be seen.

Classification	Species name	Antibiotic removal efficiencies
Emergent	*Phragmites australis*	Huang et al. (2019) used microcosms of subsurface flow CWs (1.5m length, 40 cm width) with a density of 50 per m^2 and influent containing 25.7–67.4 ng/L of enrofloxacin and 64.0–211.2 ng/L of sulfamethoxazole pumped in continuously. From September 2014-September 2016 removal efficiencies were found of 81.11% for enrofloxacin, 64.94% for sulfamethoxazole, and 56.26% for ARGs. Removal efficiencies were found using influent and effluent concentrations. Carvalho et al. (2012) used exposures of 100 µg/L of enrofloxacin and tetracycline over 2 days and fresh biomass of 44 +/- 6g. Removal efficiencies of 94% for enrofloxacin and 75% for tetracycline were found.
Emergent	*Iris pseudacorus*	Huang et al. (2019) used microcosms of subsurface flow CWs (1.5m length, 40 cm width) with a density of 50 per m^2 and influent of 25.7–67.4 ng/L enrofloxacin and 64.0–211.2 ng/L sulfamethoxazole pumped in continuously. From September 2014-September 2016 removal efficiencies were found of 77.64% for enrofloxacin, 68.7% for sulfamethoxazole, and 58.21% for ARGs. Removal efficiencies were found using influent and effluent concentrations.
Emergent	*Typha latifolia*	
Emergent	*Scirpus lacusris*	
Emergent	*Cyperus papyrus*	

Classification	Species name	Antibiotic removal efficiencies
Emergent	*Schoenoplectus tabernaemontani*	
Emergent	*Eleocharis macrostachya / palustris / rostellata / acuta*	
Emergent	*Acorus calamus*	
Emergent	*Carex rostrate Stokes*	
Emergent	*Juncus effusus*	
Submerged	*Vallisneria natans*	
Submerged	*Myriophyllum spicatum*	
Submerged	*Hydrilla verticillata*	
Submerged	*Potamogeton crispus*	
Submerged/ Floating	*Ceratophyllum demersum*	Thuy Hoang et al. (2012) found removal efficiencies of 40% for norfloxacin and 39-44% for ciprofloxacin with 10mg/L exposures over 10 days and 4-5 plants used.

Classification	Species name	Antibiotic removal efficiencies
Free Floating	*Lemna minor*	Iatrou et al. (2017) found removal efficiencies of 100% for cefadroxil, 96% for metronidazole, 73% for sulfamethoxazole and 59% for trimethoprim. Each antibiotic had 250 µg/L concentrations and were exposed to a starting mass of 2g of *Lemna minor* for 24 days.
Free Floating	*Spirodela polyrhiza*	
Free Floating	*Salvinia natans*	
Free Floating	*Hydrocharis dubia*	
Free Floating	*Nymphaea odorata Aiton*	
Free Floating	*Nuphar lutea*	
Free Floating	*Nymphoides peltata*	
Free Floating	*Trapa bispinosa Roxb*	
Free Floating	*Marsilea quadrifolia*	

1.3. Brief overview of the study and the following chapters

This study investigated *Lemna minor* being used in antibiotic remediation in CWs treating WWTP effluent, with regards to the effects of antibiotic exposures on its growth and metabolome. This study was conducted under lab-controlled conditions, with the exposures occurring over a 9-day period. The following chapter explores the methods that were used in this study, with in depth justifications behind the methodology and its development.

Chapter 2. Method development

2.1. Selecting the study plant *Lemna minor*

To allow a study to be conducted on the effects of antibiotic exposure on plants in CWs, firstly a study plant needed to be selected (objective 1). A range of criteria needed to be met when selecting the plant to make this study useful for researchers looking at plant chemistry in CWs globally. As such it was desirable that the plant was: used in CWs frequently and applicable to CWs world-wide and so important in CW ecology, have a high remediation potential for a range of contaminants including antibiotics if possible, have a relatively short growth time of days/weeks not months (due to time constraints), and robust for growth in a lab-based setting (objective 1). It was also ideal if the plant had a strong background in scientific literature to give a solid base for informing lab work and to make the study and its findings more reliable. To determine which plant to use in this study a literature review was conducted which can be seen in Table 1, where plant possibilities were investigated and narrowed down to several potential species due to reasons such as time constraints of the study and the growth rates of certain species.

Emergent plants were quickly removed from consideration for this study due to several issues that would likely be encountered in lab work such as their relatively larger size and slower growth rates in comparison to other classes, especially free floating. For example, Mauchamp et al. (2001) found that *Phragmites australis* (emergent) shoots grow slowly up until the age of 1.5 months, whereas Adesina et al. (2005) found that *Lemna minor* (free- floating) has a doubling time of roughly 48 hours. While the use of plugs could have helped counter this issue, the slow growth rate paired with the time the study needed to be completed would have made the study less able to adapt to any issues in lab work that arose. While submerged plants can be found in CWs, high nitrates in WWTP effluent results in eutrophication, reduced sunlight and reduced oxygen and aerated water, presenting many issues for submerged plants (Wu et al., 2016, Saeed and Sun 2012). Because of such issues submerged plants are often used further away from the effluent input (Saeed and Sun 2012), and while still vital in CWs, for this study a plant that is more widely applicable across much of a CW is favoured. As shown in Table 1 the most applicable plant options were put forward and, as a free-floating species were preferred, *Lemna minor* was then selected from this classification due to a large range of reasons and positive attributes and applicability outlined below.

Due to its rapid reproduction that results in large biomass production (especially with high nutrient concentrations), there is currently research in developing *Lemna minor* as a low input biomass animal feed, a bioethanol fuel, and in the generation of electricity (Anderson et al., 2011; Appenroth et al., 2013; Cui and Cheng, 2015; Lemon et al., 2001; Sree et al., 2015; Stout et al., 2010; Ziegler et

al., 2015; Ziegler et al., 2016; Ziegler et al., 2017). Other *Lemna* species have been found to outperform conventional land crop plants in biomass production and a similar performance can be expected from *Lemna minor* (Ziegler et al., 2016). While *Lemna minor* has great potential to help solve numerous problems, issues surrounding its implementation persist, especially with regards to financial costs. However, *Lemna minor* does have a long history of use in aquaculture as fish feed and livestock feed, though further development is required to optimise this and make it useable on larger scales (Ekperusi et al. 2019; Khan et al., 2014; Iqbal, 1999).

Lemna minor has been used to remediate contaminants and improve water quality has been utilised in both natural and constructed wetlands globally and has been used either individually or in combination with other plant species (Ekperusi et al. 2019). *Lemna minor* was found to successfully remove iron from coalmine effluents forty years after operation (Teixeira et al. (2014) and further study findings for contaminant removal rates are covered below. Using *Lemna minor* to remediate contaminants and then the biomass being extracted and used as biofuel or fodder could help reduce many of the financial barriers preventing its wider use in remediation, fuel, and food (Ekperusi et al. 2019). Unfortunately, the biomass once used to remove contaminants can often be unusable; while solving this may be possible to allow its use as a biofuel, it seems highly unlikely it could then be used as fodder (Zeigler et al., 2016). Producing a useable crop while also remediating wastewater without appropriating productive land for terrestrial crops would be of great value to a world facing climate change and food insecurity. While development is underway, this is not yet feasible.

Lemna minor was determined to be the most appropriate for this study due to its small size, simple structure, and rapid duplication rates resulting in it being highly useful in laboratory-based settings. For such reasons *Lemna minor* is often used as a model organism in laboratory toxicity tests (OECD, 2006). *Lemna minor* is a free-floating aquatic plant found around the world, from temperate to tropical regions; it is native to Africa, Asia, Europe, and North America and invasively in South America and Australia (Ali et al., 2020; Ekperusi et al., 2019; Ziegler 2017). Its wide distribution has likely been aided by *Lemna minor*'s ability to stick to migratory water birds (Ekperusi et al., 2019; Mbagwu and Adeniji, 1988) and as such its study is applicable to many regions (OECD, 2006), including those where CWs may be a viable water treatment option. Identifying the taxonomy of *Lemna minor* was a complex issue for hundreds of years due to its few morphological characteristics, global distribution, and high adaptability (Ekperusi et al., 2019). It resides within the *Lemnoideae* subfamily, which contains 5 genera: *Wolffia*, *Wolffiella*, *Spirodela*, *Landoltia* and *Lemna*, however, its position is still disputed among taxonomists (Ali et al., 2020; Ekperusi et al., 2019). Many species exist within the *Lemna* genus, but the most studied are those of *Lemna gibba* and *Lemna minor* (Ali

et al., 2020; Balarak and Chandrika, 2019; Philippot et al., 2013; Radulović et al., 2020; Ziegler et al., 2015).

Lemna minor are composed of several (1-4) attached fronds 2-4mm in diameter, with simple hairless roots (Ali et al., 2020; Ekperusi et al., 2019; Mkandawire et al., 2014; Ziegler 2017). Fronds consist of spongy mesophyll and large air spaces giving the *Lemna minor* its buoyancy (Ziegler et al., 2016). While the majority of the *Lemna minor* only has contact with a water body, its surface can exchange carbon dioxide and oxygen directly with the atmosphere (Mkandawire et al., 2014). *Lemna minor* reproduces via asexual reproduction, with fronds duplicating and the newer paler daughter fronds budding off to form new *Lemna minor* (OECD, 2006). The doubling time for *Lemna minor* is roughly 48 hours (Adesina et al., 2005) and under lab conditions can grow indefinitely, however, a frond usually dies after six generations (Ziegler et al., 2015). As such *Lemna minor* has been named a "Darwinian Demon" due to its ability to seemingly "live forever" (Ekperusi et al., 2019). Daughter fronds often remain near parent fronds, forming large colonies (Ekperusi et al., 2019; Ziegler et al., 2016). Such colonies usually occur over still water in freshwater ponds with high nutrient levels but can also be found in brackish water and slow-moving streams (Ali et al., 2020; Balarak and Chandrika, 2019; Ekperusi et al., 2019; Ziegler et al., 2016). The colonies can form mats that reduce sunlight to submerged aquatic plants and can result in dramatic changes to the surrounding environment (Ekperusi et al., 2019) by increasing denitrification rates due to the inhibition of oxygen production by submerged macrophyte photosynthesis (Veraart et al., 2011).

Lemna minor is often used in CWs to remediate contaminants from WWTP effluent (Almuktar et al., 2018, Saeed and Sun 2012, Wu et al., 2016) due to its ability to survive under such harsh conditions and rapidly adapt to changes in climate (Ali et al., 2020; Grenni et al., 2019). *Lemna minor* is very effective in the remediation of contaminants ranging from heavy metals and organics to pharmaceuticals, antibiotics, and even radioactive waste (Ekperusi et al., 2019; Zeigler et al., 2015; Ziegler et al., 2016). It has been found to have a >90% removal efficiency for chromium, zinc, aluminium, arsenic, cadmium, cobalt, copper, lead and nickel (Ekperusi et al., 2019). Iatrou et al. (2017) found high antibiotic removal efficiencies with antibiotic exposures of 250 µg/L and a *Lemna minor* starting mass of 2g. Removal efficiencies of 100% for cefadroxil, 96% for metronidazole, 73% for sulfamethoxazole and 59% for trimethoprim were found. Cascone et al. (2004) investigated flumequine removal rates by *Lemna minor*, with concentrations as high as 1000 ug/L over 5 weeks, with a starting mass of 5g of *Lemna minor*. They found removal rates as high as 96% with *Lemna minor* compared to 80% in controls without *Lemna minor*. Zhou et al. (2018) found large declines in the presence of pharmaceuticals with *Lemna minor.* The relative concentrations of atenolol and ibuprofen (at concentrations of 10 µg/L over 15 days, with a *Lemna minor* starting mass of 3-5

fronds) saw declines between controls and those with *Lemna minor*, of 97.7% to 52.1% and 80.9% to 65.1% for atenolol and ibuprofen, respectively. Though Zhou et al. (2018) does state not all the removal is due directly to the *Lemna minor* but also other mechanisms that are supported by the presence of *Lemna minor* such as microbial degradation. It is important to note that many studies that find successful remediation of a range of contaminants using *Lemna minor* often take place in a laboratory microcosm, with highly regulated conditions (Ekperusi et al. 2019). The remediation performance of *Lemna minor* may change in the field and as such more studies using mesocosm experiments and investigations in CWs are needed to fully determine the overall performance of *Lemna minor*. *Lemna minor* can achieve high growth rates in highly contaminated waters (Grenni et al., 2019) and recover from harsh exposures within days due to its many effective anti-stress responses including an increased production in enzymes and anti-shock proteins that protect its tissues and organs (Drost et al., 2007; Ekperusi et al., 2019). Alongside reducing the concentrations of contaminants, it can also improve water quality further by reducing algal and fungal growth with its presence (Ali et al., 2020). However, *Lemna minor* can experience death with high concentrations of contaminants; even at 1mg/L of erythromycin, its growth can be inhibited by 20% as the antibiotic effects its cell division and photosynthesis (Gomes et al., 2020; Pomati et al., 2004). As it is important and frequently used in CW remediation, it is useful as a study organism to investigate the effects of antibiotic exposure on the physiology of plants remediating WWTP effluent contaminants.

2.2. *Lemna minor* sampling and culturing

2.2.1. Source and sampling

The *Lemna minor* used in this study was sourced from a pond in memorial gardens in York city centre; its location can be seen below in Figure 1 and an image of the pond in Figure 2. Sampling was conducted in August 2021 during a week of high temperatures (approximately 25-35°C) with little cloud cover. *Lemna minor* was present as a mat in the pond and was extracted by skimming across the pond surface with plastic containers. *Lemna minor* was then separated from the surrounding plants and acclimatized and sterilised (covered in section 2.2.2). Leeches were present in the *Lemna minor* samples, approximately over 100 in volumes of 1 litre. This along with the visual signs of pollution in the pond such as plastic rubbish including crisp packets and bottles may indicate the pond has poor water quality. The pond is paved in concrete and located in a small, enclosed grass area approximately 20 metres from a traffic junction and appeared to be stagnant with little water movement. A gaggle of geese were observed around the pond area, potentially a source of nutrient inputs via faeces into the pond.

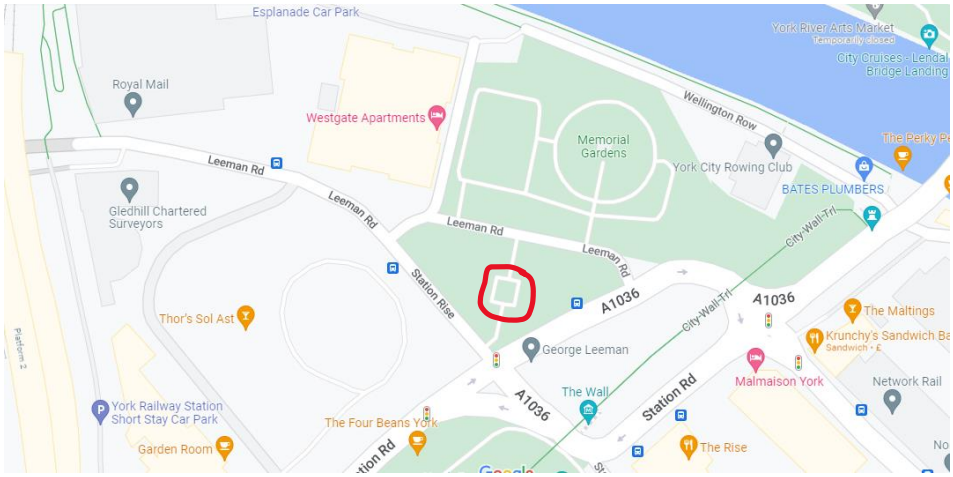


Figure 1. Location of the pond (York memorial gardens) used to source *Lemna minor* for the study. The pond is circled in red.

Figure 2. Images of the pond used to source *Lemna minor*, in memorial gardens, York. Taken early January 2023, left; the full pond and right; *Lemna minor* frozen within the pond.

2.2.2. Sterilization and acclimatization of stock culture

Following the collection of *Lemna minor (*see section 2.2.1) a sterilisation and acclimatization method was derived from many toxicity studies that used *Lemna minor* including Baciak et al. (2016), Cascone et al. (2004), Ebert et al. (2011), Ekperusi et al. (2019), Gomes et al. (2017), Gomes et al. (2020), González-Renteria et al. (2020), Grenni et al. (2019), Iatrou et al. (2017), Lima et al.

(2021), Mkandawire et al. (2014), OECD (2006), Radulović et al. (2020), Sree et al. (2015) and Stout et al. (2010). A 0.5% sodium hypochlorite sterilisation solution followed by rinsing with sterile deionised water was recommended by the OECD (2006) and a similar method can be seen in Radulović et al. (2020) and Grenni et al. (2019). This study used a 0.25% sodium hypochlorite solution due to higher concentrations resulting in total frond death, while this solution had a frond survival rate of approximately 75%. This sterilisation process was repeated every 7 days, with healthy fronds transferred to new sterile flasks and growth media (see section 2.2.4). Healthy fronds were identified by their rich green colour (see section 2.2.5, Figure 3) and the presence of at least 2 fronds per *Lemna minor*, with single fronds potentially showing stress (OECD, 2006). *Lemna minor* stock cultures were grown in a Versatile Environmental Test Chamber (model: MLR-351H, Sanyo, Osaka, Japan) with an effective capacity of 294 L and with 800 W cool white fluorescent tubes (Sanyo, Osaka, Japan) mounted behind clear glass barriers. The same chamber was also used to conduct the exposure study. Over several weeks high growth rates of sterile *Lemna minor* cultures were produced, which was then followed by a 4-week acclimatization period. This period was selected based on the methods of other studies that ranged from 25 days (Gomes et al., 2017; Gomes et al., 2020), 4 weeks (Iatrou et al., 2017) to 8 weeks (OECD, 2006; Grenni et al., 2019). As recommended by the OECD (2006), *Lemna minor* was grown in media with minimum depths of 20mm and containers that allow for a large surface area to prevent fronds overlapping, while also being sterile and having minimal shadows affecting light and growth. This was achieved by growing *Lemna minor* hydroponically in sterile, stoppered Erlenmeyer flasks of 1 litre containing 300ml of sterile media. Similar methods can be seen in studies such as Gomes et al., (2017) and Gomes et al., (2020). The culturing process was kept sterile as recommended by the OECD (2006) toxicity study guidelines, to reduce the impact of microorganisms on the study. During the acclimatization period the *Lemna minor* was transferred to fresh media and flasks, approximately every 7 days as used in studies such as Ebert et al. (2011), Iatrou et al. (2017) and Baciak et al. (2016). This was conducted to ensure the optimum productivity and health of the *Lemna minor* as the biomass can double every 48 hours (Adesina et al., 2005) and as such overwhelm their containers. Replenishing the culturing medium is also of importance to prevent the build up of harmful products or depletion of nutrients impacting the *Lemna minor* growth. This was investigated in Cascone et al., (2004) and Sree et al., (2015).

2.2.3. Environmental conditions

The environmental conditions used in the cultivation of *Lemna minor* and the exposure study can be seen below in Table 2, which were selected based on the conditions used in several other studies. By reviewing the methodology of several studies that used *Lemna minor* in toxicity investigations, a

table was generated containing the key information from such studies regarding the environmental conditions and growth media used to culture and study *Lemna minor*, the frond numbers studied during experimental periods and the variables analysed to assess toxicity effects. These details can be seen in the appendix in Table 15. *Lemna minor* can survive a large range of environmental conditions for example, pH ranges of 5.6-10.44 and temperature ranges of 6-33°C (Ekperusi et al., 2019). However, optimum conditions were selected for this study to produce sufficient *Lemna minor* biomass to allow for an exposure study.

Table 2. Environmental conditions used for cultivation of *Lemna minor* and the exposure study.

Environmental Variable	Environmental condition
pH of media	6.9
Temperature	23°C
Relative Humidity	70-75%
Photoperiod	15hrs light / 9 hrs dark

2.2.4. Culturing medium

Swedish Standard Sterile media was used as a growth medium to culture *Lemna minor*, as recommended by the OECD (2006) and used by Iatrou et al. (2017). Similar media were used by other studies and can be seen in Table 15 in the appendix. Firstly, a series of stock solutions outlined in Table 3 were created and then used to make the final media, as shown in Table 4 and sterilised using a Monarch 50 Autoclave (by The Rodwell Autoclave Company, Chester, UK) as required. The pH of the media was measured using a Accument AB150 benchtop pH meter (Fisher Scientific) under sterile conditions and adjusted using 1ml of hydrochloric acid to give a media with a pH of 6.9, within the optimum growth range.

Table 3. The stock solutions used to create the Swedish Standard Sterile media. For stocks A-E compounds were added to deionised water and autoclaved to sterilise. For stock F compounds were added to sterilised deionised water, mixed and a sterile membrane filtration system used to extract the stock mixture.

Stock Solution	Compounds	Concentration (mg/L)
A	$NaNO_3$ (Sodium nitrate)	8500
A	KH_2PO_4 (Potassium dihydrogen phosphate	1340
B	$MgSO_4.7H_2O$ (Magnesium sulfate heptahydrate)	15000
C	$CaCl_2.2H_2O$ (Calcium Chloride Dihydrate)	7200

Stock Solution	Compounds	Concentration (mg/L)
D	Na_2CO_3 (Sodium carbonate)	4000
E	H_3BO_3 (Boric Acid)	1000
E	$MnCl_2.4H_2O$ (Manganese (II) chloride tetrahydrate)	200
E	$Na_2MoO_4.2H_2O$ (Sodium molybdate dihydrate	10
E	$ZnSO_4.7H_2O$ (Zinc sulfate heptahydrate)	50
E	$CuSO_4.5H_2O$ (Copper (II) sulfate pentahydrate	5
E	$Co(NO_3)_2.6H_2O$ (Cobalt(II) nitrate hexahydrate)	10
F	$FeCl_3.6H_2O$ (Iron (III) chloride hexahydrate)	170
F	Na_2-EDTA.$2H_2O$ (Ethylenediaminetetraacetic acid disodium salt dihydrate)	280

Table 4. The volume of each stock solution (ml) added to 1,969ml of sterilised deionised water to create 1L of Swedish Standard Sterile media.

Stock Solution	Volume (ml)
A	10
B	5
C	5
D	5
E	1
F	5

2.2.5. Confirmation of Lemna minor growth in media

To confirm the growth of *Lemna minor* with the cultivation conditions and Swedish Standard Sterile media, changes in the surface area of the *Lemna minor* were investigated using the computer programme Image J (153-win-java8, Rasband, W.S., Maryland, USA). Images of the *Lemna minor* were taken and using the programme converted to 9 bit, binary and set to scale. *Lemna minor* fronds were selected using the 'analyse particles total area' function, changes in the total area covered by *Lemna minor* and average frond diameter over time could be determined. Paint programmes were also used to remove algal interference when required and computer image enhancing employed to improve the quality of the results. An example of the formatting of images using Image J and paint programmes can be seen below in Figure 3. The results showing an increase in surface area with time can be seen below in section 2.4.6, Table 12; the same data was used to

determine frond addition per test vessel for the exposure study. This method was also used to measure the percentage change in surface area of *Lemna minor* with antibiotic exposures during the exposure study, the method of which can be seen in section 3.1.4 and the results in Chapter 4.

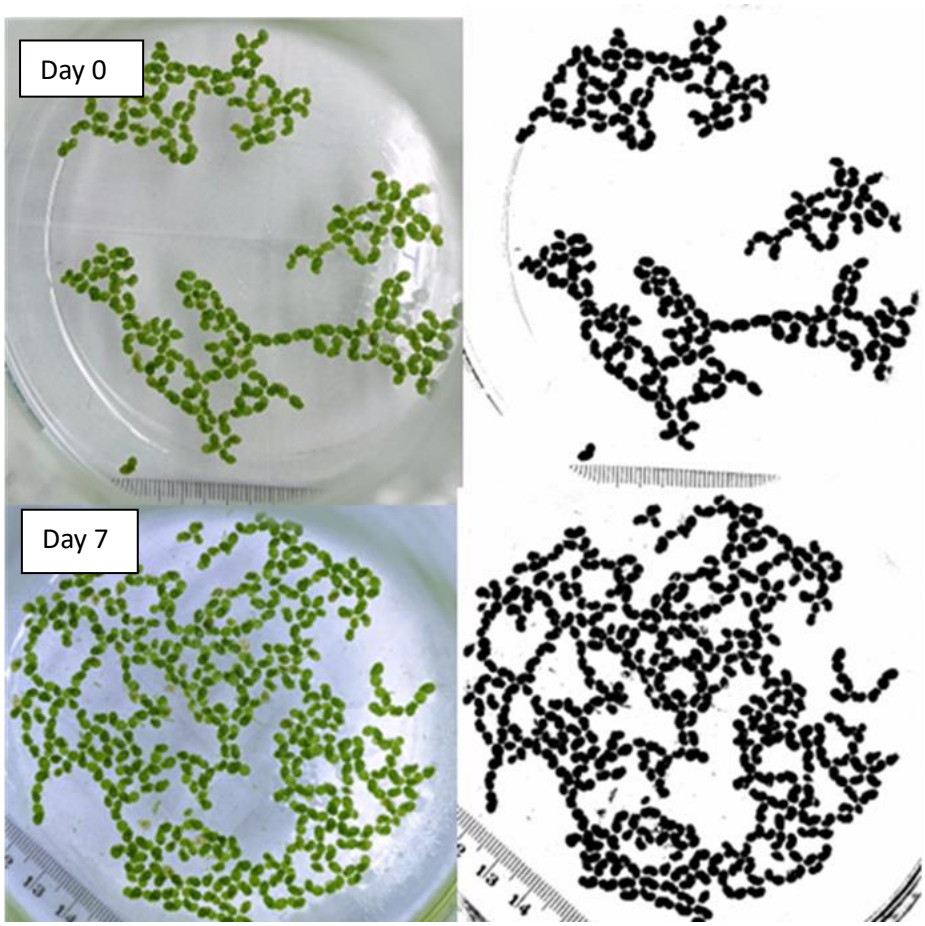


Figure 3. *Lemna minor* growth analysed with Image J over a 7-day period, original (left) and processed (right).

2.3. Selecting the antibiotics used in the exposure study

Antibiotic resistance (AR) originating from a global abuse of antibiotics has led to a rise in antibiotic resistant bacteria (ARB) and antibiotic resistant genes (ARGs) in the environment, resulting in a growing threat to human and ecological health (Ávila et al., 2021; Huang et al., 2015). Even with the global threat of AR to human and ecosystem health, the movement of AR in the environment and relation to human exposure is still not fully understood (Abou-Kandil et al., 2021). As such there are many studies that could be conducted in this area of research. This thesis provides a number of antibiotics and antibiotic exposures selected by a risk based prioritisation method using both predicted environmental concentrations in CWs while also considering the selection of antibiotics for ARB (objective 2). By incorporating ARB pressure into the selection of the antibiotics, the

methodology outlined below and the antibiotics and their exposures determined in this study could be of potential use for studies investigating AR in CWs.

2.3.1. Overview of data collection and calculations

Data was gathered from multiple sources and used in calculations to provide an unbiased method to select the antibiotics used in this study, while also prioritising antibiotics that select for AR (objective 2). Tables containing all data gathered and their references can be seen in the appendix, Tables 18 and 19. Predicted Environmental Concentrations (PECs) for all antibiotics entering CWs via WWTP effluent were calculated and used with Predicted No Effect Concentration-Minimum Inhibitory Concentrations (PNEC-MIC) to create a risk quotient value for all antibiotics prescribed in England. This method allowed for both their threat for AR and their presence in CWs to be central in antibiotic selection. Antibiotics with a risk quotient above 1 were selected for the study and as such this method reduced the potential list from > 80 antibiotics to 10, a number feasible considering the cost and time constraints of the study.

2.3.2. Data collection

2.3.2.1. Total antibiotic mass prescribed in England

The total antibiotic masses prescribed in England were sourced from a 2019 NHS data base and then amended by Alice Marshall to create a concise data set for environmental studies. The original data set can be seen at NHSBSA Statistics and Data Science (2019) and includes the mass of the active ingredients for all antibiotics prescribed in England, as well as the number of prescriptions, and the number and form (tablet or solution) in each prescription. Using this data the total mass of each compound prescribed could then be determined, which can be seen in the Appendix, Table 17. The prescribed mass for all antibiotics prescribed in England (assuming all antibiotics prescribed were administered) could then be used for further calculations covered in section 2.3.3 to find the PECs for all antibiotics.

2.3.2.2. Antibiotic families

Due to a lack of available data on human excretion rates, WWTP removal rates and PNEC-MICs for many antibiotics, all the antibiotics prescribed in England were sorted into classes to allow for averages to be calculated using the most studied antibiotics values for each class based on similar physicochemical properties of the compounds within a class and its mode of action. The behaviour of antibiotics within families should be similar enough to allow for averages to be used for families as antibiotics are grouped into their families based on their behaviour and mechanisms of action against bacteria (Kapoor et al., 2017). A table of the antibiotics prescribed, and their families can be seen in the appendix in Table 16, with references.

2.3.2.3. Human excretion rates of antibiotics, antibiotic removal rates by WWTPs and PNEC-MICs

For all prescribed antibiotics their human excretion rates, removal rates in WWTPs and PNEC-MICs were found; the values and references can be seen in the appendix, Tables 16 and 17. Conventional treatments using conventional activated sludge (CAS) systems removal rates were used to calculate WWTP removal rates for each antibiotic. This is because CAS systems are commonly used as a key treatment in WWTPs (Modin et al. 2016). Averages were generated, when possible, using values for other antibiotics within their family. However, when data was not sufficient to calculate averages, a 100% excretion rate and a 0% WWTP removal rate was used, to avoid potentially underestimating an antibiotics presence in CWs. Due to the complex nature of establishing PNEC-MICs, if there was no data for an antibiotic family, this was left blank, and the antibiotic removed from further analysis, to avoid influencing the data with bias.

2.3.3. Calculations

Following data collection, a series of calculations were conducted for every antibiotic to find the worst case scenario, PEC and risk quotient values, the results of which can be seen in the appendix, Table 17.

To find the total water volume released by WWTPs per total population per year in England (L/yr), the volume of wastewater per person per day (L) was multiplied by the days in a year and multiplied by the population of England. To calculate this variable data was collected for the water volume released from WWTPs per person per day (Department for Environment, Food & Rural Affairs (Defra), 2020) and the population of England in 2019 (Office for National Statistics, 2020). The equation used to find the total water volume released by WWTPs per total population per year in England can be seen below. (Equation (1)):

$$Total\ volume = Volume\ x\ 365\ x\ Population \quad (1)$$

To find the Worst Case Scenario for each antibiotics exposures in CWs (µg/L), the total mass of antibiotic prescribed in England in 2019 (µg/yr) was divided by the total volume of water released from WWTP per total population per year (L/yr). The Worst Case Scenario can be seen in the following equation. (Equation (2)):

$$Worst\ Case\ Scenario = \frac{Mass}{Total\ volume} \quad (2)$$

The PEC (µg/L) for each antibiotic in CWs were calculated by multiplying the total mass of antibiotic prescribed in England in 2019 (µg /yr) with the percentage excreted (decimal form) and the percentage not removed in WWTPs (decimal form). This was then divided by the total volume of water released from WWTPs per total population per year (L/yr). The equation used to find PECs can be seen below. (Equation (3)):

$$PEC = \frac{(Mass\ x\ Excretion\ x\ Not\ removed\ in\ WWTPs)}{Total\ volume} \quad (3)$$

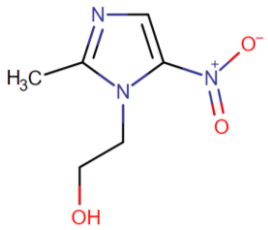
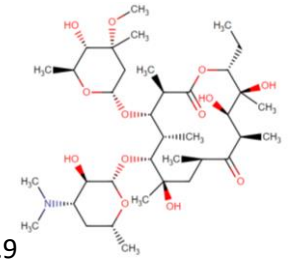
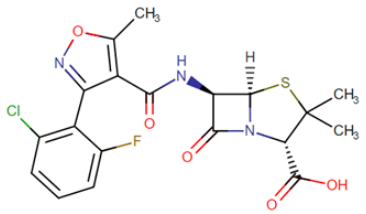
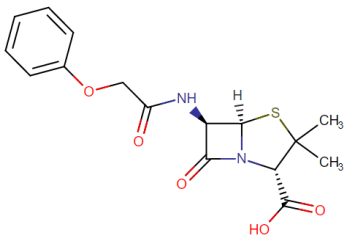
To find the Risk quotients (RQs) for all antibiotics prescribed in England in 2019, the PEC (µg/L) for each antibiotic was divided by the PNEC-MIC (µg/L) for the antibiotic. This can be seen in the following equation. (Equation (4)):

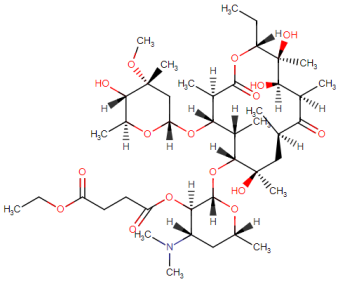
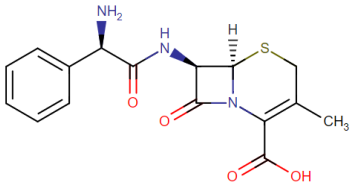
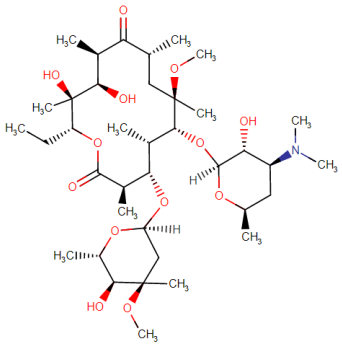
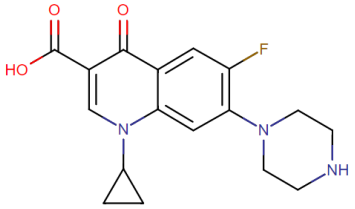
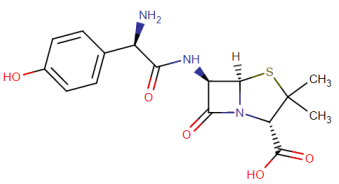
$$RQ = \frac{PEC}{PNEC-MIC} \quad (4)$$

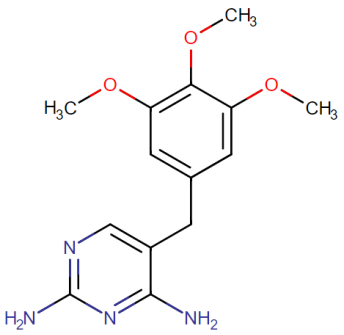
2.3.4. Final selection of antibiotics

Following the calculations of risk quotients, antibiotics with a value above 1 were selected to be used in the study. Of the 13 suggested, only 10 could be used in the study due to issues of supply and costs which resulted in rifaximin, methenamine hippurate and lymecycline not being used in the study. The antibiotics used in this study can be seen below in Table 5, with concentrations for different scenarios and chemical data. The persistence, movement and toxicity of the antibiotics used in this study vary along with the frequency of their use in toxicity studies. Of all the antibiotics used in this study metronidazole likely persists and accumulates the most in the environment (Tiwari et al., 2019). It adsorbs weakly to soils and as such is found in high concentrations in leachates entering ground and surface waters (Rabølle and Spliid, 2000). Its low degradability and high solubility in water makes its removal from water difficult and sometimes even impossible (Nasseh et al., 2016). Fluoroquinolones such as ciprofloxacin and macrolides such as erythromycin, also have high stability and do not easily degrade, persisting in the environment from months to years and as such accumulating (Hamscher et al., 2009; Rosendahl et al., 2012; Topp et al., 2016). However, not all the antibiotics used in this study behave as such. Penicillins (amoxicillin, penicillin v, and floxacillin sodium) and the cephalosporin cefalexin, hydrolytically and biologically degrade within hours to days in soil (depending on soil moisture) (Braschia et al., 2013) and often break down quickly in surface waters (Polianciuc et al., 2020). They have a reduced tendency to adsorb to soils and wash into ground and surfaces waters and as such are found more in aquatic environments than soils (Polianciuc et al., 2020). However, penicillin's and cefalexin are usually found in lower environmental concentrations than other antibiotic families (Watkinson et al., 2009; Jechalke et al., 2014). The antibiotics used in this study also have a range of toxicity effects, however, they all can select for resistant bacteria in the environment (Chen et al., 2010; Tell et al., 2019). Further effects from antibiotic exposures can also occur, for example, fluoroquinolones (ciprofloxacin) are very toxic to aquatic organisms resulting in genotoxic effects (Polianciuc et al., 2020), morphological deformities in plants and changes to a plants photosynthetic pathways (Opris et al., 2013; Polianciuc et al., 2020; Wang et al., 2015a). Metronidazole may be both carcinogenic and mutagenic in both humans and animals (Nasseh et al., 2016; Tiwari et al., 2019) and exposures may damage the DNA of lymphocytes in a range of species (Nasseh et al., 2016). As such investigating the effect of such antibiotic exposures on *Lemna minor* used to remediate such contaminants in CWs is of great importance.

Table 5. A list of the antibiotics used in this study, with values for Worst Case Scenario concentrations (µg /L), PECs (µg/L) and risk quotients included. The chemical formula, molar mass and an image of the antibiotic's structure is also included.

Antibiotic and Antibiotic family	Worst Case Scenario (µg/L)	PEC (µg/L)	Risk quotient	Chemical formula, molar mass, and structure
Metronidazole (Nitroimidazole)	3.11	1.95	97.63	C6H9N303 171.15  (DrugBankOnline, 2023)
Erythromycin (Macrolide)	2.54	1.143	57.14	C37H67NO13  733.9 (DrugBankOnline, 2023)
Flucloxacillin sodium (Penicillin)	21.68	8.07	32.27	C19H17ClFN3O5S 453.872  DrugBankOnline, 2023)
Phenoxy-methylpenicillin / Penicillin V (Penicillin)	17.34	5.20	20.80	C16H18N2O5S 350.39  (DrugBankOnline, 2023)

Antibiotic and Antibiotic family	Worst Case Scenario (µg/L)	PEC (µg/L)	Risk quotient	Chemical formula, molar mass, and structure
Erythromycin ethylsuccinate (Macrolide)	2.51	1.13	14.09	C43H75NO16 862.064  (DrugBankOnline, 2023)
Cefalexin (Cephalosporin)	2.81	1.11	13.93	C16H17N3O4S 347.389  (DrugBankOnline, 2023)
Clarithromycin (Macrolide)	6.17	1.33	13.32	C38H69NO13 747.9534  DrugBankOnline, 2023)
Ciprofloxacin (Fluoroquinolone / Quinolone)	1.78	0.43	7.10	C17H18FN3O3 331.3415  DrugBankOnline, 2023)
Amoxicillin (Penicillin)	52.92	16.92	2.53	C16H19N3O5S 365.404  (DrugBankOnline, 2023)

Antibiotic and Antibiotic family	Worst Case Scenario (µg/L)	PEC (µg/L)	Risk quotient	Chemical formula, molar mass, and structure
Trimethoprim (Antifolate)	2.13	0.75	1.50	C14H18N4O3 290.3177  (DrugBankOnline,2023)

2.3.5. Antibiotic Resistance Genes of interest for further study

Following the selection of the antibiotics above, a literature review was conducted of the antibiotics used in the study and their associated ARGs. The antibiotics and ARGs of great interest can be seen below in Table 6, with a wider list of potential ARGs considered in Table 19 in the appendix. Of an initial 51 genes considered in analysis (see Table 19), 8 were identified as of great interest for further studies (see Table 6 below), approximately 15%. The ARGs chosen were based not just on their reported association with bacteria exposed to the antibiotics used in this study but also several other considerations impacting the final selection shown in Table 6, including the often high costs associated with AR and genetic (qPCR) analyses, which could potentially limit a larger number of ARGs from being investigated. This study aimed to select a range of ARGs that would reflect the wide effects of all the antibiotics used in this study, while also being concise and cost effective. Once a list of all the ARGs commonly associated with each antibiotic in scientific literature was assembled (see Table 19), the ARG often considered the most important for each antibiotic was then determined. Not all antibiotics were assigned a single ARG solely associated with the single antibiotic due to predicted cost restrictions with genetic analyses and also a lack of information for the antibiotics less used in AR studies.

There are no clear ARGs often cited in literature associated with Penicillin V as it is not frequently studied with AR, with amoxicillin instead being the primary focus. However, BlaCTX-M is often stated as being very important for altered genes with amoxicillin exposures (Hayward et al., 2019; Lien et al., 2017; Sidrach-Cardona et al., 2014) and as they are both in the penicillin family this gene was applied to both. Erythromycin is often associated with erm genes (Hayward et al., 2019; Guo et al., 2015; Karaolia et al., 2018; Preethi et al., 2017; Shen et al., 2019; Wang et al., 2020), with ermB_3 being selected as the ARG due to primer access. Clarithromycin exposures are often linked to 23S

rRNA gene variations (Geng et al., 2017; Vala et al., 2016; Hussein et al., 2022; Pina et al., 1998; Zhang et al., 2021), however, to reduce costs as erythromycin and clarithromycin are both of the same family, ermB genes have been associated with both erythromycin and clarithromycin and their macrolide family (Mosleh et al., 2014; Wang et al., 2020), and so was chosen for both. The key ARGs for metronidazole and ciprofloxacin are nimE (Alauzet et al., 2019; Baaity et al., 2021; Gal and Brazier, 2004) and qepA (Cheng et al., 2021; Lien et al., 2017), respectively, and so included on the final select list of ARGs to analyse. A dfr ARG was also selected for trimethoprim, with dfrA27 being selected partially due to prominence in literature (Kraupner et al., 2020; Mukherjee and Chakraborty, 2006; Park et al., 2003; Stange et al., 2016; Suhartono et al., 2016) but also the availability and cost of primers. As the antibiotics used in this study could not be grouped further to reduce costs, the ARGs for metronidazole, ciprofloxacin and trimethoprim remained. 16S rRNA can be seen included in Table 6 below, as a housekeeping gene which is commonly used in genetic (qPCR) analyses to confirm accurate analysis (Wang et al., 2015b). Two class 1 integrons were also included due to their well-established role and importance for the acquisition and dissemination of ARGs between ARB (Hardwick et al., 2008). By including the most important associated ARG for each antibiotic where possible, alongside mobile genetic elements (integrons) and where possible multiple drug resistant genes (BlaCTX-M and ermB), a valuable picture of the range of effects caused by the antibiotic exposures used in this study for AR studies could be created.

Table 6. Genes of interest for antibiotic resistance studies, with reason for their selection included with their associated antibiotic or purpose.

Selected genes	Associated antibiotic or purpose	References
16S rRNA	Housekeeping gene	Hayward et al. (2019), Meng et al. (2017), Wang et al. (2015b), Wang et al. (2020)
Class 1 integrons (intl1 and intl1_2)	Mobile genetic elements	Hardwick et al. (2008), Meng et al. (2017), Schmitz et al. (2020), Stange et al. (2016), Suhartono et al. (2016), Wang et al. (2020)
BlaCTX-M	Multidrug resistance gene associated with penicillin's, particularly amoxicillin	Hayward et al. (2019), Lien et al. (2017), Sidrach-Cardona et al. (2014)
nimE	Metronidazole	Alauzet et al. (2019), Baaity et al. (2021), Gal and Brazier (2004)

Selected genes	Associated antibiotic or purpose	References
qepA	Ciprofloxacin	Cheng et al. (2021), Lien et al. (2017)
dfrA27	Trimethoprim	Kraupner et al. (2020), Mukherjee and Chakraborty (2006), Park et al. (2003), Stange et al. (2016), Suhartono et al. (2016)
ermB_3	Erythromycin and clarithromycin	Hayward et al. (2019), Guo et al. (2015), Karaolia et al. (2018), Preethi et al. (2017), Shen et al. (2019), Wang et al. (2020)

2.4. Experimental design of the exposure study

2.4.1. Antibiotic and time exposures

The antibiotic exposures used in the study can be seen below in Table 7 and time exposures in Table 8, with replicate number included. A total of 5 antibiotic mixture concentrations and 3 time durations were selected for this study (see Table 8) and were selected to include environmentally relevant (PECs) and elevated concentrations (x10PEC and x100PEC) to give results applicable to *Lemna minor* in CWs but also confirm exposures and potentially establish toxicity limits via a dose response. A worst case scenario exposure was considered (see Table 17 in the appendix), however it was not included as such precise concentrations may have resulted in significant error when spiking solutions. The time exposures were determined using studies' such as Ebert et al., (2011), (Iatrou et al., 2017), Baciak et al., (2016) and OECD (2006) which often used 7 days as the maximum exposure time in their toxicity studies. For this study 24 hours, 5 day and 9 day exposures were determined to capture both rapid and longer term effects on the *Lemna minor*. The number of replicates per exposure were selected by balancing the need for many replicates to give greater confidence and statistical power to results but also feasibility with time and cost constraints. As such for control treatments (24hrs, 5 days and 9 days) 7 replicates were used in the exposure study and for the remaining treatments 5 replicates were used (see Table 8). Aside from the different antibiotic and time exposures, all replicates were treated identically during the exposure study, with identical test vessels, frond addition, and environmental conditions.

Table 7. Antibiotic exposures with the concentrations (µg/L) for each antibiotic, ordered by risk quotients.

Antibiotic	PEC/10	PEC	X10 PEC	X100 PEC
Metronidazole	0.12	1.95	19.53	195.26
Erythromycin	0.11	1.142773	11.42773	114.2773
Flucloxacillin sodium	0.81	8.07	80.66188	806.6188
Phenoxymethylpenicillin (Penicillin V)	0.52	5.20	52.01	520.07
Erythromycin ethylsuccinate	0.11	1.13	11.27	112.70
Cefalexin	0.11	1.11	11.14	111.42
Clarithromycin	0.13	1.332069	13.32	133.21
Ciprofloxacin	0.04	0.43	4.26	42.59
Amoxicillin	1.69	16.92	169.22	1692.22
Trimethoprim	0.08	0.75	7.51	75.06

Table 8. Antibiotic (control and PECs) and time (24 hrs, 7 days and 9 days) exposures used in the exposure study with the number of replicates per exposure.

	Control	1/10th PEC	PEC	X 10 PEC	X100 PEC
Removal after 24 hrs	~~IIII~~ II	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~
Removal after 7 days	~~IIII~~ II	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~
Removal after 9 days	~~IIII~~ II	~~IIII~~	~~IIII~~	~~IIII~~	~~IIII~~

2.4.2. Antibiotic stock preparation and antibiotic spiking during the exposure study

To create the antibiotic exposures shown above in Table 7, a series of steps had to be taken to be able to spike the experimental medium with the required antibiotics. Firstly, a stock solution for each antibiotic was made up at a concentration of 1µg/µl in methanol. A mixture was then made at the x100PEC concentration level for each compound in methanol. The x100PEC solutions were evaporated using a miVac sample concentrator, Model: DNA (Genevac, Ipswich, UK) to remove methanol and the vials stored in foil with the stock solutions at -20°C to reduce antibiotic degradation. When required during the exposure study to spike the experimental medium, deionised water was added to a x100PEC vial, vortexed to ensure dissolution and added into the experimental medium. This was repeated 3 times to create a x100PEC concentration. Serial dilutions of the x100PEC solutions using the experimental medium was used to form the other antibiotic exposures (x10PEC, PEC and PEC/10). This process was carried out 48 hours before the spiked medium was used in the renewal schedule (see section 2.4.4) to allow the solution to stabilise. For such procedures sterile equipment was used to reduce potential contamination.

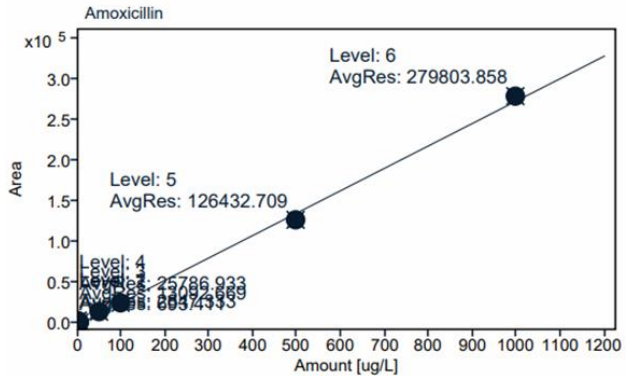
2.4.3. Confirmation of antibiotic spiking during the exposure study

To confirm that accurate antibiotic spiking was conducted during the exposure study (described above in 2.4.2), water samples of the spiked experimental medium used during the exposure study were collected and antibiotic concentrations determined using Liquid Chromatography-Mass Spectrometry (LC-MS). In liquid chromatography, the samples are passed through a column where chemicals interact with the stationary phase of the column and the complex mixture separates out, depending on the physiochemical properties of each chemical. Such separation results in increased accuracy in mass spectrometry, in which the chemicals are ionised and sorted by mass to charge ratios, which are then used to quantify compounds.

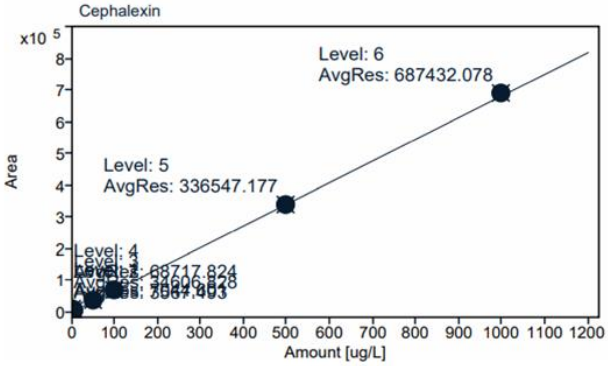
To quantify the antibiotics, calibration standards were created to optimise the instrument for antibiotic analysis and to create calibration curves that are used to transform intensity readings from the LC-MS to antibiotic concentrations in µg/L. A mixture of antibiotics was made at a 1000 µg/L from individual stock solutions. The calibration standards 1000, 500, 100, 50, 5, 1, and 0 µg/L were created using a series of dilutions with a methanol: water mixture (20:80). Calibrations standards were run lowest to highest, to reduce carry over affecting results and blanks analysed in between standards to confirm minimal transference. While attempts were made to optimise the LC-MS to be able to analyse all 10 antibiotics simultaneously, it was determined that using a single optimization programme, the antibiotics amoxicillin, cephalexin, and metronidazole could be analysed together with high accuracy. Due to budget and time constraints, further analysis was not conducted, and the three antibiotics used to confirm accurate spiking. The calibration curves for each antibiotic, with high R^2 values and linear lines can be seen below in Figure 4. Samples of x100PEC spiked experimental medium, with 3 replicates per sample, were diluted with methanol to create a methanol: water mixture identical to standards. Samples were run after the standards with a programme series of 7 samples, blank, 7 samples, repeating until analysis was complete.

When accounting for antibiotic degradation and the reduced concentrations in the samples, the results (see averages in Table 9 below) show consistent accurate spiking. Antibiotic concentrations between different samples are similar, with standard deviation overlapped. While there were lower concentrations found for all antibiotics in the final 9 day experimental media batch, the findings were consistent, and as replicates were all treated identically, this should not affect the findings of the study.

Signal: MS1 +SIM(366.1) ESI Frag=110V Gain=1.0 (rt: 3.79 - 4.39 min)
Exp. RT: 4.089
Residual STD: 5459.18709
R: 0.99888
R^2: 0.99775
Formula: y = ax + b
a: 275.7584
b: -1142.7242
c:
d:



Signal: MS1 +SIM(348.3) ESI Frag=110V Gain=1.0 (rt: 6.77 - 7.37 min)
Exp. RT: 7.072
Residual STD: 3952.86178
R: 0.99990
R^2: 0.99981
Formula: y = ax + b
a: 683.4624
b: 871.7523
c:
d:



Signal: MS1 +SIM(172.3) ESI Frag=110V Gain=1.0 (rt: 4.87 - 5.47 min)
Exp. RT: 5.170
Residual STD: 3853.46345
R: 0.99996
R^2: 0.99992
Formula: y = ax + b
a: 1037.8181
b: 3827.4273
c:
d:

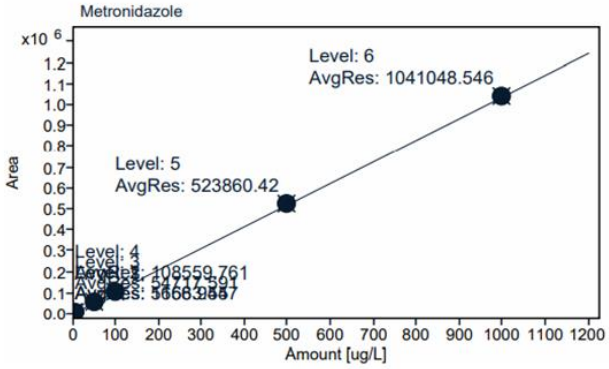


Figure 4. Calibration curves for LC-MS analysis of the antibiotic's amoxicillin, cefalexin, and metronidazole.

Table 9. Concentrations of the antibiotic amoxicillin, cephalexin, and metronidazole in samples of x100PEC spiked experimental medium used in the exposure study, with averages and standard deviations.

Antibiotic	Sample	Average concentration (µg/L) with standard deviation (+/-)
Amoxicillin	48 hours after *Lemna minor* addition to petri dish (removed day 2)	101.13 (+/-1.92)
Amoxicillin	Day 0, before *Lemna minor* addition	107.43 (+/- 5.62)
Amoxicillin	Day 5, before *Lemna minor* addition	88.84 (+/- 13.29)
Amoxicillin	Day 9, before *Lemna minor* addition	81.84 (+/- 4.85)
Cephalexin	48 hours after *Lemna minor* addition to petri dish (removed day 2)	20.33 (+/- 1.63)
Cephalexin	Day 0, before *Lemna minor* addition	23.67 (+/- 0.99)
Cephalexin	Day 5, before *Lemna minor* addition	21.52 (+/- 2.57)
Cephalexin	Day 9, before *Lemna minor* addition	17.65 (+/- 0.80)
Metronidazole	48 hours after *Lemna minor* addition to petri dish (removed day 2)	47.55 (+/- 1.29)
Metronidazole	Day 0, before *Lemna minor* addition	46.61 (+/- 1.62)
Metronidazole	Day 5, before *Lemna minor* addition	46.81 (+/- 4.16)
Metronidazole	Day 9, before *Lemna minor* addition	42.10 (+/- 0.45)

2.4.4. Experimental medium and renewal schedule

In order to replicate realistic conditions whilst maintaining consistency throughout the experiment, a medium of artificial effluent was initially chosen for the exposure study. Using effluent from a CW could lead to unknown contaminants influencing the results of the study and as such artificial effluent would allow for the closer replication of conditions in CWs treating WWTP effluent while also maintaining a controlled study. The artificial effluent used (see Table 10) was based on methods from the studies Liu et al., (2000), Duran et al., (2011), Expositio et al., (2017) and OECD (2001). A preliminary test was conducted to determine whether *Lemna minor* could survive in the artificial effluent. Exposure to the effluent was found to result in *Lemna minor* stress and total death during a 7 day period, with all fronds turning a pale white (see Figure 5). The pH of the effluent was found to be within survivable ranges and as such the components of the artificial effluent were likely causing the stress. By exposing the *Lemna minor* to gradual increases in compound concentrations over time, it may have been possible to acclimatize the *Lemna minor* to the artificial effluent. However, due to the time constraints of this study, and no guarantee of successful acclimatization, it was decided that artificial effluent could not be used in the exposure study. Deionised water was then selected as the experimental medium for the exposure study. Efforts were made to maintain minimal microbial contamination, although sterilisation was not conducted. Following antibiotic

spiking, pH tests were performed to confirm the ranges were within survivable conditions. As recommended by the OCED (2006) the experimental medium spiked with antibiotics was replaced every 48 hours to remove the build-up of toxic substances produced by the *Lemna minor* and mimic the constant flow of effluent in CWs.

Table 10. Compound concentrations used with deionised water to create artificial effluent.

Compound	Concentration (µg/L)
Peptone	23,529
Beef Extract	16,177
Urea	4,412
Sodium chloride (NaCl)	7,000
CaCl2. 2H2O	4,000
K2HPO4	28,000

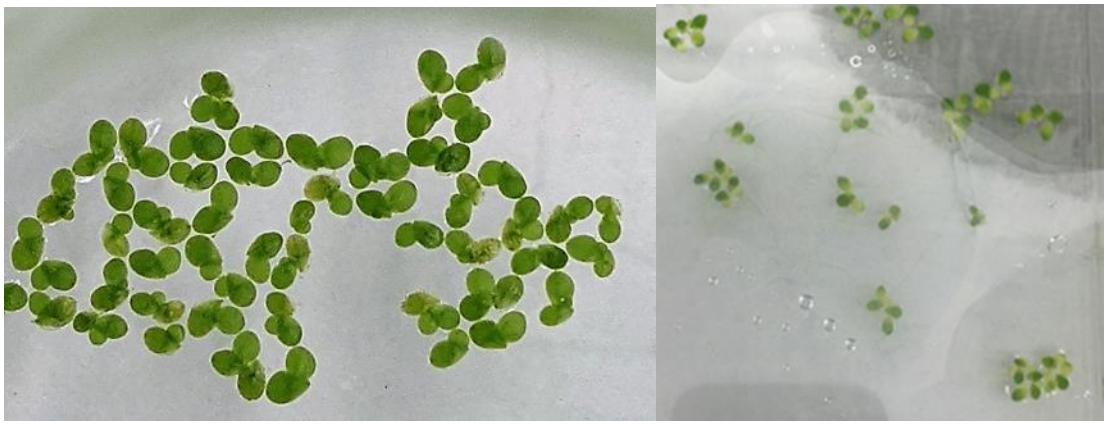


Figure 5. Images of *Lemna minor* before (left) and 3 days after (right) introduction to artificial effluent.

2.4.5. Test vessel

Square petri dishes (11349273 Fisher scientific 100 mm) with lids were used as the test vessel for the exposure study. Petri dishes with lids were determined to be most appropriate for this study as they would allow clear images to be captured for surface area analysis and result in minimal contamination. The dishes provided 10 mm of depth and a surface area of 100 mm x 100 mm. While 20 mm of depth is recommended by the OECD (2006), few petri dishes with appropriate depth and surface area are available. As such surface area was prioritised for the study to prevent fronds overlapping and allow for roots to extend horizontally. 80 ml of experimental medium was added to each petri dish to allow for *Lemna minor* to interact with an atmosphere inside the petri dishes, exchanging carbon dioxide and oxygen. The renewal of experimental medium every 48 hours also replenished the atmosphere of the petri dishes during the exposure study. Petri dish location in the

environmental chamber during the exposure study was randomised and rotated daily to minimise the effect of light intensity and temperature from light sources in the chamber impacting the growth of *Lemna minor* in certain replicates and as such impacting the study.

2.4.6. Determining frond addition

Prior studies use a range of 12 to 20 fronds per replicate (Ebert et al., 2011, Iatrou et al., 2017, Gomes et al., 2017, Sree et al., 2015, OECD, 2006), however in their analysis requirements, environmental conditions and growth rates were different to this study. A frond addition that would provide adequate masses for analysis (chlorophyll a content and metabolomics) while not overwhelming the petri dish, was required. One frond of *Lemna minor* was found to be approximately equivalent to 1 mg (fresh weight). For metabolomic analysis a minimum of 0.1 mg of fresh material was required (preferably 1 mg). A test was conducted to determine the minimum mass required for accurate chlorophyll a content analysis, for the method see section 3.1.5. Several samples of different weights and time exposures (see Table 11) were extracted and analysed, the raw fluorescence values were found to not fluctuate for all the different masses and as such 5 mg with a 48 hour, 3.5 ml acetone extraction, with 2 ml decanted for analysis with a fluorometer was selected for the study as this balanced accurate measurements with minimal mass requirements. A third variable was also considered during method development, and while ultimately not included in the exposure study, 50 mg was set aside during frond determination calculations. This excess 50mg was then included in excess biomass for additional or repeat analyses. A minimum fresh mass of 56 mg or 56 fronds was determined to be appropriate per replicate. Surface area changes over time were then determined for *Lemna minor* using the methods described in 2.5.5. The percentage increase in surface area can be seen below in Table 12, with an average percentage increase in surface area per day of 19.2% and an increase of 172.7% over a 9 day period. With an approximate 173% increase, a frond diameter as high as 5 mm (see Table 12), with a petri dish 100 cm^2, and a minimum requirement of 56 fronds, a total of 120 fronds per petri dish was determined to be appropriate for the study, and allow for excess biomass for additional or repeat analysis if necessary. This allowed for larger quantities of material to be stored at -80°C and used if the masses for different variables were found to not be sufficient for analysis, while also not overwhelming the petri dishes during the exposure study. An experimental stock culture was grown prior to the exposure study and was used to inoculate all replicates with approximately 120 fronds. A small stock culture was then maintained alongside the experiment.

Table 11. Raw fluorescence values for mass and acetone time exposures for *Lemna minor*.

Time treatment	Mass treatment	Raw fluorescence values	Raw fluorescence values	Raw fluorescence values
24 hrs	5mg	3913.67	3905.18	3899.83
24 hrs	10 mg	5790.7	5771.35	5768.39
24 hrs	20 mg	12714.63	12736.71	12725.46
48 hrs	5mg	5352.29	5361.57	5362.52
48 hrs	10 mg	7182.9	7183.77	7183.49
48 hrs	20 mg	14142.08	14162.1	14165.92

Table 12. Surface area (cm), average frond diameter (cm), percentage increases in surface area over a 6-7 day period and the percentage increase in surface area per day for *Lemna minor*.

	Surface area (cm)	Surface area (cm)	Surface area (cm)	Average frond diameter (cm)	Average frond diameter (cm)	Average frond diameter (cm)	Percentage increase in surface area	Percentage increase in surface area	Percentage increase in surface area per day
Replicate	Day 1	Day 6	Day 7	Day 1	Day 6	Day 7	For 6 days	For 7 days	
1	11.23	26.66		0.12	0.16		137.45		22.91
2	14.94	27.81		0.20	0.16		86.19		14.37
3	23.92		36.94	0.50		0.40		54.44	7.78
4	16.01		51.53	0.5		0.15		221.82	31.69

2.4.7. Determining the dry weight: fresh weight ratio

To allow for the statistical analysis of chlorophyll a content in samples a dry weight: fresh weight ratio for *Lemna minor* was required. The fresh weights of three samples of *Lemna minor* were recorded and then dried for 48 hours. The dry weights were recorded and used to calculate a dry weight: fresh weight ratio, the results of which can be seen below in Table 13. An average dry weight: fresh weight ratio of 0.07 was found.

Table 13. The fresh weight, dry weight, and dry weight: fresh weight ratios for *Lemna minor*.

Replicate	Fresh Weight (mg)	Dry Weight (mg)	Dry weight: fresh weight ratio
1	19153.6	1298.2	0.0778
2	19931.2	1695.6	0.0851
3	17748.3	1003.2	0.0565

2.5. Conclusion

The decisions and knowledge used to form the basis of this study have been laid out above; in the following chapter an outline of the methods used in the exposure study and the following data analysis can be found.

3. Methods

3.1. The exposure study

3.1.1. Overview

During the exposure study *Lemna minor* was exposed to a mixture of antibiotics and the effects on its growth, chlorophyll a content and metabolome investigated at various time intervals. The following chapter includes information on the exposure study from chemical and surface area monitoring to sampling and chlorophyll a, metabolomic, and statistical analyses carried out to provide results to meet objectives 3, 4 and 5.

3.1.2. Chemical monitoring

With the replenishment of experimental medium every 48 hours, samples of the x100PEC spiked medium were taken prior to *Lemna minor* exposure in order to confirm accurate spiking (see section 2.4.3). Samples were filtered using a 0.2 µm filter and stored at -20°C until analysis.

3.1.3. Sampling

At the end of a replicates time exposure (24 hours, 5 days or 9 days), the *Lemna minor* (fronds and roots) were collected, rinsed with deionised water and blotted dry. The *Lemna minor* were separated into 3 sections (with the precise masses recorded); ≈5 mg for chlorophyll a extractions (see section 3.1.5 for more extraction method details), ≈50 mg for biomass storage for additional or repeat analyses, and the remaining mass for metabolomic analysis flash frozen using liquid nitrogen to stop enzymatic processes. For chlorophyll a and metabolomic extractions the full *Lemna minor* organism (fronds and roots) were not separated and combined for analysis, as separate analysis was not possible with the time and cost constraints of the study. Samples for metabolomic analysis were ground down with liquid nitrogen using a mortar and pestle, their new weights recorded and then stored at -80°C. The equipment was carefully cleaned between each replicate to avoid contamination between samples.

3.1.4. Surface area images

Images were taken of each replicate (placed on a light box to improve image quality) on days 0, 1, 2, 4, 5, 6, 8 and 9. Such images were used to find percentage changes in the surface area of *Lemna minor* with antibiotic and time exposures (for Image J methods see section 2.2.5).

3.1.5. Chlorophyll a extractions and measurements

5mg of plant material for every replicate was extracted in 3.5ml of 80% acetone for 48 hours, and then 2ml decanted (without visible fronds) into cuvettes, which were cleaned between measurements and handled carefully to avoid spots interfering with the results. The samples were

analysed using a Trilogy Laboratory Fluorometer (Turner designs, San Jose, USA) with a CHL-A-NA module. During extraction samples were wrapped in foil to reduce photodegradation of chlorophyll a and stored in 4°C during extraction. Raw fluorescence values provided by a chlorometer were transformed to chlorophyll a concentrations (µg/L) using the calibration curve below in Figure 6. Calibration standards of 1000, 500, 300, 200, 100, 50, 20, 10, 5, 2 and 1 µg/L were made and their intensity readings recorded and used to generate Figure 6, which can be seen to have a high R^2 value of 0.9994. During the data analysis of the chlorophyll a content results, some were found to reach as high as 3000 µg/L, which is higher than the highest calibration standard of 1000 µg/L. To allow the use of the calibration standards and equation produced below in Figure 6 it was confirmed that chlorophyll a content remains linear against fluorescence measurements at concentrations greater than 1000 µg/L. The study Pham Phu (2014) found that the relationship between fluorescence and chlorophyll a content remained linear to 5000 µg/L, the studies highest standard. As such the calibration curve could still be used to transform the data.

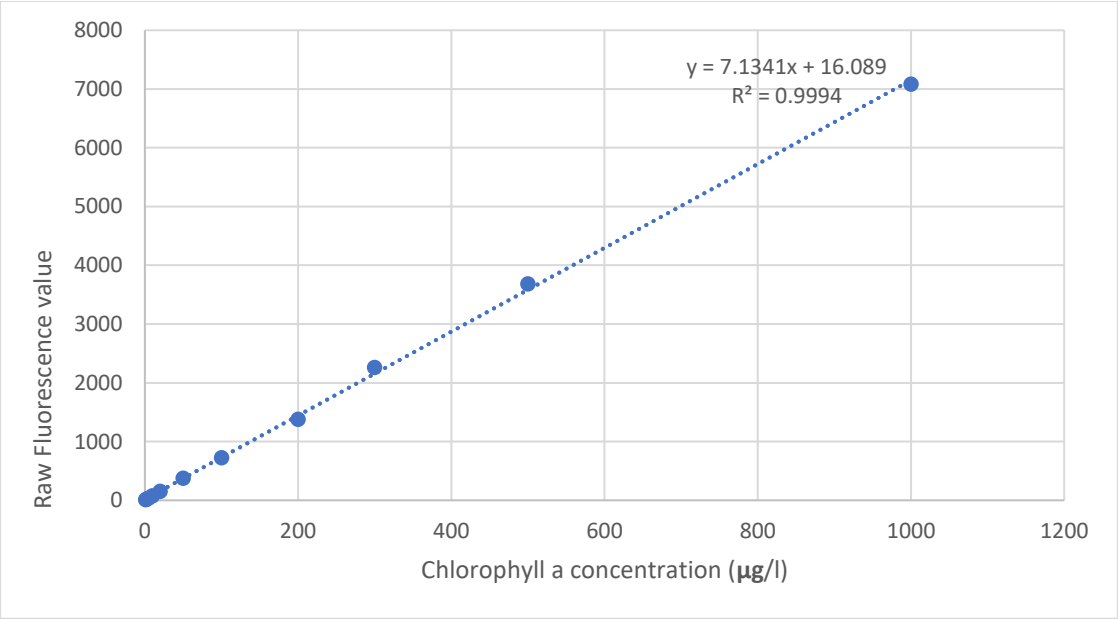


Figure 6. Calibration curve for chlorophyll A concentration (µg/L) against raw fluorescence values, with R^2 value and linear equation.

3.1.6. Metabolome extractions

The method for obtaining the extractable metabolome of *Lemna minor* was derived from Gaffney et al. (2021). Samples of *Lemna minor* were submerged in 100 µl of a methanol: water mixture (70:30), wrapped in foil and shaken at 500rpm for 30 minutes, then centrifuged at 15,000 rpm for 10 minutes. Supernatants were transferred and dried using a SpeedVac Concentrator (PD111V, Thermo Savant, Waltham, USA) under cool conditions. Once full evaporation was confirmed, samples were

reconstituted in 100 µl of the methanol: water mixture and stored at -80°C until analysis using High Performance Liquid Chromatography-Mass Spectrometry (HPLC-MS). The extraction method used in this study is often considered the most appropriate for metabolomic analysis according to the studies Gaffney et al. (2021) and Fiehn (2002) which investigated which methods are the most effective at stopping enzymatic activity and extracting metabolites. Metabolomic analysis was performed on samples from the highest exposure level (x100PEC), predicted environmental exposure level (PEC) and the control. A total of 45 samples were analysed including remained with control, PEC and x100PEC exposures for 24 hours, 5 days and 9 days with 5 replicates each.

3.1.7. High Performance Liquid Chromatography-Mass Spectrometry analysis of the metabolome

LC-MS analysis (see section 2.4.3) is often recommended and used in metabolomic studies (De Vos et al., 2007; Gaffney et al., 2021); in this study the extracted metabolites were analysed using high performance liquid chromatography – high resolution mass spectrometry (HPLC-HRMS). HPLC-HRMS was deployed for metabolomic analysis in order to achieve the necessary separation and accurate mass needed to analyse the complex plant metabolome (Gaffney et al., 2021; Fiehn, 2002). HPLC was performed using an Agilent 1200 HPLC (Agilent, Santa Clara, California, USA). This was fitted with an Atlantis T3 column (Waters, cortecs 2.7 µm, 3 × 150 mm, Milford, Massachusetts, USA), with a column temperature of 25 ◦C. The HPLC was then coupled to an Orbitrap Fusion Mass Spectrometer (ThermoScientific, Waltham, USA). The mobile phase was composed of water (A) and acetonitrile (B) both with 0.1% (v/v) formic acid, and the following gradient was used: 5% B increasing linearly to 95% over 22 min and held for 2 min. B was then returned to 5% over 0.33 min and held for 5.33 min to allow column equilibration. The flow rate was 300 µL/min and the injection volume was 20 µL. Analysis was conducted in positive-ion-mode, with an ion transfer temperature of 325 °C and a vaporiser temperature of 350 °C. The sheath gas was 50 (arb); the aux gas was (arb) 10; time between master scans was 1 s; the isolation window was 1.6; the collisional energy was stepped and the HCD collision energies (%) were 20, 35, and 60. This instrumental method was previously used in Gaffney et al. (2021).

A pooled aliquot (20 µL from every replicate) was used as a quality control and injected 20 times to equilibrate the column. The quality control (pooled aliquot) and a blank were injected after every 5 sample injections to monitor instrument performance, to determine the carryover between samples (found to be minimal), monitor instrument reproducibility and to allow for batch drift corrections in statistical analysis if required (this was not required). Five biological replicates (80 µL) per treatment were analysed, with 1 technical replicate per biological sample. Three technical replicates per biological replicate improves data analysis reliability and are used to monitor instrument reproducibility, however, cost constraints prevented this. Ultimately, the presence of 5 biological

replicates reduced this issue. While samples should be injected in a randomised order to reduce the effect of instrument drift, unfortunately this was not carried out and samples were injected in order of controls, PECs and x100PECs. However, instrument drift could be corrected for in statistical analysis if it was present, which it was not.

3.2. Data analysis

3.2.1. Percentage change in surface area, average frond size and chlorophyll a content

Once the percentage change in surface area and the average frond size for each replicate was found using image analysis, averages and standard errors were calculated for the antibiotic and time exposures. For percentage change in surface area the data was combined and split to create several graphs and conduct several statistical tests. Four data sets were created; the first containing only the first '24 hour antibiotic exposures' which were then destructively harvested ('Day 1 dataset', Control: n = 7; PEC/10, PEC, X10PEC and x100PEC: n = 5), the second containing only antibiotic exposures for the '5 day exposures' at which point this dataset were destructively harvested ('Day 5 dataset'; Control: Day 1-5 n=7; PEC/10, PEC/10, PEC, X10PEC and x100PEC: Day 1-5 n = 5), the third containing only antibiotic exposures for the 9 day exposures at which point this dataset were destructively harvested ('Day 9 dataset'; Control: Day 1-9 n=7; PEC/10, PEC/10, PEC, X10PEC and x100PEC: Day 1-9 n = 5), and finally a data set with all data from the 24 hours (Day 1 dataset), 5 day (Day 5 dataset) and 9 day exposures (Day 9 dataset) combined ('Combined dataset'; Control Day 1 n = 21, Day 2-7 n = 14, Day 8-9 n =7; PEC/10, PEC, x10PEC and x100PEC Day 1 n =15, Day 2-7 n = 10, Day 8-9 n =5). The averages and standard errors were also found for the chlorophyll a concentration (µg/mg of dry mass) for the antibiotic and time exposures following the transformation of raw fluorescence values to concentration (µg/L). A series of calculations were conducted to find these results.

Firstly, the fresh weight of a replicate (mg) was multiplied by the dry weight: fresh weight ratio to find the dry weight (mg) for each sample. This can be seen below in the following equation. (Equation (5)):

$$Dry\ weight = Fresh\ weight\ x\ Dry\ weight{:}\ Fresh\ weight \qquad (5)$$

The total chlorophyll mass (µg) was then found for each sample by multiplying the sample volume (L) by the concentration (µg/L) of each sample. This calculation is shown by the following equation. (Equation (6)):

$$Total\ chlorophyll\ a\ mass = Volume\ x\ Concentration \qquad (6)$$

The average total chlorophyll mass (µg) for each replicate was then found by averaging the three samples measured for each replicate. The concentration (µg /mg of dry mass) of chlorophyll a in *Lemna minor* was then found by dividing the average total chlorophyll mass (µg) per replicate by the dry weight (mg) for each replicate. This can be seen in the following equation. (Equation (7)):

$$Concentration\ of\ chlorophyll\ a = \frac{Total\ chlorophyll\ a\ mass}{Dry\ weight} \quad (7)$$

Graphs were made from the averages and standard errors for the variables to help determine if any patterns in the data could be seen. Statistical tests were conducted using SPSS (IBM Corp, 2021, Version 28.0, Armonk, NY) to determine if significant differences could be found between antibiotic and time exposures with percentage change in surface area and chlorophyll a content. Normality tests were conducted; much of the data was found to not be normal and as such attempts to transform the data were made and normality tests repeated. The transformed data was also found to not be normal and as such the original untransformed data was separated and used in 2 way repeated measures Anova's. Multiple comparisons, homogeneity, Tukey, Dunnet, Levene's equal variances and Mauchly's test of sphericity were also conducted. If the data was found to have a Mauchly's Test of Sphericity result of <0.05, Greenhouse-Geisser values were used. Such tests should be performed on normally distributed, continuous data with equal variances; however, the test is robust and the most applicable to analyse the data sets in an objective manner and provide results with an adequate confidence.

3.2.2. Metabolomics analysis

The full chemical profiles from all samples (with peaks showing the intensity of unique chemicals) were entered into progenesis QI (Waters, Milford, MA, USA) in centroid mode. To compensate for small variations between runs which arise during HPLC-MS analysis and to improve the reliability of the data, a quality control run (QC 5 for this study) was chosen against which all remaining runs are aligned against. All runs with an alignment within 80% of the reference run were accepted, which for this study included all runs. Careful filtering was conducted to reduce background machine noise while avoiding the removal of valuable data or altering the results. Peaks under 0.05 minutes and retention times < 1 minute and >21 minutes were excluded from further analysis to reduce the influence of poorly separated and resolved compounds. Compounds with a >30% coefficient of variation in the quality control runs were excluded from further analysis as the compounds had too much variability within samples and were not of high confidence. Such filtering resulted in a decrease from 30,000 features to 15,000 features per sample.

A PCA plot was generated, which is made using chemicals that are most responsible for variations between treatments (antibiotic and time exposures), by using the abundance levels for every

chemical across all samples (Saccenti et al., 2014; Worley and Powers, 2013). After confirming that the variability in the plot was not due to the influence of sample run order and the clustering of quality controls, indicating instrumental reproducibility and accuracy is strong (Saccenti et al., 2014; Worley and Powers, 2013), the PCA plot could then be accepted into the study. The top 20 chemicals responsible for the variation in the first and second principal component were analysed further using ChemSpider (The Royal Society of Chemistry, London, UK, 2023a; Version 1.0.5540.51345), a mass spectrum library used for chemical identification. Identifications were investigated to determine if the chemical is a plant metabolite and its role within the *Lemna minor*. Only chemical identifications with a mass error (ppm) between ± 5 were accepted and isotope similarity scores >90. The chemical identifications made in the study are tentative as confirmation with analytical standards was not conducted. For some chemical features, no identifications were possible due to the ambiguous data, with multiple potential identifications. These results were then used to assess whether the antibiotic treatment and exposure time affected the chemical metabolome of *Lemna minor*. More details and the results of such findings can be seen in more detail in chapter 5.

3.3. Conclusion

The above chapter has laid out the methodology of this study. In the following chapter the impacts of the antibiotic and time exposures used in the exposure study on the physiology of Lemna minor are investigated.

Chapter 4. Effects of antibiotic exposure on the physiology of *Lemna minor*

4.1. Introduction

It is of great importance to determine whether plants in CWs are experiencing stress with antibiotic exposures. Monitoring changes to a plants growth is a useful and commonly used variable and such methods can be frequently seen in *Lemna minor* toxicity studies. Growth rate and chlorophyll a content are frequently used in *Lemna minor* toxicity studies to indicate health and toxicity limits, with the OECD (2006) *Lemna minor* toxicity study guide recommending them as reliable indicators for plant health. Toxicity studies such as Baciak et al. (2016), Ebert et al. (2011), Iatrou et al. (2017), Lima et al. (2021) and Sree et al. (2015) investigate changes in *Lemna minor* growth, though the exact measurements can vary from growth rate to frond mortality rate and biomass changes. Changes in such variables are found by either frond numbers by eye or by using computer imaging programmes (such as Image J, used in this study, see section 2.2.5) and then calculating changes with time and treatments. Here, percentage change in frond surface area is reported. This variable was chosen for this study as exposures did not result in frond mortality and unlike biomass changes it is a useful non-disruptive option for finding growth changes according to the OECD (2006).

Chlorophyll a content is often used in toxicity studies such as Baciak et al. (2016), Cascone et al. (2004), Kalaji et al., 2016; Pavlovic et al., 2014; and Zeigler, 2016 as an indicator of the photosynthesis within the plant. With abiotic stresses such as temperature and salinity changes and contaminants such as heavy metals, the processes required for photosynthesis can be disrupted and as such may be used as an indicator of plant stress (Kalaji et al., 2016 and Kalaji et al., 2012). Chlorophyll a and b pigments as intermediaries in the process of converting light to organic matter in photosynthesis and as such is crucial for plant survival (Pavlović et al., 2014). As chlorophyll absorbs and re emits light in photosynthesis, absorption and fluorescence monitoring can be conducted to measure their abundance (Pavlović et al., 2014), the method of which is covered in section 3.1.5 for this study. The majority of *Lemna minor* toxicity studies do not investigate average frond size. Cleuvers and Ratte (2002) is one of the only *Lemna minor* toxicity studies that included frond size as a measurement. In a dye toxicity study, they investigated the effect of exposures on total frond area and mean frond size, using image analysis software similar to the methods used in this study. They found that with certain dyes and concentrations (blue dye with concentrations > 3.2 mg/L), significant decreases in mean frond size could be found when compared to controls. Changes in frond size can easily be found along side changes in frond surface area using Image J software. As such it was included as an ancillary measurement. By establishing the effects of the antibiotic exposures on the growth rate and chlorophyll a content of *Lemna minor* (objectives 3 and 4) it may

be possible to determine whether *Lemna minor* in CWs are likely to be experiencing stress and as such whether antibiotic exposures are potentially impacting CW remediation.

4.2. Results

4.2.1. Percentage change in surface area

A series of Anova tests were carried out for the split data sets to determine whether there were any significant differences in percentage change in frond surface area between antibiotic exposures and also over time. No statistically significant differences in percentage change in frond surface area between antibiotic exposures for the 24 hour data set ($F(2) = 1.475$, $P = 0.244$), the 5 day data set ($F(7.438) = 0.774$, $P = 0.619$) or the 9 day data set ($F(4.818) = 0.582$, $P = 0.708$) were found using a One Way Anova and Two Way Repeated Measures Anovas, respectively. Statistically significant differences in percentage change in frond surface area were found with time, finding increases in surface area with time, using the 5 day data set ($F(1.86) = 253.779$, $P = <0.001$) and the 9 day data set ($F(1.204) = 158.431$, $P = <0.001$), using Two Way Repeated Measures Anovas.

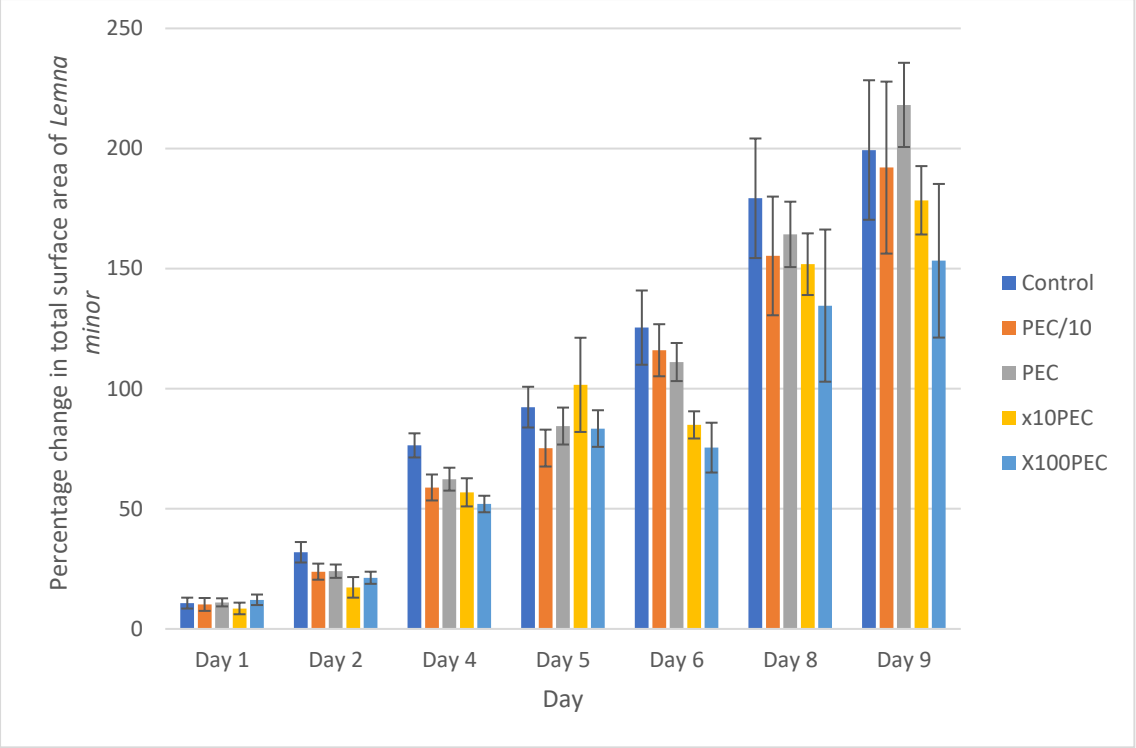


Figure 7. Average percentage changes in surface area of *Lemna minor* relative to the start of the experiment in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). All data sets for different time exposure groups (24hours, 5 days and 9 days) were combined for each antibiotic exposure to give a Combined dataset, with the following replicates per exposure: Control (Day 1 n = 21, Day 2-7 n = 14, Day 8-9 n =7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, Day 1 n =15, Day 2-7 n = 10, Day 8-9 n =5).

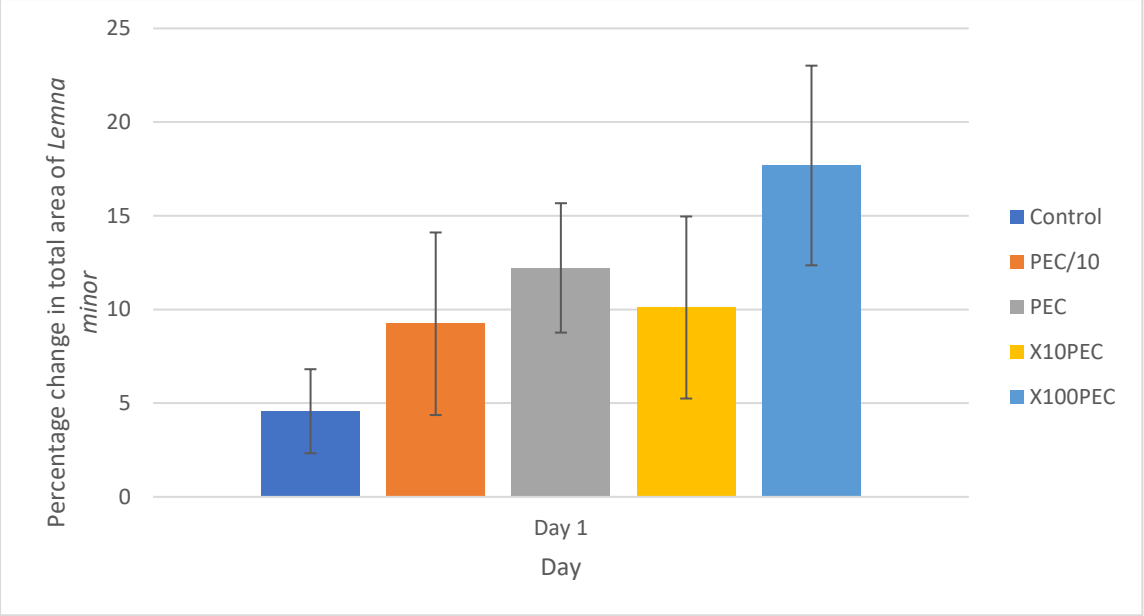


Figure 8. Average percentage changes in surface area of *Lemna minor* relative to the start of the experiment in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). Only data sets for the 24 hour exposure group were included for each antibiotic exposure to give the Day 1 dataset, with the following replicates: Control (Day 1 n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, Day 1 n = 5).

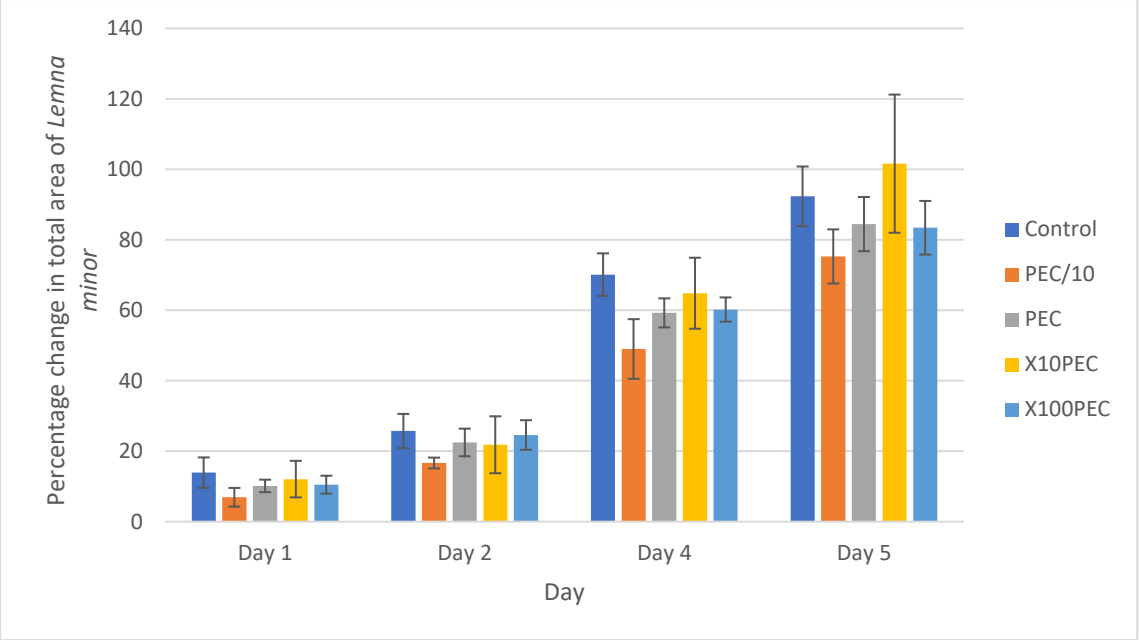


Figure 9. Average percentage changes in surface area of *Lemna minor* relative to the start of the experiment in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). Only data sets for the 5 day exposure group were included for each antibiotic exposure to give the Day 5 dataset, with the following replicates: Control (Day 1 n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, Day 1 n = 5).

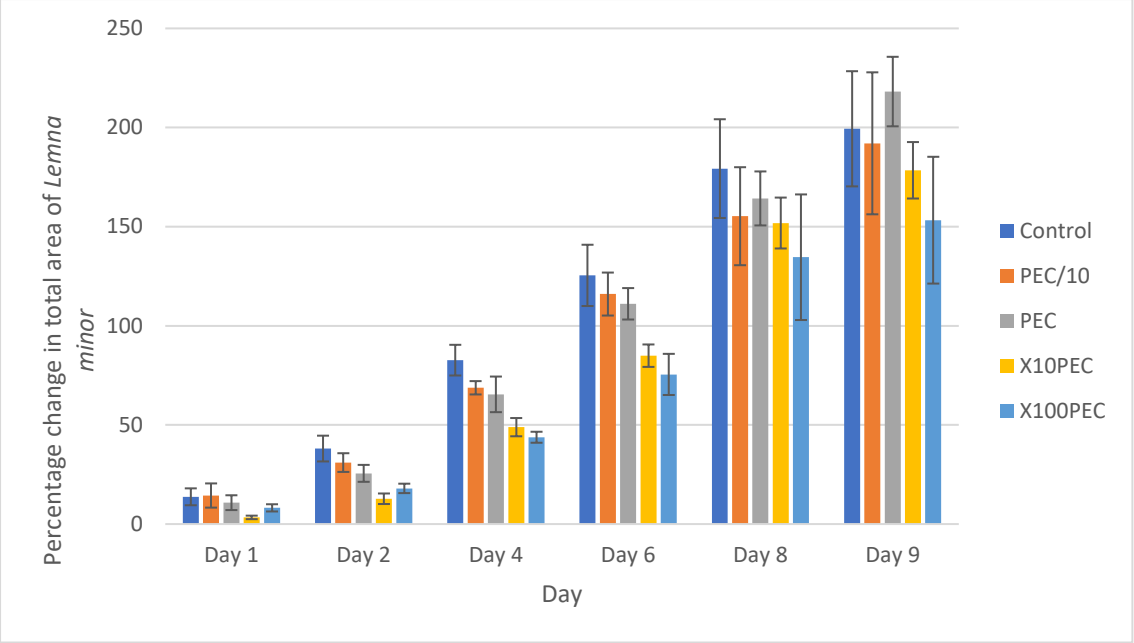


Figure 10. Average percentage changes in surface area of *Lemna minor* relative to the start of the experiment in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). Only data sets for the 9 day exposure group were included for each antibiotic to give the Day 9 dataset, with the following replicates: Control (Day 1 n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, Day 1 n = 5).

4.2.1.1. Average frond diameter

The average frond size (mm) for *Lemna minor* with different antibiotic exposures can be seen below in Figure 11 (Combined dataset), 12 (Day 1 dataset), 13 (Day 5 dataset) and 14 (Day 9 dataset). The average frond size can be seen to vary greatly between antibiotic and time exposures, with much standard error bar overlap. In Figure 11, the average frond diameter across all antibiotic exposures can be seen to increase from Day 0 to Day 2 and then decline over Days 4, 5 and 6, until another increase in Day 8 and further declines in Day 9. This can be seen most clearly for the PEC exposure, increasing from 2.09 mm on Day 0 to 6.69 mm by Day 2. The average frond size then declines to 3.61 mm on Day 4, and then declines further to 2.61 mm by Day 6. An increase can then be seen for Day 8 to 4.98 mm, which is followed by a drop to 2.82 mm on Day 9. The average frond size for the PEC exposure on Day 2, can be seen to be strongly impacted by the inclusion of the Day 9 data set. In Figure 14, the PEC exposure on Day 2 can be seen to exceed 8.71 mm in average frond size, with a standard error value of 2.68 +/-. When compared to the Day 5 dataset in Figure 13, the average frond size drops to 4.656 mm and a standard error of 0.66 +/-. A trend towards an increase or decrease in

average frond size with time or differences between antibiotic exposures is difficult to distinguish in the Figures.

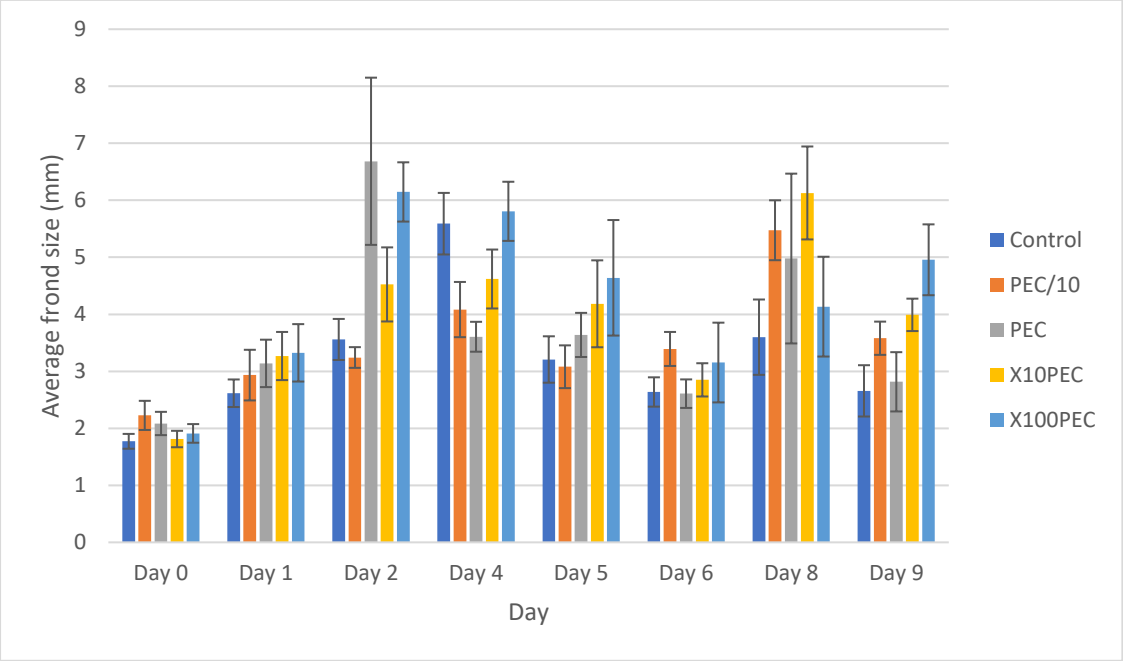


Figure 11. Average frond size / diameter (mm) of *Lemna minor* fronds in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). All data sets for different time exposure groups (24hours, 5 days and 9 days) were combined for each antibiotic exposure to give a Combined dataset, with the following replicates per exposure: Control (Day 0-1 n =21, Day 2-5 n =14, Day 6-9 n =7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, Day 0-1 n =15, Day 2-5 n = 10, Day 6-9 n =5).

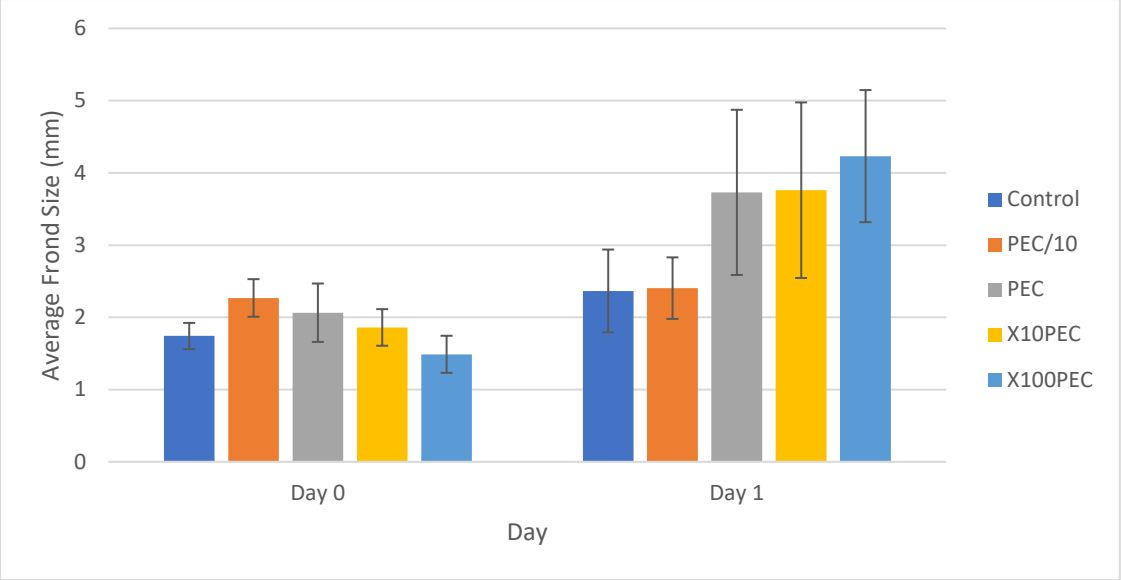


Figure 12. Average frond size / diameter (mm) of *Lemna minor* fronds in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars).Only data sets for the 24 hour exposure group were included for each antibiotic exposure to give the Day 1 dataset, with the following replicates for Day 0 and 1: Control (n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, n = 5).

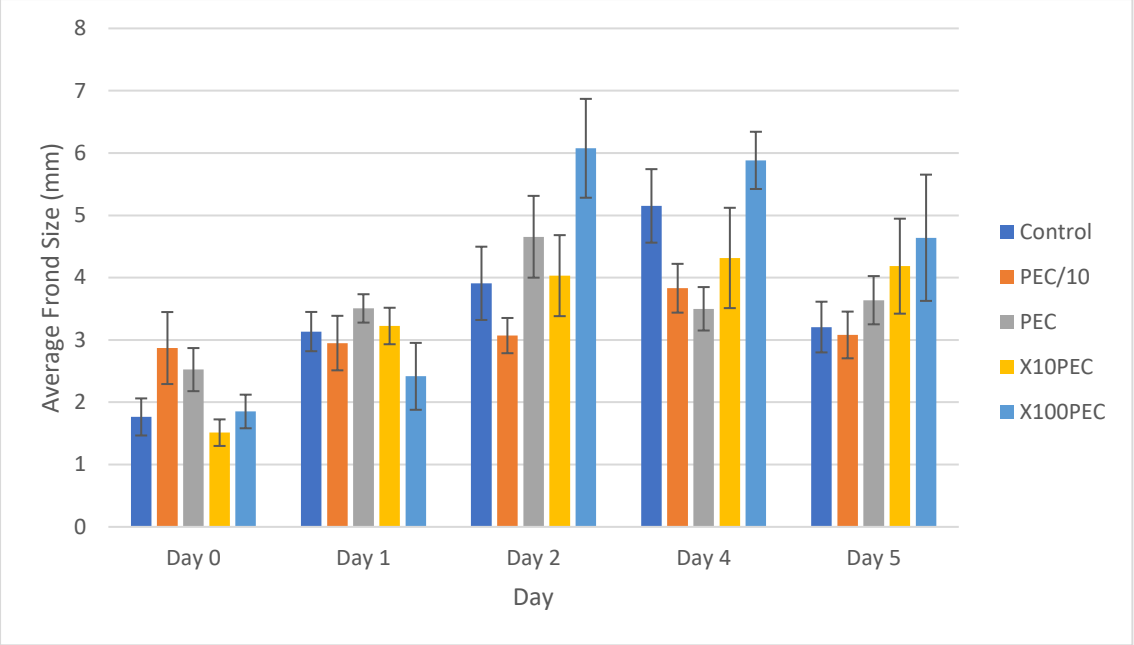


Figure 13. Average frond size / diameter (mm) of *Lemna minor* fronds in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars).Only data sets for the 5 day exposure group were included for each antibiotic exposure to give the Day 5 dataset, with the following replicates for days 0-5: Control (n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, n = 5).

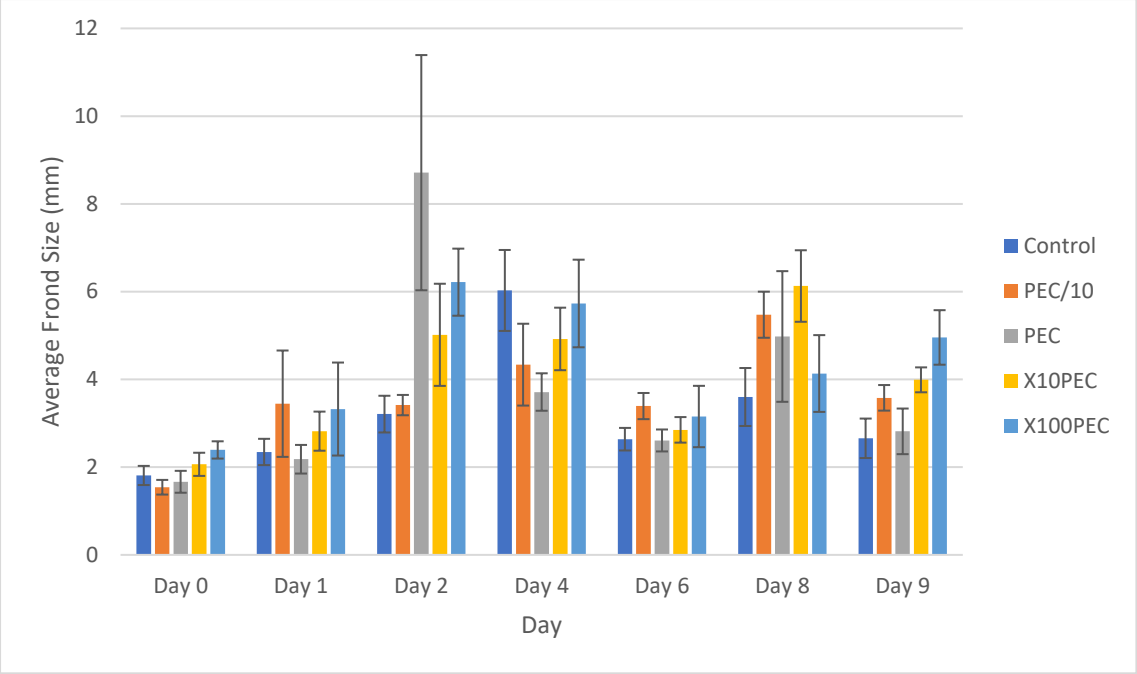


Figure 14. Average frond size / diameter (mm) of *Lemna minor* fronds in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). Only data sets for the 9 day exposure group were included for each antibiotic exposure to give the Day 9 dataset, with the following replicates for days 0-9: Control (n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, n = 5).

4.2.2. Chlorophyll a changes

The concentration of chlorophyll a (µg/mg of dry mass) of *Lemna minor* for each antibiotic exposure can be seen below in Figure 15 (Combined dataset) and in Figure 16 (with Day 1, Day 5 and Day 9 datasets separated). In Figure 15, the highest chlorophyll a concentration for the antibiotic exposures is 5.63 µg/mg of dry mass for the PEC exposure, followed by x100PEC (with 5.3), PEC/10 (with 5.25), x10PEC (with 5.1) and lastly the control (with 4.95). With the data separated in Figure 16, the highest average can be seen for the Day 5 control with a concentration of 6.52 µg/mg of dry mass, which contrasts Figure 15, where the control exposure had the lowest chlorophyll a concentration of 4.95 µg/mg of dry mass. In Figure 16, for the Day 1 dataset (24 hours), the highest chlorophyll a concentration for the antibiotic exposures is PEC with 6.17 µg/mg of dry mass, followed by x10PEC (5.77), x100PEC (5.74), PEC/10 (5.68) and lastly the control (4.46). This closely resembles the data of Figure 15; the control has the lowest concentration for the exposures and the highest exposure is the PEC in both Figures.

The concentrations for each antibiotic group vary further from Figure 15 with the Day 5 and Day 9 datasets in Figure 16. The control in both Figure 15 and 16 for the Day 1 dataset has the lowest concentration relative to the other exposures, however, in Figure 16 for the Day 5 dataset, the control has the highest concentration with 6.52 µg/mg of dry mass, followed by the PEC (with 6.49), PEC/10 (with 6.14), x10PEC (with 5.32), and the lowest concentration for x100PEC (with 6.1). The Day 9 dataset in Figure 16, finds the control has the lowest concentrations of the exposures with 3.73 µg/mg of dry mass, contrasting the Figure 16, Day 5 dataset, but complementing Figure 15 and the Figure 16 Day 1 dataset. The Figure 16 Day 9 dataset does have different concentrations for the exposures to Figure 15 and Figure 16 Day 1 dataset, finding the highest concentration with the exposure x10PEC (with 4.25), followed by PEC (with 4.22), and PEC/10 (with 3.91). A decrease in chlorophyll a content can be seen over time in Figure 16, though not in Figure 15 due to the nature of the combined datasets. An initial increase in chlorophyll a content can be seen from 24 hours to 5 days for all antibiotic exposures, except x10PEC, which decreases by 0.42 µg/mg of dry mass. The control can be seen to have the highest increase in chlorophyll a concentration from 24 hours to 5 days, increasing by 2.06 µg/mg of dry mass. The chlorophyll a concentrations decline from Day 5 to Day 9 to be below the 24 hour levels for all the antibiotic exposures. The largest decline between Day 5 and Day 9 datasets can be seen for the control, declining from 6.52 µg/mg of dry mass to 3.73, lower than 4.46 µg/mg of dry mass for the control at 24 hours.

A series of Anova tests were carried out to determine whether there were any significant differences in chlorophyll a concentrations between antibiotic exposures and over time. No statistically significant differences in chlorophyll a content between antibiotic exposures for a combined dataset was found ($F(8) = 0.689$, $P = 0.691$) using a Two Way Repeated Measures Anova. A significant decrease in chlorophyll a concentration was found with time for a combined data set ($F(2) = 13.71$, $P = <0.001$) using a Two Way Repeated Measures Anova. Post Hoc tests determined a significant decline in chlorophyll a concentration from day 5 to day 9 ($F(1) = 22.772$, $P = <0.001$).

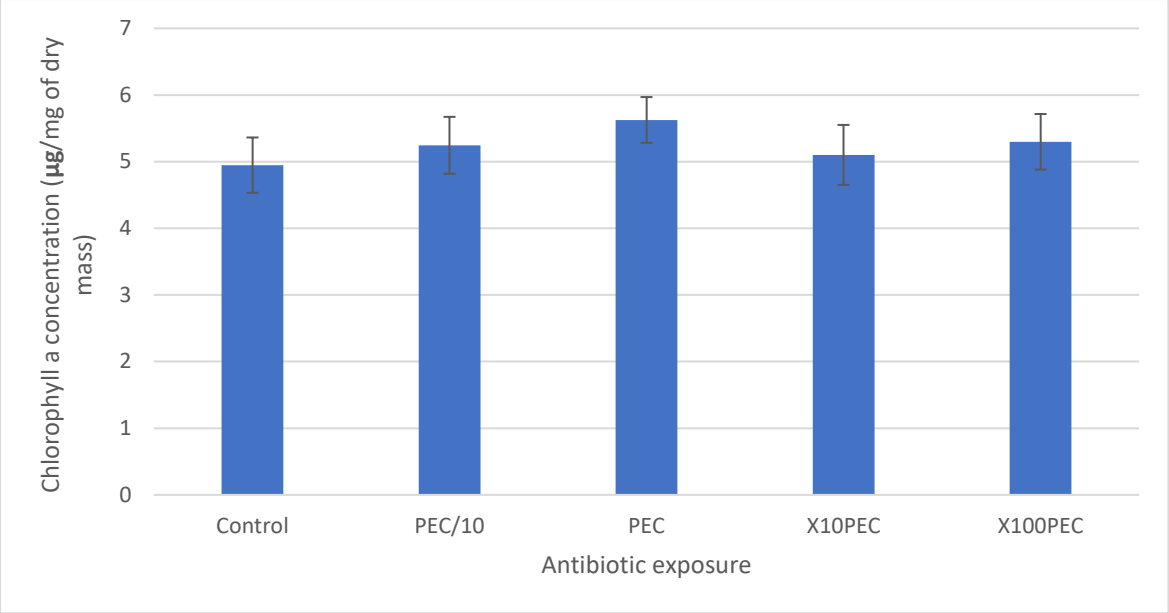


Figure 15. Average chlorophyll a concentrations (µg/mg of dry mass of *Lemna minor*) in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). All data sets for different time exposure groups (24 hours, 5 days and 9 days) were combined for each antibiotic exposure to give a Combined dataset, with the following replicates per exposure: Control (n = 21), PEC/10, PEC, x10PEC and x100PEC (for each exposure, n =15).

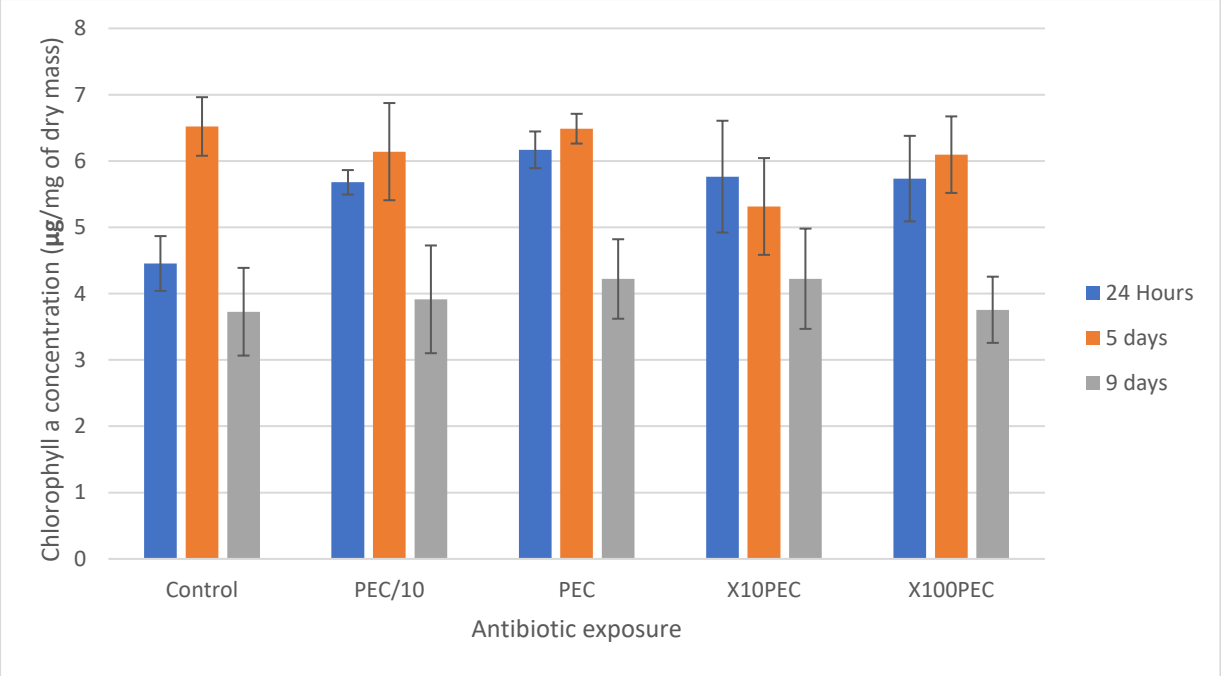


Figure 16. Average chlorophyll a concentrations (µg/mg of dry mass of *Lemna minor*) in response to predicted environmental concentrations (PEC) of antibiotics in UK Waste Water Treatment Plant effluent entering Constructed Wetlands (with customised standard error bars). The data sets for Day 1 dataset (24 hours), Day 5 dataset and day 9 dataset were kept separate and included for each antibiotic exposure, with the following replicates for all datasets: Control (n = 7), PEC/10, PEC, x10PEC and x100PEC (for each exposure, n=5).

4.3. Discussion

4.3.1. Interpretation of the results of this study

A clear pattern can be seen for an increase in percentage change (5 day data set; $F(1.86) = 253.779$, $P = <0.001$, 9 day data set; $F(1.204) = 158.431$, $P = <0.001$) in the surface area of *Lemna minor* with time for all antibiotic exposures; this can be seen in Figures 7 (Combined dataset), 9 (Day 5 dataset) and 10 (Day 9 dataset). The effect of antibiotic exposure on percentage change in surface area is less clear. In Figure 7, with the x100PEC exposure the percentage increase does not appear to be as great as other exposures by the end of the 9 day exposure period, even with the greatest percentage increase in the first 24 hours. This may suggest that while initial exposure to a x100PEC exposure over a short term does not affect growth or perhaps aids it, growth then slows down and may be impacted by the high exposure. In Figure 7, the control and PEC exposures often have the highest percentage increase in surface area, with the PEC exposure leading by day 9. This may suggest that with prolonged exposure to PECs the *Lemna minor* may experience higher growth rates. However, a percentage increase over a 9 day period with PECs does not necessarily produce a positive impact in the *Lemna minor*. It may be experiencing stress which may be visible with a longer exposure period.

Drawing such conclusions from Figures 7, 8 (Day 1 dataset), 9 and 10 to the influence of antibiotic exposures on the percentage change in surface area is not possible, with much standard error bar overlap between exposures for all Figures. As such statistical tests were conducted. Such tests found a significant difference in percentage change in surface area with time over the 9-day period ($F(1.204) = 158.431$, $P = <0.001$), showing that *Lemna minor* growth occurred during the exposure study. However, no significant differences (24 hour data set; $F(2) = 1.475$, $P = 0.244$, 5 day data set; $F(7.438) = 0.774$, $P = 0.619$, 9 day data set; $F(4.818) = 0.582$, $P = 0.708$) were found in percentage change in surface area between antibiotic exposure treatments, indicating that exposing the *Lemna minor* to the antibiotic exposures over 9 day periods does not affect the percentage increase in surface area. However, with a prolonged exposure period, this may not hold true. Overall, the results indicate that the growth rates of *Lemna minor* in CWs treating WWTP effluent are likely to not be affected by the antibiotic exposures present, which is positive news for the remediation of WWTP effluent and CW ecology.

The average frond diameters collected during the exposure study were initially planned to be analysed in the same process as the percentage change in surface area data, to determine if antibiotic exposures effect frond rate duplication. In the Figures 11 (Combined dataset), 12 (Day 1 dataset), 13 (Day 5 dataset) and 14 (Day 9 dataset), the average frond size can be seen to vary greatly between antibiotic and time exposures, with much standard error bar overlap. No clear patterns in the data can be seen for any of the Figures. This is most likely due to frond duplication; a frond diameter will increase in size and form several connected fronds that then split off from the original parent frond resulting in several daughter fronds and a decrease in the average frond diameter. The process then repeats. While duplication results in the increases in surface area seen above, for a single replicate the average frond size varies greatly over time with duplication rates. Each replicate used to calculate an average for the antibiotic and time exposure will be at different duplication stages and as such no clear pattern will emerge between antibiotic and time exposures and statistical tests will not provide useable results.

The chlorophyll a concentration of *Lemna minor* shown in Figures 15 (Combined dataset) and 16 (Separated dataset) provide no clear patterns for differences in concentration with antibiotic exposures and no significant differences ($F(8) = 0.689$, $P = 0.691$) between antibiotic exposures and chlorophyll a content were found with statistical tests. These are promising results for the survival of *Lemna minor* in CWs, with antibiotic exposures appearing to have no significant impact on the chlorophyll a content of the *Lemna minor*. The significant declines ($F(2) = 13.71$, $P = <0.001$) found for chlorophyll a content with time shown in Figure 16 and found to be statistically significant between 5 and 9 days ($F(1) = 22.772$, $P = <0.001$) is interesting, and may be due to the exposure

study time period used not allowing for the stabilisation of the chlorophyll a within the *Lemna minor*, following introduction to a new environment.

4.3.2. Comparison of this study's findings with the findings of other studies

This study found no significant differences (24 hour data set; F(2) = 1.475, P = 0.244, 5 day data set; F(7.438) = 0.774, P = 0.619, 9 day data set; F(4.818) = 0.582, P = 0.708) in growth rate with antibiotic exposures to the control, indicating that such antibiotic exposures do not impact the growth of *Lemna minor*, with the 9 day exposure period used in this study. The study Iatrou et al. (2017) had similar findings, even with higher antibiotic concentrations; 10,000 µg/L of metronidazole and cefadroxil, 2,000 µg/L of trimethoprim and sulfamethoxazole with 24 day exposures, no significant effects on the growth rate of *Lemna minor* was found. In comparison this study for the highest exposures used 195 µg/L (metronidazole) and 75 µg/L (trimethoprim). Other *Lemna minor* toxicity studies such as Cascone et al. (2004) and Mao et al (2023) have conflicting findings, with *Lemna minor* growth rate being significantly altered by antibiotic exposures. However, it is important to note that the exposures of such studies are much greater than this study and with longer time exposures. As such while these studies findings may be different, their findings may perhaps lend support to the lack of significant differences found for this study's antibiotic exposures and time exposures. Mao et al. (2023) with 2,000 µg/L ciprofloxacin exposures for 15 days (compared to 42.5 µg/L and 9 days in this study) found a significant reduction in growth rate, with exposure having only a 50% growth rate compared to 500% for their control by day 7. Cascone et al. (2004) found that with flumequine exposures as low as 50 µg/L with a 5 week exposure result in significant reduction in *Lemna minor* biomass. While this pharmaceutical was not used in this study, ciprofloxacin belongs to the quinolone family and the closest antibiotic used in this study. The ciprofloxacin used in this study was slightly lower than flumequine and the possible differences in behaviour and exposure period may account for the discrepancy in the results.

While this study found no significant differences (F(8) = 0.689, P = 0.691) in chlorophyll a content between antibiotic exposures, a significant decrease (F (2) = 13.71, P = <0.001) was found with time. Similar to this study Grenni et al. (2019) found that with exposures of 500 µg/L of sulfamethoxazole for 28 days, no significant differences in chlorophyll a content of *Lemna minor* were found. However, Cascone et al. (2004) found that with flumequine exposures (potentially similar to ciprofloxacin) a significant decrease in chlorophyll a content occurred. Studies such as Alkimin et al. (2019) and Kummerová et al. (2016) found differences in the chlorophyll a content of *Lemna minor* with diclofenac exposures as low as 4 µg/L for 14 days and 0.1 µg/L for 10 days, respectively. However, Alkimin et al. (2019) found increases in chlorophyll a content, while Kummerová et al. (2016), like this study, found decreases, from 19-53% with a range of diclofenac exposures. While finding significant

differences between exposures, Alkimin et al. (2019) also found that for all exposures, the chlorophyll a content stabilised by 14 days, with increases levelling off. With a longer exposure period, this study may have also seen a stabilisation in the decline in chlorophyll a content. Alkimin et al. (2019) stated that a longer exposure period was required to fully determine the effects of exposure on *Lemna minor* as while the *Lemna minor* appeared to cope with the exposures, the plant efforts required to recover and adapt to such exposures may ultimately compromise its survival.

4.4. Conclusion

With regards to the possible health of *Lemna minor* in CWs treating WWTP effluent with antibiotic exposures; overall, the results of growth rate and chlorophyll a content suggest that *Lemna minor* is not experiencing stress, with no physiological changes occurring even at x100PEC, 9 day exposures (objectives 3 and 4). However, while physiological changes may not be occurring within the exposures of the study, metabolomic changes may occur. The sensitivity of the growth rate and chlorophyll a content variables in comparison to metabolomics will be explored in following chapter.

Chapter 5. Effects of antibiotic exposures on the metabolome of *Lemna minor*

5.1. Introduction

There are several knowledge gaps regarding the use of macrophytes in CWs, including whether plants remediating antibiotics in CWs treating WWTP effluents are experiencing stress, and in the measurements used in plant health assessments. *Lemna minor* can be used as a test organism to help address such issues. It is important to note that many studies that find successful remediation of a range of contaminants using *Lemna minor* often take place in a laboratory microcosm, with highly regulated conditions (Ekperusi et al. 2019). The remediation performance of *Lemna minor* may change in the field in CWs and as such more studies using mesocosm experiments and investigations in CWs are needed to fully determine the overall performance of *Lemna minor* and whether they are experiencing stress with antibiotic exposures in CWs treating WWTP effluents. Prior to field investigations into antibiotic exposures in CWs, the measurements we use to determine plant health should be investigated along with controlled experiments of antibiotic exposure effect on *Lemna minor*. In lab based *Lemna minor* toxicity study's growth rates and occasionally metabolic variables such as chlorophyll a content are the most frequently used measurements to assess plant health. Determining whether such measurements are adequate to accurately assess plant health is of great importance for lab and field studies of CW macrophyte remediation. Metabolomics could potentially be used as an early indicator of *Lemna minor* stress with antibiotic exposures, that may appear before growth or chlorophyll content changes.

Some toxicity studies such as Aliferis et al. (2009), Kim et al. (2017) and Gomes et al. (2017) have found that while no significant effects on the growth and chlorophyll a content of *Lemna minor* occur, significant metabolic changes can. Such metabolic changes may indicate a threat to long term plant survival, which could have potential knock on effects from individuals to community levels, reducing plant species diversity, ecosystem functions and CW remediation. Such studies suggest that certain metabolic variables may be more sensitive than physiological (growth rates) and the metabolic variable of chlorophyll a, and could be used as an early warning of plant stress. Few studies extract and analyse the full metabolic profile of *Lemna minor*, with Wahman et al. (2022), Kostopoulou et al. (2020) and Aliferis et al. (2009) being the only available studies. However, there are several studies that look at targeted metabolic variables such as enzymatic biomarkers and other sensitive variables including Kim et al. (2017), Kummerová et al. (2016), Forni et al. (2012) and Alkimin et al. (2019). As such an investigation into the metabolome of *Lemna minor* in a toxicity study could provide several valuable insights.

Metabolomics is the non-biased identification and quantification of all extractable metabolites in a biological system (from organism, tissue, cell or even cell compartments), (Fiehn, 2002). Metabolites are the end products of cellular regulatory processes (Fiehn, 2002) and include many different compounds including amino and fatty acids, carbohydrates, vitamins, and lipids (Dunn and Ellis, 2005). The metabolome is made up of all the extractable chemical metabolites present in the plant (Gaffney et al., 2021). The size of a metabolome varies greatly depending on the organism of study; a simple yeast can contain approximately 600 metabolites while plants can reach 200,000 and the human metabolome even greater (Dunn and Ellis, 2005). While metabolomes can be great in size, they can also be of a lower sample complexity than other variables providing easier analysis, for example a yeast can contain over 6000 genes but only around 600 metabolites (Dunn and Ellis, 2005). HPLC-HRMS analysis can determine the entire chemical profile (metabolome) of a sample (Fiehn et al., 2002), as is used in this study to determine the profile of *Lemna minor* (see section 3.1.7). Metabolites are very sensitive to stressors such as antibiotics and so can be used to draw conclusions into the status of a system (a cell, plant, or ecosystem) (Dawid and Hille, 2018; Fiehn 2002; Piasecka et al., 2019). The profile can then be investigated to determine whether the *Lemna minor* is showing characteristic stress responses to the antibiotic exposures (Fiehn et al., 2002). This can be done by determining which chemicals have the greatest different abundances with different exposures, followed by attempts to identify the most important chemicals. It is then possible to link certain metabolic changes/ identified chemicals to a specific biochemical pathway in an organism (Fiehn, 2002). Such links can be used to assess whether the processes within *Lemna minor* are being affected or undermined by the antibiotic and time exposures.

The methods used in this study and the metabolomic approaches have been used in many other studies to investigate plant stress responses due to environmental stress such as drought (O'Harrigan et al., 2007; Richter et al., 2015; Röhlig et al., 2009; Sun et al., 2015; Witt et al., 2012) and even pharmaceuticals (Xue et al., 2022). The usefulness of metabolomic analysis cannot be understated, however, interpretation of the data is complicated and must be done with care due to the convoluted nature of plant metabolism and to prevent bias entering the chemical identification process (Fiehn, 2002). While specific metabolites can be identified, caution must be taken with such identification, there are assumptions and confidence is limited, until repeat analyses can be conducted alongside known concentrations of suspected chemicals (Fiehn, 2002). The influence of bias on studies can be reduced by using appropriate preparation and analytic procedures with an untargeted metabolomics approach which considers all metabolites, excluding none, as was conducted in this study (Dunn and Ellis, 2005; Fiehn 2002). Targeted metabolomics approaches do not consider unknown metabolites instead focussing on metabolites chosen prior to analysis, using

known reference standards, as such this method can miss important metabolomic changes (Gaffney, 2022). Another issue of bias that can occur is focussing on chemicals with highest abundances, instead of analysing changes in relative abundance between samples (Fiehn, 2002). Such considerations mentioned above in both the lab method and statistical analysis provide more reliable and environmentally relevant findings.

Investigating the effects of antibiotic exposures on growth rate, chlorophyll a content and the metabolome (objectives 3, 4 and 5, respectively) could be used to help establish whether *Lemna minor* in CWs treating WWTP effluent could be experiencing stress with antibiotic exposures and as such potentially impacting CW remediation. As outlined above metabolomics is a highly useful method to determine whether a plant may be showing signs of stress, which may not yet be visible with physiological changes. In this study the full metabolic profile of *Lemna minor* was analysed to provide a highly sensitive variable that could be used to help assess whether growth rate and chlorophyll a content are adequate variables in determining plant stress. By assessing the results provided in this study, an indication into whether the current practices of *Lemna minor* toxicity studies which only analyse growth rate and chlorophyll a content can possibly be made and be used to inform further toxicity studies, with regards to the variables required to accurately determine long term *Lemna minor* health and survival.

5.2. Results

5.2.1. Principal Components Analysis

The PCA plot generated for this study can be seen below in Figure 17; the chemicals used accounted for 72.7% of the variability between exposures for PC 1 and 10.4% for PC 2. While there was much overlap between many of the antibiotic and time exposures it is possible to draw indications to whether certain antibiotic and time exposures resulted in changes in the metabolome of *Lemna minor*. Two groups of clustering can be seen; the blue and red groupings and a possible anomaly (9 day control). Most of the antibiotic and time exposures clustered together with controls and quality controls in the blue grouping, indicating little change to the metabolome of *Lemna minor* with certain antibiotic and time exposures. Changes to the metabolome can be seen with the red grouping, in which 4 out of 4 replicates for 9 day x100PEC and 3 out of 5 for 5 day x100PEC clustered separately from the main grouping. Two out of 5 replicates for 5 day x100PEC clustered with the main clustering group in blue along with exposures to x100PEC for shorter time periods and lower antibiotic exposures across all time periods. This indicates that changes to the metabolic profile begin to emerge at exposures to x100PEC after 5 days and are clearly pronounced after 9 days and as such *Lemna minor* may have experienced changes to its metabolome.

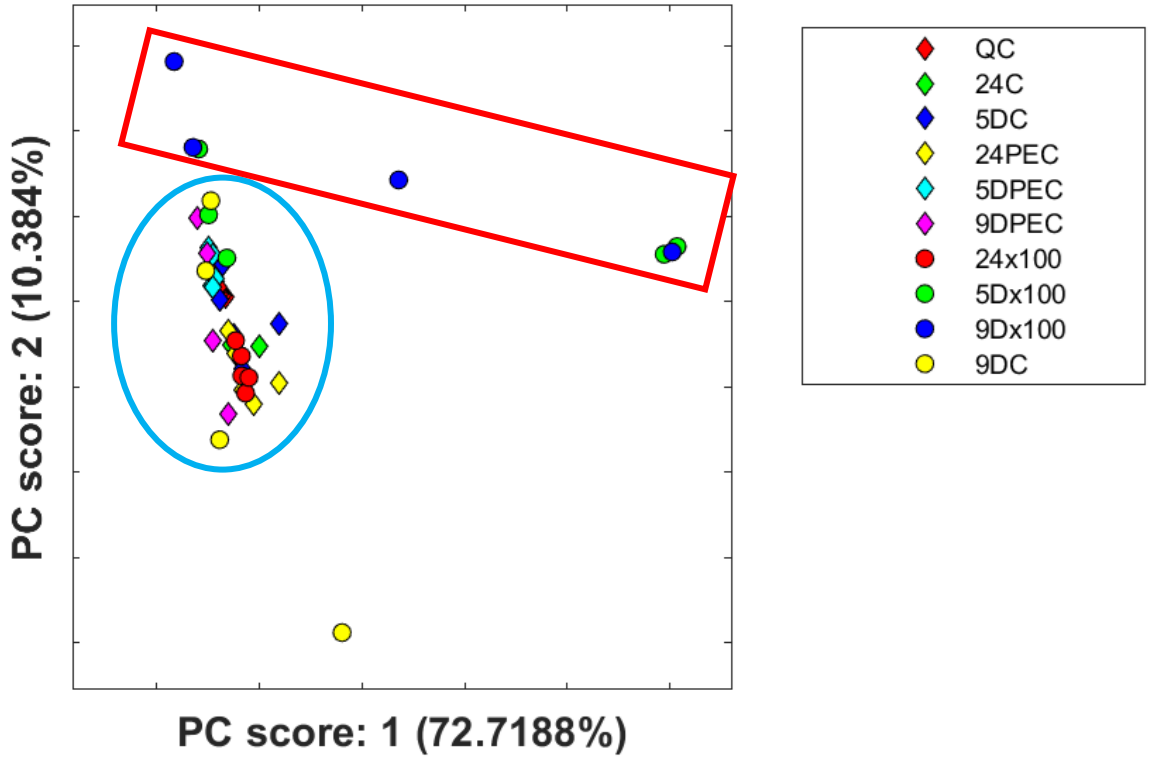


Figure 17. Principal Component Analysis plot. Chemicals used to generate axis 1 account for 72.7% of variation between the exposures and axis 2 10.4%. Clustering has been circled with blue and red to aid discussion. Key: Quality Control (QC), 24 hour Control exposure (24C), 5 Day Control exposure (5DC), 9 Day Control exposure (9DC), 24 hour Predicted Environmental Concentration (PEC) exposure (24PEC), 5 Day PEC exposure (5DPEC), 9 Day PEC exposure (9DPEC), 24 hour x100PEC exposure (24x100), 5 Day x100PEC exposure (5Dx100), 9 Day x100PEC exposure (9Dx100).

5.2.2. Identifications of chemicals

Further analysis using a loadings plot identified the top 20 chemicals most responsible for variations between exposures for PC1 and PC2. These were investigated further using Progenesis and ChemSpider software. Using the potential chemical formulas corresponding to their accurate mass, chemicals were tentatively annotated and the determined to be plant metabolites and its potential role in the *Lemna minor*. Fifteen tentatively identified plant metabolites can be seen below in Table 14 and in the appendix Table 18 possible identifications for other chemicals, with mz ratio and retention times can be seen. For some chemicals suggested identifications were unlikely, for example, chemical PC1.12 had two suggestions, a gingerglycolipid (a plant metabolite, see Table 14) and another for a product associated with a parasitic fungus. For PC1.1 and PC2.6 (see Tables 14 and 18), 4/6 suggestions were for phalates (a plant metabolite) and the remaining 2 for types of bile acids. Phalates are a plant metabolite but also a type of contaminant (see Table 14 for further

details). Due to the identical treatment of replicates from all exposures and procedures to maintain minimal contamination, it was determined that the presence of phalates were likely plant metabolites. The phalate chemical differences between treatments could possibly be due to effect of the small amount of solvent used when making antibiotic stock dilutions, with more solvent being present for higher x100PEC solutions, which may account for the differences between exposures. However, this amount is negligible and is likely to not be driving the differences. However, such suggestions undermine the confidence of chemical identifications made using the programmes and as such the identifications made in Table 14 below are not certain. Possible plant metabolite identifications (see Table 14) have a range of important roles in the *Lemna minor* including plant defence from UV radiation with flavonoids and disease resistance with neolignans. Some metabolites identified are fundamental to the survival of the *Lemna minor*, for example, gingerglycolipids are fundamental building blocks in cell membranes.

Table 14. Possible plant metabolite identifications from the top 40 chemicals responsible for the chemical variation between exposures on the PCA plot (Figure 17).

PCA axis and rank number	Chemical name and/or Formula suggestions	Confirmation as a plant metabolite and function within plants	Possible Identifications
PC1.1 and PC2.6	$C_{24}H_{38}O_4$ (Isooctyl phthalate) $C_{24}H_{38}O_4$ (Dioctyl phthalate $C_{24}H_{38}O_4$ (Bis(2-ethylhexyl) phthalate) $C_{24}H_{38}O_4$ (Diethylhexylphthalate)	All suggestions are similar chemicals of the phthalate group. These are a type of plant metabolite that may have antimicrobial properties in plants (Saleem et al., 2009). Dioctyl phthalate is found in *Eleutherococcus sessiliflorus, Ekimia bornmuelleri*, and other plants (PubChem, 2004-) and Diethylhexylphthalate found in *Ageratina altissima, Chromolaena odorata*, among other plants (PubChem, 2004-). However, phthalates are also an emerging contaminant from human activity (Przybylińska and Wyszkowski, 2016) and as such these chemicals could be a natural plant metabolite or a contaminant.	Phthalate
PC1.3 and PC2.4	$C_{29}H_{30}O_{17}$ (Malonylapiin)	Malonylapiin is a type of plant metabolite called a flavonoid, functionally related to apiin (PubChem, 2004-). Flavonoids regulate plant development, pigmentation, UV protection, and are involved in defence and signalling between plants and microorganisms (Eckey-Kaltenbach et al., 1993; Mathesius, 2018; Pradas del Real et al., 2017).	Flavonoid
PC1.3 and PC2.4	$C_{29}H_{30}O_{17}$	6 possible suggestions were provided by ChemSpider (2023b), all of which were different flavonoids. Due to the ambiguity, these have not been included and instead a classification as a flavonoid is suggested.	Flavonoid
PC1.7 PC1.20	For both separate chemicals $C_{19}H_{37}NO_3$ was suggested	This chemical formula is likely to be Palmitoyl alanine (PubChem, 2004-), which is a type of plant metabolite (Lynch and Fairfield, 1993). It is possibly a sphingolipid or an enzyme that catalyses the first step in the synthesis of sphingolipid assembly (Lynch and Fairfield, 1993).	Palmitoyl alanine
PC1.9 and PC2.2	$C_{35}H_{34}O_{16}$	This is likely a type of glucosiduronic acid called [(2R,3S,4S,5R,6S)-3,4-dihydroxy-6-[5-hydroxy-2-(4-hydroxyphenyl)-7-oxochromen-3-yl]oxy-5-[(2S,3R,4S,5R)-3,4,5-trihydroxyoxan-2-yl]oxyoxan-2-yl]methyl (E)-3-(4-hydroxyphenyl)prop-2-enoate (ChEBI, 2022a; PubChem, 2004-). These acids occur in many biological organisms including plants and as such are	Glucosiduronic acid

PCA axis and rank number	Chemical name and/or Formula suggestions	Confirmation as a plant metabolite and function within plants	Possible Identifications
		fundamental to their existence and so there is little specific information regarding their roles in plants.	
PC1.12	$C_{33}H_{56}O_{14}$	This chemical is a gingerglycolipid A, which occurs in range of organisms including plants such as *Guapira graciliflora* (PubChem, 2004-). Lipids are essential in plants for maintaining cells, organelles and acting as hydrophobic barriers in membranes (Kim, 2020). Lipids can also store energy as seeds and act as a signal molecule to regulate cell metabolism (Kim, 2020).	Gingerglycolipid
PC1.13	$C_{26}H_{28}O_{14}$ (Glucofrangulin B)	This chemical is a type of plant metabolite called a quinone (PubChem, 2004-). Quinones are a biological pigment found in plants as well as bacteria, fungi, and some animals (Devi and Mehendale, 2014). Quinones may also be released from plant roots, potentially to inhibit the growth of surrounding plants and their competition (Matvienko et al., 2001).	Quinone or flavonoid
PC1.13	$C_{26}H_{28}O_{14}$ (Apiin)	Apiin is a type of plant metabolite called a flavonoid (Saleh and Yousaf, 2018) and is found in plants such as *Crotalaria micans* and *Limonium axillare* (PubChem, 2004-). Flavonoids regulate plant development, pigmentation, UV protection, and are involved in defence and signalling between plants and microorganisms (Eckey-Kaltenbach et al., 1993; Mathesius, 2018; Pradas del Real et al., 2017).	Quinone or flavonoid
PC1.13	$C_{26}H_{28}O_{14}$ (Neoschaftoside) $C_{26}H_{28}O_{14}$ (Schaftoside)	Neoschaftoside and Schaftoside are flavonoids found in plants such as Radula complanate and Artemisia judaica (PubChem, 2004-) and Glycyrrhiza macedonica and Silene firma (PubChem, 2004-) respectively. Flavonoids regulate plant development, pigmentation, UV protection, and are involved in defence and signalling between plants and microorganisms (Eckey-Kaltenbach et al., 1993; Mathesius, 2018; Pradas del Real et al., 2017).	Quinone or flavonoid
PC1.15 and PC2.3	$C_{35}H_{34}O_{17}$	This chemical formula has two possible plant metabolite identifications; Cyanidin, a type of plant pigment found in species such as Salix atrocinerea (Dormán et al. (2016); PubChem, 2004-; PubChem, 2004-) and vitexin a flavonoid, with a role as a platelet aggregation inhibitor (PubChem, 2004-; PubChem, 2004-).	Cyanidin, Vitexin (flavonoid) or Iridoid glucoside
PC1.15 and PC2.3	$C_{30}H_{40}O_{18}$	This chemical formula is likely depressine, a type of iridoid glucoside, found in *Gentiana depressa* (Chulia et al., 1996; PubChem, 2004-). It is involved in plant defence by giving the plant a bitter taste and potentially has growth inhibitory activities against insects (Liu and Mander, 2010).	Cyanidin, Vitexin (flavonoid) or Iridoid glucoside

PCA axis and rank number	Chemical name and/or Formula suggestions	Confirmation as a plant metabolite and function within plants	Possible Identifications
PC2.5	$C_{21}H_{37}N$	This chemical is a type of Benzylamine called n-Tetradecylbenzylamine, found in plants such as *Moringa oleifera* and is involved in regulating the hydrolysis of nitrile compounds (PubChem, 2004-; PubChem, 2004-).	Benzylamine
PC2.9	$C_{24}H_{30}O_6$ (Magnoshinin)	Magnoshinin is a type of plant metabolite found in the plant *Magnolia salicifolia* (PubChem, 2004-). It is a type of neolignan, involved in plant disease resistance defences (de Castro Oliveira et al., 2017; Kadota et al., 1987).	Neolignan
PC2.17	$C_{21}H_{28}F_2O_2$ (4,4-Difluoropregn-5-ene-3,20-dione)	This chemical a type of 3-oxo-Delta (5)-steroid found in plants and is involved in regulating the brassinosteroid biosynthesis pathway (Ephritikhine et al., 1999; PubChem, 2004-). Brassinosteroids are involved in plant growth and environmental adaptation (Ohnishi, 2018).	Chemical involved in regulating brassinosteroids

5.3 Discussion

5.3.1. Using the findings of this study to address key questions

Using this study's findings, two important questions can potentially be answered. Firstly, whether the *Lemna minor* in CWs exposed to antibiotics are likely to be experiencing stress according to the results from growth rate, chlorophyll a content and metabolomic investigations. Due to the importance of plants including *Lemna minor* in CW remediation of contaminants both directly with uptake and indirectly by supporting other ecosystem functions including microbial degradation, it is of interest to determine whether antibiotic exposures in CWs is likely to be affecting the health of such plants. Secondly, whether the variables of growth rate and chlorophyll a content alone are sufficient to accurately determine whether *Lemna minor* is experiencing stress in toxicity studies, with metabolomics used in this study to help determine this. Determining whether these variables are sufficient is of great importance as many toxicity studies using *Lemna minor* often only use growth rate and chlorophyll a content as the key indicators of plant health with this practice visible in studies such as Cascone et al. (2004), Gomes et al. (2020) and the OECD (2006) guidelines for *Lemna minor* toxicity tests that focusses on growth rates as a key measure of toxicity. If these variables alone are not an adequate indicator of overall plant health, this may have implications for the validity of the findings for many different studies that include an assessment of *Lemna minor* health.

5.3.2. Interpretation of the results of this study

With elevated and prolonged x100PEC exposure, the metabolome of *Lemna minor* was found to be altered. Potential identification for the chemicals changing in abundance were found to be of great importance in *Lemna minor* survival and as such their change in abundance could have a detrimental effect on the health, growth, and survival of the *Lemna minor* over time. While elevated and prolonged exposure to x100PEC of antibiotics may result in changes to the metabolome of *Lemna minor*, the data did not suggest that *Lemna minor* experience metabolomic changes with PECs during the 9 day exposure period. PEC exposures cluster strongly with controls and quality controls in the blue group suggesting that the metabolomes of *Lemna minor* in CWs exposed to WWTP effluent were not altered. Metabolomic changes could be seen to occur with elevated exposures, giving greater confidence to the results indicating PEC exposures do not result in metabolomic alterations. While the results indicate that metabolomic changes do not occur with PEC exposures, this cannot be stated for certain as changes may have occurred with exposure periods longer than 9 days. As such PECs may require even longer exposure periods to induce effects. This study also cannot determine whether antibiotic concentrations accumulate in certain areas of CWs with slow throughflow, which may alter the metabolome of the *Lemna minor* over time. While the *Lemna*

minor may not show metabolomic changes with PEC exposures, other plant species may experience effects. However, the findings of this study do indicate that *Lemna minor* and potentially other plants in CWs treating WWTP effluent do not experience metabolomic changes at the concentrations predicted based upon prescription rates in the United Kingdom. This is a positive finding for WWTP effluent remediation in CWs but also plant ecology and ecosystem services.

5.3.3. Comparison of this study's findings with the findings of other studies

This study was not alone in finding metabolomic changes in *Lemna minor* with antibiotic exposures. The metabolomic changes found (at x100PEC at 5 and 9 day exposures) indicate that the *Lemna minor* experienced stress with the exposures, with abundance changes in possible chemicals such as flavonoids and gingerglycolipids. However, as no effects on growth rate and chlorophyll a content were found, the *Lemna minor* may be able to cope with such exposures. But such metabolomic changes may have long term effects on *Lemna minor* health and survival, as mentioned previously, Alkimin et al. (2019) states that additional adaptive efforts due to such exposures may ultimately compromise plant survival. Furthermore, the findings of metabolomic changes at x100PEC, with 5 days and even more strongly at 9 days indicate that similar metabolic changes could potentially also occur at PEC exposures with a longer exposure period. The study Wahman et al. (2022) also found metabolomic changes with pharmaceutical exposures when analysing the full metabolome of *Lemna minor.* With diclofenac exposures of 3,000 µg/L and 30,000 µg/L for 96 hours, the profile changed with key indicators of stress. Alterations including the increase of abundance of several fatty acids such as oleamide (at 30,000 µg/L) and changes in the abundances of flavonoids such as vicenin-1 and dipeptides such as N-alanyl-alanine (at 3,000 µg/L) were identified using a mixture of un-targeted and targeted analysis. While there are not many studies that extract and analysis the full chemical profile of *Lemna minor* like this study and Wahman et al. (2022), there are several studies that analyse the effect of pharmaceuticals and contaminants on certain key chemicals. Kostopoulou et al. (2020) found that with exposure to the herbicides metribuzin (at concentrations of 500, 1,000, 5,000, 10,000 and 25,000 µg/L) and glyphosate (at concentrations of 5,000, 10,000, 25,000, and 50,000 µg/L) for 72 hours, several effects on key metabolites in *Lemna minor* were observed, primarily focussed on amino acids in a targeted analysis. Exposures were found to increase amino acid abundance including γ-aminobutyric acid, salicylate, caffeate, α,α-trehalose, and squalene. These amino acids are of great importance, playing multiple roles in plant metabolism including signalling and structure protection (Kostopoulou et al. 2020).

5.3.4. The implications of this studies findings for Lemna minor in Constructed Wetlands

With regards to the possible health of *Lemna minor* in CWs treating WWTP effluent with antibiotic exposures, overall the findings of this study suggest that the *Lemna minor* is not experiencing stress, with no physiological or metabolomic changes at the most environmentally relevant concentrations (PECs). No changes in percentage change in surface area and chlorophyll a content were found. While metabolomic changes can be seen at x100PEC exposures, no changes were found to occur in the *Lemna minor* with PEC exposures for any period. This is a positive finding for the remediation of pollutants in CWs along with CW plant community structure, diversity, and ecosystem functions. However, as suggested previously *Lemna minor* at PEC exposures for longer periods than 9 days may experience metabolomic changes. A further study investigating growth rate, chlorophyll a content and metabolomic analysis with a prolonged exposure period with PEC and x100PEC exposures is required to determine this. Such a study would also be of interest to determine whether chlorophyll a content stabilises with a longer period and whether over time the *Lemna minor* would adapt to the exposures or ultimately the health of the *Lemna minor* be undermined.

5.3.5. The sensitivity of physiological and metabolomic measurements

This study finds that the variables growth rate and chlorophyll a content may not be adequate to accurately determine the health of *Lemna minor*. While no changes to growth rate and chlorophyll a content could be found with the antibiotic exposures, metabolic changes occurred, with the possible chemical identifications made indicating plant stress. The results indicate metabolomic changes may be more sensitive than physiological and chlorophyll content and as such could potentially be used as an early warning of potential negative effects on plant health, growth and photosynthesis that may occur over time. Studying metabolomic changes could also be used to indicate whether a plants performance in CW contaminant remediation may decline. Other studies have also found similar findings, with metabolomic variables appearing to be more sensitive than growth rate. Gomes et al. (2020) found that with exposures to amoxicillin and enrofloxacin (at concentrations of 2 µg/L for 14 days), no significant differences on the growth rate of *Lemna minor* could be seen but significant decreases in hydrogen peroxide (for both antibiotics) and significant decreases in mitochondrial electron transport chain enzymes (for enrofloxacin) could be seen, indicating oxidative and respiratory stress. As Gomes et al. (2020) found significant declines in frond number with oxytetracycline exposure (at concentration of 1 µg/L for 14 days) along with metabolic changes, this further emphasises that growth rate and visible changes may not be the most accurate variable to assess long term plant health and response to antibiotic and pharmaceutical exposures. Furthermore, Kim et al. (2017) found that with coronatine exposures (of 300 µg/L over 32 days), no significant differences in the growth rate of *Lemna paucicostata* could be found with exposures but

significant changes in metabolite abundances including amino acids, alcohols, fatty acids, phenolics and sugars were found. However, not all studies find such clear data. Forni et al. (2012) found that alongside significant metabolic changes to henylalanine ammonia-lyase (key enzyme in shikimic acid pathway) and guaiacol peroxidase, visible stress symptoms of yellowing leaves and dehydrated fronds could be seen with a sodium dodecyl sulphate *Lemna minor* toxicity study. However, such changes could only be seen at exposures of 50,000 µg/L and 100,000 µg/L for 7 days. With lower concentrations of 10,000 µg/L and 25,000 µg/L, visible stress symptoms could no longer be seen, but metabolic still could. Overall, the results of Forni et al. (2012) suggest that metabolic variables are more sensitive than physiological.

While growth rate may not be as sensitive as metabolomic analysis, chlorophyll a content may be a useful compromise between these variables, possibly being more reliable than growth rate to capture stress effects but not requiring as extraction intensive methods as many metabolic variables and full metabolome extractions. Kummerová et al. (2016) found that while no significant changes in growth rate could be found for their *Lemna minor* exposures, chlorophyll a content and other metabolic changes were found to be more sensitive indicators of stress. With significant differences in chlorophyll a content with diclofenac exposures, significant increases in malondialdehyde content (indicating a reduction in plasma membrane integrity) was also found. However, while chlorophyll a content may be a more sensitive indicator than growth rate, the findings of several studies (including this study) strongly suggest that alone or only with growth rate results, it may not be a reliable indicator for long term plant health. Gomes et al. (2017) found that with ciprofloxacin exposures (of 750, 1050, 2250, 3050 µg/L for 5 days), while no significant differences were found for chlorophyl content, significant differences for several other metabolic variables could be seen at 1050 µg/L and higher. Changes in variables such as maximal PSII photochemical efficiency, photochemical quenching and electron transport rates indicated important changes to photosynthesis that could have long term impacts on the *Lemna minor* that chlorophyll content did not capture.

Furthermore, Krupka et al. (2021) investigated the effects of tetracycline exposures (of 4.44 10^5, 1.11 10^6 and 4.44 10^6 µg/L for 7 days with a following recovery time of 7 days) on *Lemna minor*. The growth rate and chlorophyll a content were found to recover quickly during the recovery time, no longer being significantly different to the controls, with growth recovering from inhibition rates as high as 70%. However, the mitochondrial activity, while it improved never fully recovered. By the end of the recovery period there was significant reduction in dead mitochondria frequency (23% for 4.44 10^6 µg/L) but such exposures were still found to be significantly different to the control, unlike growth rate and chlorophyll a content. The findings of Krupka et al. (2021) indicated that growth rate and chlorophyll a content may be sufficient to determine *Lemna minor* health with antibiotic

exposures, but that other metabolomic variables such as mitochondrial activity were more sensitive to exposures. The study by Alkimin et al. (2019) also found similar results. While significant changes in chlorophyll a content were found with diclofenac exposures (mentioned previously), other metabolic variables such as enzymatic biomarkers were more sensitive. For example, the biomarkers catalase, ascorbate peroxidase and glutathione-S-transferases were found to be significantly different in abundance to the controls with diclofenac exposures as low as 4 µg/L, while chlorophyll a content only became significantly different with higher exposures of 20 and 100 µg/L. While many studies such as those mentioned above find that chlorophyll a and other metabolic variables are more sensitive than growth rate, this has not been found by all studies. Grenni et al. (2019) found that with sulfamethoxazole exposures (of 500 µg/L for 28 days) no significant differences in chlorophyll a content in *Lemna minor* were found, but a significant decline in total frond area was, of up to -33.7%, indicating stress. Though the findings of this study appear to be an outlier, the conflicting findings of studies show that more research is required into the adequacy of variables in establishing short and long term plant health. The findings of both this study and others indicate that alongside growth rate and chlorophyll a content, other metabolic variables should possibly be considered to provide a more reliable indicator of overall plant health.

5.4. Conclusion

To determine whether metabolic variables should be included in plant health assessments along side growth rate and chlorophyll a content, research is necessary regarding if the health and survival of *Lemna minor* is detrimentally effected by exposures that result in metabolomic changes but no significant changes to growth rate and chlorophyll a content. It would be of great interest to investigate if such effects occur with long term exposures. Some of the variables mentioned previously such as mitochondrial activity and enzymatic biomarkers could be investigated to determine the reliability of the growth rate and chlorophyll variables and help us determine whether they should be incorporated into *Lemna minor* toxicity studies. Full metabolic extraction and analysis as carried out by this paper may not be possible for all studies regarding due to the instruments required to perform such analysis (HPLC-MS may not be widely available to all researchers). However, such analysis could be of great use to help determine which metabolic variables are most reliable for determining *Lemna minor* health. A longer exposure study using *Lemna minor* investigating the effects of antibiotics on growth rate, chlorophyll a content, specific metabolic variables and full metabolomic analysis would be of great value. A longer exposure period would enable an investigation into whether certain variables stabilise over time indicating an ability of the *Lemna minor* to cope with such exposures or if the *Lemna minor* ultimately begins to show signs of being undermined with such exposures. A longer exposure study would also enable further

investigation into whether PEC exposures in CWs may cause metabolomic changes in *Lemna minor* over prolonged periods, potentially compromising the survival of *Lemna minor* in CWs over the long term. Such findings could potentially have direct applications to CW remediation performance. Studies into the sufficiency of different variables in determining plant health is required, not just to inform *Lemna minor* toxicity studies but also for wider plant assessments and research which then influence environmental policy across a broad range of areas.

Chapter 6. General discussion

Key knowledge gaps and potential avenues of research include the design of CW research studies, the mechanisms and relationships involved in microbial and macrophyte contaminant removal and the use of new technologies such as microbial fuel cells. Answering such questions is of great importance, to adequately inform policy and CW design and implementation.

6.1. Scaling of studies (size and time)

Recently published studies over the last decade often use small test scales, both in size (laboratory and micro/mesocosms) and time scales (days to months) (Liu et al., 2019a; Vymazal et al., 2021). Reasons behind such decisions range from cost, feasibility, and the desire for a high degree of control over variables, to allow for strong causal links to be proven or disproven. In the review paper Vymazal et al. (2021), of the 131 studies published between 2019-2020 investigating the future of CW design and performance, less than 10 used full scale CWs. Most studies were found to be laboratory or micro/mesocosm based. While results from such studies are valuable, their applicability to full scale CW systems around the world may be limited, as such small-scale studies may struggle to accurately recreate the complex nature of CWs (Liu et al., 2019a; Vymazal et al., 2021). Vymazal et al. (2021) also found that most studies do not exceed one year, with the majority lasting only a few months. Due to slow macrophyte growth rates, such short studies often cannot exceed more than one growth season (Vymazal et al., 2021). The effect of changing plant development and competition between species over longer time periods (years to decades) that may affect CW performance are likely to not be captured in such studies. Studies with these time scales often cannot provide valid information regarding long term CW performance, which is of great importance as CWs operate for decades (Vymazal et al., 2021). Performing longer, larger scale studies will help us to create a more accurate picture of CW performance and the many complex and changeable mechanisms involved. Findings from these studies could then be used to help develop CW design and technology to improve the removal efficiencies of contaminants, reduce greenhouse gas emissions and potentially allow for the treatment of a wider range of wastewater.

6.2. Microorganisms, macrophytes and their interactions

Despite the importance of microbes, macrophytes and their relationships in CW performance, these interactions are still not fully understood (Vymazal et al., 2021). Microbial community composition is strongly related to macrophyte species presence, for example, a bacteria associated with *Phragmites australis* can promote plant growth and increase CW contaminant removal efficiencies (Riva et al., 2019). However, we do not fully understand all the processes involved in this relationship, which potentially could be exploited further with human intervention to improve contaminant removal.

This applies to a range of relationships found between macrophytes and microbial communities in CWs treating wastewater. The microbial communities present in CWs have not been studied in detail (Vymazal et al., 2021); there is little research available on why the effects recorded on microbial community structure by antibiotic exposures are occurring (Liu et al., 2019a; Liu et al., 2019b; Liu et al., 2019c). While we have found that microbial community richness, diversity and nitrogen related microorganisms can be affected by specific compounds such as organophosphate flame retardants, we still do not fully understand all the factors that influence these changes (Liu et al., 2019a; Liu et al., 2019b; Liu et al., 2019c). For example, with antibiotic exposures, plant root exudates can mediate against antibiotic toxicity on microorganisms in the rhizosphere (Tong et al., 2020). However, we still do not fully understand the mechanisms involved in these processes.

6.3. Greenhouse gas emissions

Greenhouse gas emissions from CWs have been well studied and current consensus is that they are a sink for carbon dioxide (Mander et al., 2014; Maucieri et al., 2017; Søvik et al., 2006). However, changes to certain conditions, contaminant concentrations and CW design can change certain emissions greatly. Zheng et al., (2021) found that with the presence of sulfamethoxazole in integrated vertical flow CWs nitrogen transformation processes shift, favouring anammox. An intermittent loading regime of wastewater has been found to produce high nitrous oxide emissions (Filai et al., 2017). Biochar application to CW substrate can reduce nitrogen emissions (ammonia and nitrous oxide) while simultaneously increasing methane emissions (Feng et al., 2020; Guo et al., 2020) and in highly aerated and high influent CWs increase nitrous oxide emissions (Feng et al., 2020). The use of polyethylene biofilm carriers can potentially decrease emissions of nitrous oxide by 7-59% and methane by 11-70% (Sun et al., 2019), but its potential is still disputed. Luo et al. (2020) found that increasing plant species richness may potentially reduce emissions of nitrous oxide and methane, while Han et al. (2019) did not find a correlation.

The potential use of microbial fuel cells in antibiotic remediation could have many impacts on the greenhouse gas emissions of CWs. Xu et al. (2021) investigated microbial fuel cells, finding > 90% ciprofloxacin removal rates and a decline in methane fluxes of 15.29%. Such data suggests that using microbial fuel cells in antibiotic remediation in CWs could also reduce methane emissions, a finding of great potential value to contribute to global efforts to reduce greenhouse gas emissions. However, such investigations are still in their initial stages and further research regarding the many potential impacts on CW remediation processes that could occur from the use of microbial fuel cells is required. With conflicting findings and potentially many factors in CW design that can change greenhouse gas emissions, contributing to climate change, further investigation is of great importance for the effects of many different contaminants including antibiotics on greenhouse gas

emissions in CWs. Thought must also be given to the impact of the specific type of emission, while also considering quantity to determine if some emission states are preferable to others and perhaps justifiable to remove certain contaminants from the environment.

6.4. Conclusion of Chapter 5 and thesis

There are many areas of research that can be explored in CWs, as has been outlined by this thesis. As a result of the findings of this study, further investigations are required to establish whether *Lemna minor* experiences physiological and metabolomic effects at PECs over prolonged periods. Such results could be used to help determine whether plants in CWs treating WWTP effluent are likely to be experiencing stress. To improve the usefulness of such findings for informing policy, and the use and design of CWs, careful consideration to experimental design in size and time scale should be given, with field studies also conducted alongside highly controlled laboratory studies, if possible due to the limited use of such studies. Determining whether the long-term health and survival of *Lemna minor* is detrimentally affected by antibiotic exposures that result in metabolomic changes but no significant changes to growth rate and chlorophyll a content would also be of great value. Such findings could provide insight into the adequacy of plant health assessments and inform future *Lemna minor* toxicity studies and potentially a wider range of plant assessments. Metabolomics may offer a unique and valuable tool to identify plant stress prior to signs manifesting physiologically, providing crucial time to address the underlying causes responsible before potentially irreparable damage to plants. However, further study is required, with a wider range of plants and pollutants to determine its usefulness and to establish its potential in plant health assessments, ecotoxicology research and influence in environmental protection.

7. Appendix

Table 15. Key notes from several toxicity studies using *Lemna minor*, including the environmental conditions, growth medium, fronds added to test vessels in experimental periods, replicate numbers, and variables monitored and analysed.

Study	Key Notes
Baciak et al. (2016)	Replicate number: 3-5 replicates per treatment Calculations: percentage growth rate inhibition (by frond number), percentage yield reduction, Chlorophyll a and b content, carotenoid content, fresh weights, and dry weights. Test Length: 7 days Medium: Steinburg Temperature: 16-20 °C Light Intensity: fluorescent lights (140 µmol photon m−2 s−1 PAR) in a light-to-dark cycle of 16 h/8 h
Cascone et al. (2004)	Calculations: fresh and dry weights and Chlorophyll a content Test Vessel and volumes: Plastic bowls (20cm diameter), 750ml media Medium: Hoagland (replenished weekly) pH: 6.7 Light Intensity: 140–200 µE m−2 sec−1 (PAR) Other variables: Relative Humidity 70–75%
Ebert et al. (2011)	Replicate number: 6 concentration treatments with 3 replicates for each and 6 controls. Calculations: Average frond growth rate and biomass yields Test length: 7 days Frond Number per test vessel: 12 Test vessel and volumes: 150ml Beakers (120ml media), randomised position Medium: Steinburg pH: 6.4 -6.9 Temperature: 22.5 - 24.8°C Light Intensity: Continuous, top down, light tubes (Osram L 36 W/25 universal white) at 83.0 - 98.8 µE m−2 s−1
Gomes et al. (2017)	Acclimatization and Sterilisation: All media sterile, flasks stoppered with cotton wool to minimise evaporation and contamination and 25 days to acclimate. Frond Number per test vessel: 15 Test vessel and volumes: 250ml Erlenmeyer flasks Growth Medium: CHU 10

Study	Key Notes
	Temperature: 18 - 22 °C Light intensity: continuous light (45 µmol photons m−2 s−1), cool white fluorescent lamps
Gomes et al. 2020	Acclimatization and Sterilisation: 25 days to acclimate and all media sterile Test Length: 14 days Test vessel and volumes: 250ml Erlenmeyer flasks (100 ml media Growth Medium: Bold's Basal Temperature: 22-26 °C Light intensity: 12 hour photoperiod (100 µmol photons m−2 s−1)
González-Renteria et al. (2020)	Acclimatization and Sterilisation: 2 months to acclimate Temperature: 19.6 - 20 °C
Grenni et al. (2019)	Acclimatization and Sterilisation: Repeated washing and sterilisation with surfactants and NaClO solutions until visible contamination minimized. Transference into fresh media frequently and only uncontaminated fronds transferred on. Temperature: 22 - 28 °C Light intensity: 60–80 µmol m−2 s−1, photoperiod of 14-h light/10-h dark.
Iatrou et al. (2017)	Calculations: Frond growth rate inhibition (based on frond number). Acclimatization and Sterilisation: 4 weeks to acclimatize Test Length: 7 days Frond Number per test vessel: 12 Test vessel and volumes: glass petri dish (100ml media) Growth Medium: Swedish Standard Sterile Temperature: 23.5 – 24.5 °C Light intensity: continuous, fluorescent lamps
Lima et al. (2021)	Calculations: frond mortality rates (dead fronds counted) Acclimatization and Sterilisation: culture wells sterilized using a UV light for 30 mins. Test Length: 5 days Test vessel: culture plates with wells. Growth Medium: Hoagland Temperature: 22 °C
Mkandawire et al. (2014)	pH: 5.6–7.5 Temperature: 18–24°C Light intensity: 4200 and 6700 (equiv. 85–125 µE m−2 s−1or 400–700 nm, 14–16, Photoperiod (hr day−1) Other variables: 20–24 salinity optimal range (g L-1)

Study	Key Notes
OECD (2006)	Growth Medium: Swedish standard sterile Temperature: 22 – 26 °C Light intensity: Continuous, white fluorescent lighting, 85-135 µE·m-2s -1, (400-700 nm)
Radulović et al. (2020)	Acclimatization and Sterilisation: surface sterilisation for 5 minutes in bleach solution, then washed with sterile distilled water 3 times. Frond Number per test vessel: up to 4 Growth Medium: Murashige-Skoog Temperature: 22- 26 °C Light intensity: fluorescent light of 40 µmoL m−2 s−1 with 16 h light/8 h dark photoperiod
Sree et al. (2015)	Calculations: Frond growth rates, dry weights, starch content Acclimatization and Sterilisation: all media and beakers autoclaved, and beakers covered with glass plates Test Length: 7 – 14 days Frond Number per test vessel: 10 -20 Test vessel and volumes: 400ml beaker (300ml media) Growth Medium: Schenk–Hildebrandt, replenished weekly Temperature: 24 – 26 °C Light intensity: continuous white light from fluorescence tubes (TLD 36 W/86, Philips, Eindhoven, Netherlands).
Stout et al. (2010)	Test Length: 14 days Temperature: 20 °C Light intensity: photoperiod of 16 h light/8 h dark

Table 16. All antibiotics prescribed in England in 2019 with antibiotic family, Human excretion value, WWTP removal rates percentage and references (when applicable).

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
			Value	Reference	Value	Reference
Amines	Ethambutol hydrochloride	DrugBankOnline (2023)	72	DrugBankOnline (2023)	100	
Aminosalicylic acids.	Aminosalicylic acid	DrugBankOnline (2023)	100		100	
Aminoglycosides	Amikacin	Werth (2020)	100	DrugBankOnline (2023)	100	
Aminoglycosides	Fosfomycin calcium	Ronco et al. (2017)	100	DrugBankOnline (2023)	100	
Aminoglycosides	Fosfomycin trometamol	Ronco et al. (2017)	100	DrugBankOnline (2023)	100	
Aminoglycosides	Gentamicin sulfate	Werth (2020)	70	DrugBankOnline (2023)	100	
Aminoglycosides	Neomycin sulfate	Werth (2020)	100	DrugBankOnline (2023)	100	
Antifolate	Trimethoprim	DrugBankOnline (2023)	80	DrugBankOnline (2023)	44	Batt et al., (2006), Ghosh et al., (2009), Gobel et al., (2007), Gulkowska et al., (2008), Li and Zhang (2011), Lindberg et al., (2005), Lindberg et al., (2006), Radjenovic et al., (2009), Roberts and Thomas, (2006), Sahar et al., (2011), Verlicchi et al., (2012), Verlicchi et al., (2013), Watkinson et al., (2007), Watkinson et al., (2009).

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Carbapenems	Ertapenem sodium	Werth (2020)	80	DrugBankOnline (2023)	100	
Carbapenems	Meropenem	Werth (2020)	70	DrugBankOnline (2023)	100	
Carboxylic acids and derivatives	Clavulanic acid	DrugBankOnline (2023)	90	DrugBankOnline (2023)	100	
Cephalosporins	Cefaclor	Werth (2020)	85	DrugBankOnline (2023)	6	Watkinson et al., (2007), Watkinson et al., (2009).
Cephalosporins	Cefadroxil	Werth (2020)	90	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Cephalosporins	Cefalexin	Werth (2020)	99	DrugBankOnline (2023)	40	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Cephalosporins	Cefixime	Werth (2020)	50	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012)
Cephalosporins	Cefotaxime sodium	Werth (2020)	86	DrugBankOnline (2023)	37	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012)
Cephalosporins	Cefradine	DrugBankOnline (2023)	90	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Cephalosporins	Ceftazidime pentahydrate	Werth (2020)	90	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Cephalosporins	Ceftriaxone sodium	Werth (2020)	100	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Cephalosporins	Cefuroxime axetil	Werth (2020)	86	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Cephalosporins	Cefuroxime sodium	Werth (2020)	86	DrugBankOnline (2023)	28	Gulkowska et al., (2008), Li and Zhang (2011), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Fluoroquinolones / Quinolones	Ciprofloxacin	DrugBankOnline (2023)	100	DrugBankOnline (2023)	24	Ghosh et al., (2009), (Golet et al., 2003), Li and Zhang (2011),, Lindberg et al., (2005) , Lindberg et al., (2006), Verlicchi et al., (2012), Verlicchi et al., (2013), Zorita et al., (2009), Watkinson et al., (2007), Watkinson et al., (2009).
Fluoroquinolones / Quinolones	Levofloxacin	Werth (2020)	91	DrugBankOnline (2023)	37	Chang et al., (2008), Ghosh et al., (2009), (Golet et al., 2003), Gulkowska et al., (2008), Li and Zhang (2011), Lindberg et al., (2005) , Lindberg et al., (2006),Michael et al., (2013); Radjenovic, et al., (2007), Radjenovic et al., (2009), Verlicchi et al., (2012), Verlicchi et al., (2013), Watkinson et al., (2007),

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
						Watkinson et al., (2009). Xu et al., (2007), Zorita et al., (2009),
Fluoroquinolones / Quinolones	Moxifloxacin	DrugBankOnline (2023)	45	DrugBankOnline (2023)	39	Chang et al., (2008), Michael et al., (2013).
Fluoroquinolones / Quinolones	Norfloxacin	Werth (2020)	100	DrugBankOnline (2023)	42	Ghosh et al., (2009), Golet et al., (2003), Gulkowska et al., (2008), Li and Zhang (2011), Lindberg et al., (2005), Lindberg et al., (2006), Verlicchi et al., (2012), Verlicchi et al., (2013), Watkinson et al., (2007), Watkinson et al., (2009), Xu et al., (2007), Zorita et al., (2009)
Fluoroquinolones / Quinolones	Ofloxacin	Werth (2020)	88	DrugBankOnline (2023)	42	Li and Zhang, (2011), Lindberg et al., 2005), Radjenovic, et al., (2007), Radjenovic et al., (2009), Verlicchi et al., (2012), Verlicchi et al., (2013), Xu et al., (2007), Zorita et al., (2009)
Hydantoins	Nitrofurantoin	DrugBankOnline (2023)	90	DrugBankOnline (2023)	100	
Glycopeptides, Polypeptides and Lipopeptides	Daptomycin	DrugBankOnline (2023)	84	DrugBankOnline (2023)	98	Michael et al., (2013), Zuccato et al., (2010)
Glycopeptides, Polypeptides and Lipopeptides	Teicoplanin	DrugBankOnline (2023)	92	DrugBankOnline (2023)	98	Michael et al., (2013), Zuccato et al., (2010)
Glycopeptides, Polypeptides and Lipopeptides	Vancomycin hydrochloride	DrugBankOnline (2023)	100	DrugBankOnline (2023)	98	Michael et al., (2013), Zuccato et al., (2010)

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Lincosamides	Clindamycin hydrochloride	DrugBankOnline (2023)	14	Federal Drug Agency (No. Date.)	60	Watkinson et al., (2007), Watkinson et al., (2009).
Macrolides	Azithromycin	Werth (2020)	6	DrugBankOnline (2023)	47	Ghosh et a., (2009), Yasojima et al., (2006), Verlicchi et al., (2012)
Macrolides	Clarithromycin	Werth (2020)	30	DrugBankOnline (2023)	72	Ghosh et al., (2009), Gobel et al., (2007), Sahar et al., (2011),Yasojima et al., (2006), Verlicchi et al., (2012), Verlicchi et al., (2013)
Macrolides	Erythromycin	Werth (2020)	100	DrugBankOnline (2023)	45	Gobel et al., (2007), Gulkowska et al., (2008), Li and Zhang, (2011), Radjenovic, et al., (2007) , Radjenovic et al., (2009), Sahar et al., (2011) , Verlicchi et al., (2012). Verlicchi et al., (2013)Xu et al., (2007)
Macrolides	Erythromycin ethylsuccinate	Werth (2020)	100	DrugBankOnline (2023)	45	Ghosh et a., (2009), Verlicchi et al., (2012)Gobel et al., (2007), Gulkowska et al., (2008), Li and Zhang, (2011), Radjenovic, et al., (2007) , Radjenovic et al., (2009), Sahar et al., (2011), Verlicchi et al., (2012), Verlicchi et al., (2013)Verlicchi et al., (2013), Yasojima et al., (2006),
Macrolides	Erythromycin lactobionate	Werth (2020)	100	DrugBankOnline (2023)	45	Ghosh et a., (2009), Verlicchi et al., (2012)Gobel et al., (2007), Gulkowska et al., (2008), Li and Zhang, (2011), Radjenovic, et al.,

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
						(2007) , Radjenovic et al., (2009), Sahar et al., (2011), Verlicchi et al., (2012), Verlicchi et al., (2013)Verlicchi et al., (2013), Yasojima et al., (2006),
Macrolides	Erythromycin stearate	Werth (2020)	100	DrugBankOnline (2023)	45	Ghosh et a., (2009), Verlicchi et al., (2012)Gobel et al., (2007), Gulkowska et al., (2008), Li and Zhang, (2011), Radjenovic, et al., (2007) , Radjenovic et al., (2009), Sahar et al., (2011), Verlicchi et al., (2012), Verlicchi et al., (2013)Verlicchi et al., (2013), Yasojima et al., (2006),
Macrolides	Fidaxomicin	Werth (2020)	93	DrugBankOnline (2023)	50	Ghosh et a., (2009), Verlicchi et al., (2012)Gobel et al., (2007), Gulkowska et al., (2008), Li and Zhang, (2011), Radjenovic, et al., (2007) , Radjenovic et al., (2009), Sahar et al., (2011), Verlicchi et al., (2012), Verlicchi et al., (2013)Verlicchi et al., (2013), Yasojima et al., (2006),
Monobactams	Aztreonam	Werth (2020)	100	DrugBankOnline (2023)	100	
Nitroimidazoles	Metronidazole	DrugBankOnline (2023)	95	DrugBankOnline (2023)	66	Verlicchi et al., (2013)
Nitroimidazoles	Tinidazole	DrugBankOnline (2023)	37	DrugBankOnline (2023)	66	Verlicchi et al., (2013)

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Oxazolidinones	Cycloserine	DrugBankOnline (2023)	90	DrugBankOnline (2023)	100	
Oxazolidinones	Linezolid	Werth (2020)	90	DrugBankOnline (2023)	100	
Penicillins	Amoxicillin	Werth (2020)	78	DrugBankOnline (2023)	41	Watkinson et al., (2007), Watkinson et al., (2009).
Penicillins	Ampicillin	DrugBankOnline (2023)	93	DrugBankOnline (2023)	55	Cha et al., (2006), Li et al. (2009) , Michael et al., (2013),
Penicillins	Benzylpenicillin sodium (Penicillin G)	DrugBankOnline (2023)	100	DrugBankOnline (2023)	34	Watkinson et al., (2009).
Penicillins	Flucloxacillin sodium	DrugBankOnline (2023)	93	DrugBankOnline (2023)	40	Cha et al., (2006), Li et al. (2009), Michael et al., (2013), Watkinson et al., (2007), Watkinson et al., (2009).
Penicillins	Phenoxymethylpenicillin (Penicillin V)	Werth (2020)	100	DrugBankOnline (2023)	30	Watkinson et al., (2007), Watkinson et al., (2009).
Penicillins	Pivmecillinam hydrochloride	DrugBankOnline (2023)	93	DrugBankOnline (2023)	40	Cha et al., (2006), Li et al. (2009), Michael et al., (2013), Watkinson et al., (2007), Watkinson et al., (2009).
Penicillins	Temocillin sodium	Ronco et al. (2017)	93	DrugBankOnline (2023)	40	Cha et al., (2006), Li et al. (2009), Michael et al., (2013), Watkinson et al., (2007), Watkinson et al., (2009).
Phenazines and derivatives	Clofazimine	DrugBankOnline (2023)	100		100	
Phthalimides	Thalidomide	DrugBankOnline (2023)	100		100	
Pyrazines	Pyrazinamide	DrugBankOnline (2023)	70	DrugBankOnline (2023)	100	
Pyridines and derivatives	Isoniazid	DrugBankOnline (2023ac)	70	DrugBankOnline (2023ac)	100	

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Rifamycins	Rifabutin	Werth (2020)	93	DrugBankOnline (2023)	100	
Rifamycins	Rifampicin	DrugBankOnline (2023)	30	DrugBankOnline (2023)	100	
Rifamycins	Rifaximin	Werth (2020)	97	DrugBankOnline (2023)	100	
Steroids and steroid derivatives	Sodium fusidate	DrugBankOnline (2023)	100		100	
Streptogramins	Pristinamycin	Rath et al. (2010)	100		100	
Sulphonamides	Sulfadiazine	Werth (2020)	85	DrugBankOnline (2023)	35	Garcia galan et al., (2011), Li and Zhang, (2011), Verlicchi et a., (2012), Verlicchi et al., (2013), Xu et al., (2007),
Sulphonamides	Sulfamethoxazole	Werth (2020)	85	DrugBankOnline (2023)	37	Clara et al., (2005), Garcia galan et al., (2011), Ghosh et al., (2009), Gobel et al., (2007), Li and Zhang, (2011), Lindberg et al., (2005), Radjenovic et al., (2007), Radjenovic et al., (2009), Sahar et al., (2011), Verlicchi et al., (2012), Verlicchi et al., (2013), Watkinson et al., (2007), Watkinson et al., (2009), Xu et al., (2007),
Sulphonamides	Sulfapyridine	DrugBankOnline (2023)	85	DrugBankOnline (2023)	52	Garcia Galan (2011), Verlicchi et al., (2012)
Sulfones	Dapsone	DrugBankOnline (2023)	100	PubChem (2004)	100	

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Tetracyclines	Demeclocycline hydrochloride	DrugBankOnline (2023)	90	DrugBankOnline (2023)	11	Choi et al., (2007), Michael et al., (2013)
Tetracyclines	Doxycycline hyclate	Werth (2020)	90	DrugBankOnline (2023)	38	Lindberg et al., (2005), Verlicchi et al., (2012), watkinson et al., (2007), Watkinson et al., (2009).
Tetracyclines	Doxycycline monohydrate	Werth (2020)	90	DrugBankOnline (2023)	38	Choi et al., (2007), Ghosh et al., (2009), Gulkowska et al., (2008), Li and Zhang (2011),Lindberg et al., (2005), Michael et al., (2013) Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Tetracyclines	Lymecycline	DrugBankOnline (2023)	100	DrugBankOnline (2023)	43	Choi et al., (2007), Ghosh et al., (2009), Gulkowska et al., (2008), Li and Zhang (2011),Lindberg et al., (2005), Michael et al., (2013) Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Tetracyclines	Oxytetracycline	DrugBankOnline (2023)	35	PubChem (2004)	62	Li and Zhang, (2011), Verlicchi et al., (2012), Watkinson et al., (2009).
Tetracyclines	Tetracycline	Werth (2020)	100	DrugBankOnline (2023)	64	Li and Zhang (2011), Ghosh et al., (2009), Gulkowska et al., (2008), Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).
Tetracyclines	Tigecycline	DrugBankOnline (2023)	92	PubChem (2004)	43	Choi et al., (2007), Ghosh et al., (2009), Gulkowska et al., (2008), Li and Zhang (2011),Lindberg et al., (2005), Michael et al., (2013) Verlicchi et al., (2012), Watkinson et al., (2007), Watkinson et al., (2009).

Family	Antibiotic	References for antibiotic families	Percentage of antibiotic excreted by humans	Percentage of antibiotic excreted by humans	Percentage of antibiotic not removed by WWTPs	Percentage of antibiotic not removed by WWTPs
Thiazolide	Nitazoxanide	Fox and Saravolatz (2005)	100	DrugBankOnline (2023)	100	
Triazinanes	Methenamine hippurate	DrugBankOnline (2023)	100		100	

Table 17. All antibiotics prescribed in England in 2019 with their total mass and PNEC-MIC per antibiotic (data was sourced from an NHSdatabase and Tell et al. 2019, respectively). A worst case scenario, PEC and Risk Quotients for each antibiotic can also be seen.

Antibiotic	Total mass prescribed per antibiotic (µg per yr)	Worst case scenario (µg/L)	PECx1000 (µg/L)	PNEC-MIC (µg/L)	Risk Quotient
Ethambutol hydrochloride	8.98616E+13	0.030756	0.022144535	2	0.011072268
Aminosalicylic acid	2.776E+12	0.00095	0.000950122		
Amikacin	90600000000	3.1E-05	3.1009E-05	16	1.93806E-06
Fosfomycin calcium	1.4225E+12	0.000487	0.000486869	2	0.000243435
Fosfomycin trometamol	2.43279E+14	0.083265	0.083265395	2	0.041632698
Gentamicin sulfate	9.07075E+12	0.003105	0.002173208	0.15	0.014488051
Neomycin sulfate	9.69351E+12	0.003318	0.00331773	0.03	0.11059101
Trimethoprim	6.23042E+15	2.132442	0.750619711	0.5	1.501239422
Ertapenem sodium	1.472E+12	0.000504	0.000403049	0.13	0.003100376
Meropenem	3.8985E+12	0.001334	0.000934019	0.06	0.015566976
Clavulanic acid	4.7044E+15	1.610143	1.449129012		
Cefaclor	1.48343E+14	0.050772	0.002589393	0.5	0.005178785
Cefadroxil	1.3184E+13	0.004512	0.001137124	2	0.000568562
Cefalexin	8.22053E+15	2.813585	1.114179549	0.08	13.92724436
Cefixime	4.6474E+12	0.001591	0.000222689	0.06	0.003711477
Cefotaxime sodium	2.1E+11	7.19E-05	2.28707E-05	0.1	0.000228707
Cefradine	3.11735E+14	0.106695	0.026887228	0.47	0.057206869
Ceftazidime pentahydrate	3.787E+12	0.001296	0.00032663	0.5	0.00065326
Ceftriaxone sodium	1.10925E+13	0.003797	0.001063035	0.03	0.035434486
Cefuroxime axetil	3.61588E+13	0.012376	0.002980093	0.5	0.005960186
Cefuroxime sodium	1.05756E+12	0.000362	8.71612E-05	0.5	0.000174322
Ciprofloxacin	5.18482E+15	1.774572	0.425897376	0.06	7.098289605
Levofloxacin	1.75512E+14	0.060071	0.020225999	0.25	0.080903996

Antibiotic	Total mass prescribed per antibiotic (µg per yr)	Worst case scenario (µg/L)	PECx1000 (µg/L)	PNEC-MIC (µg/L)	Risk Quotient
Moxifloxacin	3.89476E+13	0.01333	0.002339471	0.13	0.017995934
Norfloxacin	5.04E+11	0.000173	7.24502E-05	0.5	0.0001449
Ofloxacin	2.70485E+14	0.092577	0.034216413	0.5	0.068432827
Nitrofurantoin	4.87638E+15	1.669004	1.502103987	64	0.023470375
Daptomycin	9700000000	3.32E-06	2.73298E-06	1	2.73298E-06
Teicoplanin	3.0622E+12	0.001048	0.000944947	1	0.000944947
Vancomycin hydrochloride	3.30524E+13	0.011313	0.011086363	1	0.011086363
Clindamycin hydrochloride	1.05891E+15	0.362425	0.030443687	0.5	0.060887375
Azithromycin	3.7358E+15	1.278625	0.036057228	8	0.004507154
Clarithromycin	1.80183E+16	6.166985	1.332068664	0.1	13.32068664
Erythromycin	7.41972E+15	2.539497	1.142773478	0.02	57.1386739
Erythromycin ethylsuccinate	7.31755E+15	2.504525	1.127036179	0.08	14.08795223
Erythromycin lactobionate	32000000000	1.1E-05	4.92859E-06	0.5	9.85717E-06
Erythromycin stearate	4.19287E+14	0.143506	0.064577888	0.5	0.129155776
Fidaxomicin	6.298E+11	0.000216	0.000100234	0.5	0.000200468
Aztreonam	1.6455E+11	5.63E-05	5.63194E-05	0.5	0.000112639
Metronidazole	9.09898E+15	3.114244	1.952630836	0.02	97.63154182
Tinidazole	8.8265E+12	0.003021	0.000737724	0.5	0.001475449
Cycloserine	46500000000	1.59E-05	1.43237E-05	0.13	0.000110182
Linezolid	1.71336E+13	0.005864	0.005277777	0.13	0.040598287
Amoxicillin	1.54603E+17	52.91503	16.92222588	6.7	2.525705355
Ampicillin	8.63958E+13	0.02957	0.015125089	6.7	0.002257476
Benzylpenicillin sodium (Penicillin G)	6.744E+11	0.000231	7.84795E-05	0.25	0.000313918
Flucloxacillin sodium	6.33527E+16	21.6833	8.06618784	0.25	32.26475136
Phenoxymethylpenicillin (Penicillin V)	5.06503E+16	17.33571	5.20071311	0.25	20.80285244

Antibiotic	Total mass prescribed per antibiotic (µg per yr)	Worst case scenario (µg/L)	PECx1000 (µg/L)	PNEC-MIC (µg/L)	Risk Quotient
Pivmecillinam hydrochloride	8.43488E+14	0.288695	0.107394485	0.2	0.536972427
Temocillin sodium	1.53E+11	5.24E-05	1.94802E-05	0.06	0.000324671
Clofazimine	3.311E+11	0.000113	0.000113323	0.2	0.000566616
Thalidomide	18075000000	6.19E-06	6.1864E-06	0.2	3.0932E-05
Pyrazinamide	4.8255E+12	0.001652	0.001156113		
Isoniazid	6.73127E+13	0.023039	0.01612704		
Rifabutin	1.8252E+12	0.000625	0.00058097	0.095	0.006115468
Rifampicin	3.81476E+14	0.130565	0.039169513	0.13	0.301303948
Rifaximin	1.52728E+15	0.522731	0.507048881	0.06	8.450814676
Sodium fusidate	7.06714E+13	0.024188	0.024188204	0.06	0.403136732
Pristinamycin	3.54E+11	0.000121	0.000121161	0.06	0.002019352
Sulfadiazine	2.1521E+13	0.007366	0.002191338	0.5	0.004382676
Sulfamethoxazole	3.03328E+15	1.038181	0.326507825	6.75	0.04837153
Sulfapyridine	6.46E+11	0.000221	9.7727E-05	13	7.51746E-06
Dapsone	6.54667E+13	0.022407	0.022406842	0.6	0.037344736
Demeclocycline hydrochloride	6.12263E+13	0.020955	0.002074592	6.8	0.000305087
Doxycycline hyclate	4.30284E+15	1.472703	0.503664485	2.32	0.217096761
Doxycycline monohydrate	2.1179E+13	0.007249	0.00247909	1.3	0.001906992
Lymecycline	2.06502E+16	7.067816	3.039160864	2	1.519580432
Oxytetracycline	8.50277E+15	2.910184	0.631509927	2	0.315754963
Tetracycline	4.91178E+14	0.168112	0.1075916	1.3	0.082762769
Tigecycline	1500000000	5.13E-07	2.03099E-07	0.5	4.06198E-07
Nitazoxanide	6000000000	2.05E-06	2.05358E-06	1	2.05358E-06
Methenamine hippurate	5.02381E+15	1.719465	1.719465207	1	1.719465207

Table 18. The top 20 priority chemicals from both PC1 and PC2 from the PCA plot responsible for the most chemical variation between treatments, with PC1 accounting for 72.7188% and PC2 10.384%. Which Principal component axis and rank number, mz and retention time have been included for each. Some chemicals of the top 20 for each PC were not identified by progenesis and chem spider and so their rank numbers cannot be seen).

Principal Component Axis and Rank Number	mz	Retention time	Possible chemical name and/or formula	Mass Error (ppm)[b]	Isotope Similarity[b]	Description of the chemical[a]
PC1 (rank number 1) and PC2 (rank number 6)	413.2664	19.56527	$C_{24}H_{38}O_4$ (Alpha-Apocholic acid)	0.33	98.07	Apocholic acid is a type of cholanoid (PubChem, 2004-). There is a lack of data for further role identification, however, it may be a type of bile acid or bile alcohol found in mammals, fish, amphibians and reptiles (Hoshita, 1996).
PC1 (rank number 1) and PC2 (rank number 6)	413.2664	19.56527	$C_{24}H_{38}O_4$ (3alpha,12alpha-Dihydroxy-5beta-chol-6-en-24-oic acid)	0.33	98.07	This chemical is likely a type of bile acid (PubChem, 2004-).
PC1 (rank number 3) and PC2 (rank number 4)	651.1553	7.8999	$C_{26}H_{22}N_{10}O_{11}$	1.72	98.6	No reliable data
PC1 (rank number 3) and PC2 (rank number 4)	651.1553	7.8999	$C_{30}H_{26}N_4O_{13}$	-2.41	96.2	No reliable data
PC1 (rank number 5)	464.3582	21.5638	$C_{24}H_{49}NO_7$ (2-Amino-3-hydroxyoctradecyl beta-D-glucopyranoside)	0	92	This chemical is possibly a bacterial metabolite (ChEBI, 2022b)

Principal Component Axis and Rank Number	mz	Retention time	Possible chemical name and/or formula	Mass Error (ppm)[b]	Isotope Similarity[b]	Description of the chemical[a]
PC1 (rank number 9) and PC2 (rank number 2)	711.1915	7.8176	$C_{32}H_{26}N_{10}O_{10}$	1.19	98.01	No reliable data
PC1 (rank number 9) and PC2 (rank number 2)	711.1915	7.8176	$C_{36}H_{30}N_4O_{12}$	-2.59	95.45	This chemical is possibly Enduracyclinone A, a natural product found in Nonomuraea, a type of bacteria (PubChem, 2004-).
PC1 (rank number 11) and PC2 (rank number 19)	282.2793	21.8016	$C_{18}H_{35}NO$ ((9Z)-9-Octadecenamide)	0.46	97.22	No reliable data
PC1 (rank number 11) and PC2 (rank number 19)	282.2793	21.8016	$C_{18}H_{35}NO$ (Dodemorph)	0.46	97.22	This chemical is possibly a type of fungicide (PubChem, 2004-).
PC1 (rank number 11) and PC2 (rank number 19)	282.2793	21.8016	$C_{18}H_{35}NO$ (CM3995000)	0.46	97.22	No reliable data
PC1 (rank number 11) and PC2 (rank number 19)	282.2793	21.8016	$C_{18}H_{35}NO$	0.41	97.22	No reliable data
PC1 (rank number 12)	699.3556	14.2840	$C_{30}H_{48}N_{10}O_8$	1.02	98.39	No reliable data
PC1 (rank number 12)	699.3556	14.2840	$C_{29}H_{52}N_6O_{12}$	3	97.97	No reliable data
PC1 (rank number 12)	699.3556	14.2840	$C_{34}H_{52}N_4O_{10}$	-2.95	96.08	This chemical is possibly hirsutatin A, a natural product found in Hirsutella nivea, an insect parasitic fungus (PubChem, 2004-).
PC1 (rank number 13)	565.155	7.3167	$C_{26}H_{28}O_{14}$ ((1S)-1,5-Anhydro-2-O-alpha-L-arabinopyranosyl-1-[5,7-	-0.26	93.35	No reliable data

Principal Component Axis and Rank Number	mz	Retention time	Possible chemical name and/or formula	Mass Error (ppm)[b]	Isotope Similarity[b]	Description of the chemical[a]
			dihydroxy-2-(4-hydroxyphenyl)-4-oxo-4H-chromen-6-yl]-D-glucitol)			
PC1 (rank number 14) and PC2 (rank number 10)	432.3319	9.4690	$C_{23}H_{45}NO_6$	-0.16	95.57	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{30}H_{34}N_2O_{19}$	4.69	96.31	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{31}H_{36}N_8O_{14}$	0.47	97.59	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{32}H_{32}N_8O_{10}$	-1.48	97.93	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{32}H_{26}N_{10}O_{11}$	1.04	97.31	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{34}H_{32}N_4O_{13}$	0.67	96.81	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{33}H_{36}O_{17}$	2.57	97.61	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{31}H_{30}N_6O_{15}$	2.88	97.95	No reliable data
PC1 (rank number 15) and PC2 (rank number 3)	727.1863	7.351417	$C_{35}H_{28}N_8O_9$	-1.23	95.35	No reliable data

Principal Component Axis and Rank Number	mz	Retention time	Possible chemical name and/or formula	Mass Error (ppm)[b]	Isotope Similarity[b]	Description of the chemical[a]
PC1 (rank number 19)	694.4004	14.2840	$C_{29}H_{61}N_5O_{11}$	0.74	97.62	No reliable data
PC1 (rank number 19)	694.4004	14.2840	$C_{30}H_{57}N_9O_7$	-1.3	98.12	No reliable data
PC1 (rank number 19)	694.4004	14.2840	$C_{32}H_{57}N_5O_{10}$	0.95	97.31	No reliable data
PC1 (rank number 19)	694.4004	14.2840	$C_{33}H_{59}NO_{14}$	-0.63	96.72	No reliable data
PC1 (rank number 19)	694.4004	14.2840	$C_{33}H_{53}N_9O_6$	-1.05	95.84	No reliable data
PC1 (rank number 19)	694.4004	14.2840	$C_{31}H_{61}NO_{14}$	2.94	97.92	No reliable data
PC1 (rank number 19)	694.4004	14.2840	$C_{34}H_{55}N_5O_{10}$	-2.55	95.26	No reliable data
PC2 (rank number 7)	332.2795	7.2486	$C_{18}H_{37}NO_4$	-0.07	96.94	No reliable data
PC2 (rank number 9)	415.2116	14.77883	$C_{24}H_{30}O_6$ (Inspra)	0.22	97.24	No reliable data
PC2 (rank number 9)	415.2116	14.77883	$C_{24}H_{30}O_6$ (Estra-1,3,5(10)-triene-3,6beta,17beta-triol triacetate)	0.22	97.24	No reliable data
PC2 (rank number 9)	415.2116	14.77883	$C_{24}H_{30}O_6$ (Estra-1,3,5(10)-triene-3,6alpha,17beta-triol triacetate)	0.22	97.24	No reliable data
PC2 (rank number 9)	415.2116	14.77883	$C_{24}H_{30}O_6$ (Methyl (4aS,4bR,5aS,6aS,7R,9aS,9bR,10R)-4a,6a-dimethyl-2,5'-dioxo-2,4,4',4a,5',5a,6,6a,8,9,9a,9b,10,11-tetradecahydro-3H,3'H-spiro[cyclopenta[7,8]phenanthro[4b,5-b]oxirene-7,2'-furan]-10-carboxylate)	0.22	97.24	No reliable data
PC2 (rank number 9)	415.2116	14.77883	$C_{24}H_{30}O_6$	0.26	97.24	No reliable data
PC2 (rank number 9)	415.2116	14.77883	$C_{25}H_{26}N_4O_2$	-2.97	95.63	No reliable data
PC2 (rank number 11)	537.3034	16.094	$C_{24}H_{38}N_{10}O_3$	2.56	96.17	No reliable data
PC2 (rank number 14)	316.2846	8.089717	$C_{18}H_{37}NO_3$	0.02	97.04	No reliable data
PC2 (rank number 17)	351.2141	10.76273	$C_{16}H_{26}N_6O_3$	0.65	97.73	No reliable data
PC2 (rank number 18)	326.3782	18.28685	$C_{22}H_{47}N$	0.09	96.57	This chemical is possibly Diundecylamine is a natural

Principal Component Axis and Rank Number	mz	Retention time	Possible chemical name and/or formula	Mass Error (ppm)[b]	Isotope Similarity[b]	Description of the chemical[a]
						product found in Trypanosoma brucei (a type of parasite) (PubChem, 2004-).
PC2 (rank number 20)	829.5062	22.28573	$C_{42}H_{66}N_{10}O_6$	0.35	96.28	No reliable data
PC2 (rank number 20)	829.5062	22.28573	$C_{41}H_{70}N_6O_{10}$	2.01	97.56	No reliable data
PC2 (rank number 20)	829.5062	22.28573	$C_{45}H_{74}O_{12}$	-1.32	95.21	This chemical formula gives two possible identifications. Firstly, Macrotetrolide C a type of macrotetrolide antibiotic (PubChem, 2004-). Another identification is 21-Hydroxyl-oligomycin A, a type of oligomycin antibiotic (PubChem, 2004-).

[a]. All possible identifications (except plant metabolites, shown in section 5.2.2 Table 14) are included below with details on the possible chemical identification. Where there was a lack of data or no reliable data to use for some chemical identifications, No reliable data was stated. Some chemical formulas resulted in many potential chemical identifications, with the formula being ambiguous and non unique, No reliable data is also stated due to there being a lack of confidence in making a likely identification.

[b]. Only chemicals with a mass error (ppm) between + 5 and -5 were accepted and chemical identifications with low isotope similarity scores (below 90) of which there were two have been removed.

Table 19. A list of ARGs considered to be included in the qPCR analysis of *Lemna minor* samples based on a literature review of the antibiotics used in this study and the ARGs associated with exposure to the antibiotics. Due to a lack of information regarding ARGs associated with an antibiotic, antibiotics were grouped into families (for example there was a lack of Penicillin V information resulting in its grouping with amoxicillin under the penicillin family). ARGs for cefalexin exposures are not included due to a lack of literature.

Antibiotic and class	Associated ARGs	References
Amoxicillin and Penicillin V (Beta Lactam)	pbp1A	(Kwon et al., 2017; Tran et al., 2022; Tseng et al., 2009)
Amoxicillin and Penicillin V (Beta Lactam)	blaTEM-1	Hayward et al. (2019); Sidrach-Cardona et al. (2014); Tseng et al. (2009)
Amoxicillin and Penicillin V (Beta Lactam)	blaCTX-M	Hayward et al. (2019), Sidrach-Cardona et al. (2014)
Amoxicillin and Penicillin V (Beta Lactam)	blaSHV	Sidrach-Cardona et al. (2014)
Amoxicillin and Penicillin V (Beta Lactam)	blaz-R	Ramessar and Olaniran (2019)
Amoxicillin and Penicillin V (Beta Lactam)	OXA-1, OXA-2, OXA-10	Meng et al. (2017)
clarithromycin (Macrolide-Lincosamide-Streptogramin B (MLSB))	23S rRNA gene (variations of A2142G, A2143GX, A2142C, A2143G, A2143, A2144G)	Geng et al. (2017), Vala et al. (2016), Hussein et al. (2022), Pina et al. (1998), Wang et al. (2020), Zhang et al. (2021)
erythromycin and erythromycin ethylsuccinate (Macrolide-Lincosamide-Streptogramin B (MLSB))	ermF and ermF-1	Guo et al. (2015), Schmitz et al. (2021), Shen et al. (2019)
erythromycin and erythromycin ethylsuccinate (Macrolide-Lincosamide-Streptogramin B (MLSB))	ermB, ermB-1, ermB-3	Guo et al. (2015), Hayward et al. (2019), Karaolia et al. (2018), Preethi et al. (2017), Shen et al. (2019), Wang et al. (2020)

Antibiotic and class	Associated ARGs	References
erythromycin and erythromycin ethylsuccinate (Macrolide-Lincosamide-Streptogramin B (MLSB))	ermA	Guo et al. (2015), Hummel et al. (2007)
erythromycin and erythromycin ethylsuccinate (Macrolide-Lincosamide-Streptogramin B (MLSB))	ermD	Guo et al. (2015)
erythromycin and erythromycin ethylsuccinate (Macrolide-Lincosamide-Streptogramin B (MLSB))	ermT 1	Wang et al. (2015b)
ciprofloxacin (fluoroquinolone)	gyrA	Kraupner et al. (2018), Rehman et al. (2021), Sidhu et al. (2021), Sproston et al. (2018)
ciprofloxacin (fluoroquinolone)	bla(CTX-M)	Lien et al. (2017)
ciprofloxacin (fluoroquinolone)	qepA	Cheng et al. (2021), Lien et al. (2017)
ciprofloxacin (fluoroquinolone)	qnrA, qnrB, qnrC, qnrS, qnrD	Rahman et al. (2017), Wang et al. (2020)
metronidazole (nitroimidazole)	nimE genes	Alauzet et al. (2019), Baaity et al. (2021), Gal and Brazier (2004)
metronidazole (nitroimidazole)	rdxA	Chu et al. (2020), Hanafi et al. (2016), Kargar et al. (2010), Paul et al. (2001)
metronidazole (nitroimidazole)	frxA	Chu et al. (2020), Hanafi et al. (2016)
Trimethoprim (antifolate)	dfrA1, dfrA5, dfrA7, dfrA8, dfrA12, dfrA14, dfrA17, dfrA27	Kraupner et al. (2020), Mukherjee and Chakraborty (2006), Park et al. (2003), Stange et al. (2016), Suhartono et al. (2016)
Multi drug resistance and housekeeping genes	16S rRNA sequencing	Hayward et al. (2019), Meng et al. (2017), Wang et al. (2015b), Wang et al. (2020)
Multi drug resistance and housekeeping genes	class 1 integron genes (intl1)	Hardwick et al. (2008), Meng et al. (2017), Schmitz et al. (2020), Stange et al. (2016), Suhartono et al. (2016), Wang et al. (2020)

Antibiotic and class	Associated ARGs	References
Multi drug resistance and housekeeping genes	class 2 integrons (intl2)	Stange et al. (2016), Suhartono et al. (2016)
Multi drug resistance and housekeeping genes	mobP11, mobP14, mobP51, mobF11, mobF12, mobQ11, mobQu	Suhartono et al. (2016)

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