Investigating the horizontal transmissibility of bacterial metaldehyde and metazachlor-degrading genes

Amy Skye Cooke

Master of Science by Research

University of York

Biology

January 2023

Abstract

The molluscicide metaldehyde and the herbicide metazachlor have been detected in surface waters previously, posing risks to drinking water quality and the health of humans and wildlife. Soil-derived bacterial strains can degrade metaldehyde, a trait which was acquired by inferred horizontal gene transfer. The metaldehyde-degrading genes and plasmids responsible could be further disseminated to other bacterial strains by horizontal gene transfer. Novel pathways for the metaldehyde biodegradation process were also said to exist. In comparison, much less was known about the microbial biodegradation of metazachlor; however, it was predicted to occur in soils facilitated by bacterial glutathione conjugation by glutathione transferases.

In vitro and soil microcosm-based facilitated horizontal gene transfer, selective enrichment, whole genome sequencing and bioinformatics approaches were employed to investigate the hypothesis that horizontal gene transfer processes could be harnessed experimentally to identify novel metaldehyde and metazachlor-degrading organisms, genes and metabolic pathways, and to demonstrate the horizontal transmissibility of metaldehyde and metazachlor-degrading genes.

Two novel metaldehyde-degrading *Acinetobacter calcoaceticus* strains, 5.1 and 6.6, were isolated from allotment soils inoculated with the non-degrading bacterial strain *Acinetobacter calcoaceticus* RUH 2202. Two candidate metaldehyde-degrading genes, 15 and 2947, were identified in both strains. Additionally, three novel metazachlor-degrading *Pseudomonas* strains, 4.2.2, 4.5.2 and 6.4.2, were isolated from agricultural soils. Glutathione transferase enzymes, *gstB_1*, *gstB_2*, *yibF*, 4164, 3838 and 4909, were identified as candidate metazachlor-degrading genes. These findings lay the groundwork for further investigations into microbial biodegradation and bioremediation of metaldehyde and metazachlor. The methodological approach used could also provide valuable insights for future research on microbial xenobiotic biodegradation and horizontal gene transfer.

Table of Contents

List of figures

List of Tables

Acknowledgements

Thorunn Helgason and my thesis advisory panel member Ville Friman for their added support and advice. I am also thankful for the valuable support and technical expertise that was provided by our J1 research technician, Hannah Walker, throughout the year.

Author's declaration

I declare that this thesis is a presentation of original work and I am the sole author. Whole genome sequencing and *de novo* assemblies were provided by MicrobesNG (Birmingham, UK) and Sanger sequencing was provided by Source Bioscience Sanger sequencing service (Cambridge, UK), as detailed in the thesis. This work has not previously been presented for an award at this, or any other, University. All sources are acknowledged as References.

Chapter 1: General introduction

1.1 Pesticides

The application of pesticides throughout the agricultural industry is invaluable to maintaining global food security and ensuring that our growing population's demands for food can be met, especially in developing countries where poverty and hunger are most prevalent (Sarkar *et al.*, 2021). Pesticides encompass a wide range of chemicals including plant growth regulators, nematicides, rodenticides, insecticides, fungicides, herbicides, molluscicides and more, which can protect crops from varying threats (Aktar, Sengupta and Chowdhury, 2009). Many of them are classed as xenobiotic compounds, which can be defined as chemicals foreign to an organism or ecological system.

The usage of pesticides, however, is accompanied by a wealth of challenges, including environmental pollution. Such contamination often occurs post-application when pesticides spread to non-target areas—air, soil, and water bodies—via volatilization, leaching, and runoff (van der Werf, 1996). The persistence and mobility of pesticides within the environment—determined by factors such as their properties, soil conditions, application method, and weather—shape their environmental fate (Kerle, Jenkins and Vogue, 1994).

1.2 Degradation of pesticides in the environment

Pesticides, once applied, undergo degradation which is usually their environmental fate. Environmental pesticide degradation encompasses a range of different transformation mechanisms, both biotic and abiotic. Abiotic mechanisms include direct and indirect phototransformation due to sunlight which can occur in sunlit surface water, and chemical transformation due to reactions including oxidation and hydrolysis, which can occur in groundwater, (Fenner *et al.*, 2013). On the other hand, biotic degradation, also known as biodegradation, is largely mediated by microorganisms or plants (Van Eerd *et al*, 2003).

1.2.1 Microbial biodegradation of pesticides

Biodegradation refers to natural processes in which substances in the environment are converted into simpler compounds (Joutey *et al.*, 2013). Many different species of

microorganisms have been found to possess metabolic pathways which allow them to facilitate the biodegradation of pesticide compounds, which can be categorised into metabolic and co-metabolic processes. In metabolic pesticide degradation, a pesticide compound can provide a microorganism with a source of carbon, energy and/or nitrogen, supporting its growth (Nzila, 2013; Yanze-Kontchou and Gschwind, 1994). In cometabolic pesticide degradation, a pesticide is metabolised as a non-growth substrate and, therefore, bears no benefit to the growth of the organism, but is metabolised along with a second necessary growth substrate or another transformable compound (Dalton and Stirling, 1982).

Biodegradation can be categorised differently, depending on the extent to which the compound in question is degraded. Primary biodegradation of a compound (also known as biotransformation) refers to a biologically-induced structural transformation which results in changes to its molecular integrity (Raymond *et al.*, 2001). Mineralisation refers to the degradation of an organic compound into inorganic compounds and products which are associated with typical metabolic pathways, including carbon dioxide and water (Joutey, *et al*, 2013). Pesticide biodegradation can, however, can lead to the production of harmful metabolites which can, in some cases, be more toxic than the parent pesticide compound itself (Sparling and Fellers, 2007). Various microorganisms, including bacteria, fungi, and microalgae, are capable of pesticide degradation (Huang *et al.*, 2018).

1.3 Horizontal gene transfer and the evolution and dissemination of microbial pesticide degradation pathways

Microorganisms can adapt to the degradation of pesticides through the evolution of novel metabolic pathways which facilitate the process. This can benefit degrading microorganisms by allowing them to utilise pesticides as a source of carbon and energy, conferring a potential increase in bacterial fitness and a resulting selective advantage over non-degrading organisms (Arbeli and Fuentes, 2007).

A major driver for the evolution of novel microbial xenobiotic degradation mechanisms is horizontal gene transfer. Horizontal gene transfer encompasses the processes of conjugation, transformation and transduction, and it facilitates the exchange of genetic material between different strains of bacteria (Dröge, Pühler and Selbitschka, 1999). Conjugation is facilitated by bacterial cell-cell contact and is mediated by mobile genetic elements (MGEs) such as conjugative plasmids or transposons, transformation is the process by which bacteria take up foreign DNA from their environment and then integrate it

into their genome, and transduction refers to the unintentional transfer of packaged phage DNA into a host bacterium upon phage infection (Miguel, Jetten and Welte, 2020). Horizontal gene transfer can contribute to the evolution of novel xenobiotic metabolising pathways via several mechanisms. An important key example is the concept of patchwork assembly (or pathway assembly). In this process, a novel degradation pathway is constructed by assembling genes or gene modules derived from different bacteria into a single new host bacterium, which is facilitated by the genetic exchange mechanisms provided by horizontal gene transfer (Top and Springael, 2003; Copley, 2000).

The importance of the involvement of horizontal gene transfer in the innovation of novel bacterial pesticide degradation pathways is demonstrated by the presence of pesticide degradation genes on plasmids, or their location close to other MGEs such as insertion sequences (Dunon *et al.*, 2018; Don and Pemberton, 1981). Furthermore, not only can horizontal gene transfer mediate the innovation of novel metabolic pathways, but it can also facilitate their further dissemination to additional bacteria, which is demonstrated by the presence of highly conserved pesticide-degrading pathways in different bacterial species (Castro-Gutiérrez *et al.*, 2022a).

1.4 Microbial xenobiotic bioremediation

Microorganisms with efficient xenobiotic-degrading capabilities can be exploited for the design of bioremediation approaches. Bioremediation can be defined as a process where environmental pollutants are removed or neutralized by the metabolism of biological organisms (Sharma, 2020). Techniques can be applied *in-situ*, where bioremediation takes place at the contaminated site such as polluted soil or groundwater with little site disturbance, or *ex-situ*, where contaminated material is removed from the site and treated elsewhere, for example following the excavation of polluted soil or pumping of polluted water (Vidali, 2001).

There are several bioremediation approaches which utilise microbial biodegradation processes. Bioaugmentation using microorganisms involves the addition of exogenous xenobiotic-degrading microorganisms to a contaminated site, intended to increase the rates of xenobiotic degradation through the exogenous microorganisms' metabolic capabilities and their ability to contribute to increases in the genetic diversity of the site's indigenous microbial community (El Fantroussi and Agathos, 2005). This method has been trialled previously for the bioremediation of pesticide-contaminated soil (El Fantroussi and Agathos,

2005; Kong *et al.*, 2014). Bioaugmentation using microorganisms is also a promising strategy for the removal of pesticide pollution from water sources, including the bioaugmentation of water filters used for pesticide removal (Karas *et al.*, 2016; Castro-Gutiérrez *et al.*, 2022b). An alternative form of bioaugmentation is the application of plasmids containing xenobiotic-degrading genes to a polluted environment, intending to increase the number of degrading organisms via the uptake of the plasmid through horizontal gene transfer (Garbisu *et al.*, 2017).

Another microorganism-targeted approach is biostimulation, which seeks to increase the degradation capacity of the microorganisms indigenous to a polluted environment more indirectly by adding nutrients to the site, such as nitrogen sources, to stimulate microbial growth (Simpanen, 2016). The potential use of this approach for the biostimulation of pesticide-degrading organisms in polluted groundwater has been investigated previously and shows promise for the reduction of drinking water pollution (Aldas-Vargas *et al.*, 2021). Natural attenuation is a further form of microbial bioremediation; however, this simply refers to the natural, passive process of microbial biodegradation of polluted environments, devoid of human intervention (Röling and van Verseveld, 2002).

Xenobiotic bioremediation approaches can hold advantages over man-made methods due to reduced costs, reduced technological requirements, lower risk by-products such as carbon dioxide and water which are produced by microbial mineralisation, and increased efficiency in the cases of recalcitrant xenobiotics which can not be removed by man-made removal techniques (Sharma, 2012). Scientists are, therefore, looking towards these approaches as alternatives to traditional removal methods.

1.5 Metaldehyde

Metaldehyde is a synthetic molluscicide commonly used in both domestic gardens and greenhouses and on agricultural fields, to protect crops from damage by mollusc pests, including slugs and snails (Fera Science, 2020). It has a cyclic tetrameric structure made up of four acetaldehyde molecules with the formula $(CH_3CHO)_4$ (Pauling and Carpenter, 1936) (figure 1-1). In terms of its mechanism, the pesticide damages molluscs via both physical contact and ingestion, by targeting mucocytes and inducing harmful effects including excess mucus production, swelling and destruction of the endoplasmic reticulum and Golgi apparatus and reduced feeding (Triebskorn, Christensen and Heim, 1998; Bourne and Jones and Bowen, 1988). Metaldehyde is commonly applied in the form of baited

pellets, with the highest amounts being applied during the autumn and winter application season between August and December when mollusc pests can thrive due to increased moisture (Balashova *et al.*, 2021).

Figure 1-1 Chemical structure of metaldehyde made using PubChem chemical structure sketcher V2.4 (Ihlenfeldt, Bolton and Bryant, 2009).
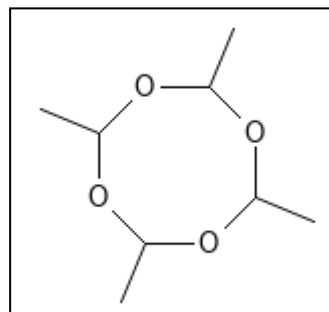
Metaldehyde has moderate solubility in water (188mg/L at 20 degrees) and it is moderately mobile in soil with an organic carbon-water partition coefficient (K_{oc}) of 240 (Lewis *et al.*, 2016). K_{oc} is a soil adsorption coefficient commonly used to express the mobility of a substance in soil, ranging from 'non-mobile' (>4000 mL/g) indicating strong soil adsorption up to 'very mobile' (<15 mL/g) indicating weak soil adsorption (Lewis *et al.*, 2016). These properties mean that after application, the pesticide can be easily transported to surface waters via drains,   flow and surface runoff and cause pollution, especially after rainfall (Asfaw, Maher, and Shucksmith, 2018). A 2018 report by the British Geological Survey on groundwater pollution stated that metaldehyde was in the top 10 most frequently detected organic micropollutant compounds found in English groundwater (Lapworth *et al.*, 2018).

Metaldehyde pollution has consistently caused drinking water compliance failures in recent years, breaching the 0.1 µg/L European Union Drinking Water Directive (EUDWD) regulatory limit for single pesticides (Council Directive 98/83/EC; DWD) (Chief Inspector of Drinking Water, 2017). Additionally, the pesticide is difficult to remove from the environment cost-effectively by using existing water treatment methods such as advanced oxidation processes, powder-activated carbon and granular activated carbon (Kay and Grayson, 2014; Autin *et al.*, 2013; Tao and Fletcher, 2013). Metaldehyde is classified by the World Health Organisation (WHO) as a 'moderately hazardous' (class II) pesticide, as it can be toxic to mammals and birds, with acute median lethal doses (the amount of a chemical which is lethal to 50% of the animals exposed to it) (LD_{50}) at 283 mg/kg and 196 mg/kg for mammals and birds, respectively (WHO, 2019; Lewis *et al.*, 2016). Although it is only lethal to humans in much higher doses such as 400 mg/kg, its toxicity and the challenges it poses for drinking water company compliance standards could not be ignored (Longstreth and Pierson, 1982).

Therefore, a ban on the use and sale of metaldehyde was implemented part-way through this study, at the end of March 2022 (DEFRA, 2020).

1.5.1 Microbial biodegradation of metaldehyde

Prior to the identification of microbial metaldehyde-degrading species, it was established that metaldehyde undergoes complete mineralisation by microorganisms in the environment to produce carbon dioxide and water, through the production of acetaldehyde and acetate intermediates (Bieri, 2003; Thomas, 2016). More recently, soil-derived metaldehyde-degrading bacterial strains were isolated previously which included *Acinetobacter calcoaceticus* E1, *Acinetobacter bohemicus* JMET-C, *Acinetobacter lwoffii* SMET-C, *Caballeronia jiangsuensis* SNO-D, *Pseudomonas vancouverensis* SMET-B, *Sphingobium sp.* CMET-H, *Sphingobium sp.* HMET-G, *Rhodococcus globerulus* HMET-A, *Rhodococcus globerulus* HNO-A and *Variovorax* sp. E3 (Thomas *et al.*, 2017; Castro-Gutiérrez *et al.*, 2020). This was achieved by performing selective enrichment procedures on various samples of domestic, agricultural and allotment soil samples involving both solid and liquid culture stages in metaldehyde-supplemented minimal salts media (MSM). This was followed by the verification of metaldehyde-degrading activity in metaldehyde-supplemented MSM during liquid growth assays.

The metaldehyde-degrading capabilities of some of the strains were also tested in a slow sand filter water treatment setting to explore their potential for bioremediation of metaldehyde-contaminated water sources (Castro-Gutiérrez *et al.*, 2022b). The model degrading bacterium *Sphingobium* CMET-H was able to remove 95% of the metaldehyde from within a slow sand filter to a concentration below the EUDWD 0.1 µg/L drinking water compliance limit, highlighting it as a promising tool for bioremediation of metaldehyde-contaminated water sources (Council Directive 98/83/EC; DWD).

Whole genome sequences were obtained for the metaldehyde-degrading strains and comparative genomics performed comparing the genomes of the metaldehyde degraders and closely related, non-degrading reference strains including the *Acinetobacter calcoaceticus* strain, RUH 2202, which was used in this study (Castro-Gutiérrez *et al.*, 2020; Castro-Gutiérrez *et al*, 2022a). These analyses revealed the presence of a potentially horizontally transferrable metaldehyde-degrading gene cluster in several of the degrading strains including *A. calcoaceticus* E1, *A. bohemicus* JMET-C, *A. lwoffii* SMET-C, *P. vancouverensis* SMET-B and *C. jiangsuensis* SNO-D. This cluster consisted of a 2-

oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily enzyme named *mahX*, a vicinal oxygen chelate family enzyme named *mahY* and an NAD(P)+-dependent aldehyde dehydrogenase enzyme named *aldH*. There were slight variations in the clusters of *C. jiangsuensis* SNO-D and *P. vancouverensis* SMET-B, in that the *aldH* gene was truncated. For the majority, however, there was a high similarity between the degrading genes between the different strains.

The proposed pathway for microbial metaldehyde degradation involving the degrading gene cluster identified above is as follows: initial oxygenation and ring cleavage of metaldehyde by *mahX* to produce a hemiacetal (1,3,5,7-tetramethyl-2,4,6-trioxa-1-hydroxy-7-octanone), breakdown of the hemiacetal by the lyase activity of *mahY* to produce acetaldehyde and a shorter chain hemiacetal and final oxidation by *aldH* to produce acetate, which can be further broken down via central metabolism. The metaldehyde-degrading cluster was missing from strains *Sphingobium sp.* HMET-G, *R. globerulus* HMET-A and *R. globerulus* HNO-A, which highlighted the fact that alternative metaldehyde degradation pathways exist in nature which could not be identified previously. In addition to this, the metaldehyde-degrading pathway was never revealed for *Variovorax* sp. E3, which was isolated earlier by Thomas *et al.* in 2017. Both the acetate and acetaldehyde metabolites demonstrate lower toxicities in terms of LD_{50} values (in rats) than metaldehyde at 4665 mg/kg and 634-687 or 1620-2240, respectfully compared to 227-690mg/kg for metaldehyde (Thomas, 2016; NCBI, 2023).

Alongside the genomic comparisons, phenotypic comparisons between the metaldehyde-degrading strains and non-degrading reference strains were performed previously. These included ethanol utilisation assays, which revealed that the non-degrading reference strain, *A. calcoaceticus* RUH 2202, was able to utilise ethanol as a carbon source, whereas the closely related degrading strain, *A. calcoaceticus* E1, could not (Thomas *et al.*, 2017; Thomas, 2016). This demonstrated an alternative characteristic that is useful for distinguishing between the *A. calcoaceticus* RUH 2202 and *A. calcoaceticus* E1 strains other than testing for metaldehyde degradation capabilities.

Combined Illumina and Oxford Nanopore whole genome sequencing and bioinformatic analysis revealed the genetic contexts of the degrading genes in several different species, which highlighted evidence of horizontal gene transfer-mediated acquisition (Castro-Gutiérrez *et al*, 2022a). The degrading gene cluster of *mahX*, *mahY* and *aldH* in *A. calcoaceticus* E1, along with an IS91 family transposase, is flanked by two ISOur1 insertion sequences, composing a probable composite transposon. Furthermore, the percentage guanine-cytosine content (GC%) for the degrading gene cluster was considerably higher

than the averages for the rest of the genome. These facts, along with the high similarity found between the degrading gene homologs in the different degrading species, suggested that the degrading genes in *A. calcoaceticus* E1 were acquired via horizontal gene transfer. The degrading cluster was found to be located on a plasmid, pAME76, in strain *A. calcoaceticus* E1. This plasmid lacks the genetic components required for conjugation, however, so it is hypothesised that it can not be disseminated further by conjugation. The natural transformation competency of *Acinetobacter* species including *Acinetobacter calcoaceticus* strains, however, suggests that further dissemination of the pAME76 plasmid and degrading genes to other bacterial strains may be possible through transformation (Johnsborg, Eldholm and Håvarstein, 2007). Interestingly, four mercury resistance genes, *merD*, *merA*, *merC* and *merR,* were identified on plasmid pAME76 in strain *A. calcoaceticus* E1 (Castro-Gutiérrez *et al*, 2022a). Experimental verification of a mercury resistance trait in this strain, however, was not provided in previous work.

Combined whole genome sequencing of strain *Sphingobium* CMET-H revealed an alternative proposed metaldehyde degradation pathway (Castro-Gutiérrez *et al*, 2022a). A metaldehyde-degrading gene, bearing resemblance to the *mahX* homologs found in other degrading strains was identified and named *mahS*. This gene belongs to the phytanoyl-coenzyme A family oxygenases. Investigation of the genetic context of this gene revealed its proximity to a cluster of genes that are involved in the acetoin degradation pathway, which facilitates the degradation of acetoin into acetyl-coenzyme A (acetyl-coA) and acetate (Dauner *et al.*, 2002). These genes include *acoA, acoB, acoC, acoD, acoX (partial)* and *acsA.* Both the metaldehyde and acetoin degradation pathways described previously produce an acetaldehyde intermediate, which can be broken down by enzymes in the acetoin degradation pathway. This led to a proposed degradation pathway which could combine both *mahS* and two of the acetoin-degrading genes. Only the initial first step could be confidently predicted, however, which would involve degradation by oxygenation and ring cleavage mediated by *mahS* to produce a hemiacetal (1,3,5,7-tetramethyl-2,4,6-trioxa-1-hydroxy-7-octanone). After the subsequent breakdown of the hemiacetal to acetaldehyde by an unknown enzyme, the acetoin degradation gene *AcoD* could convert acetaldehyde into acetate, which could be converted to acetyl-coA by *AcsA*, which could then be processed further by central metabolism.

No role of horizontal gene transfer in the acquisition of *mahS* or the acetoin-degrading genes was found in *Sphingobium* CMET-H. The metaldehyde-degrading genes for *Sphingobium* CMET-H, however, are present on a potentially conjugative plasmid named pSM1, which

displays the required genetic components for conjugative transfer including a MOB$_P$ class relaxase, a type IV coupling protein and two type IV secretion systems corresponding to the MPF$_T$ and MPF$_F$ types (Cabezón *et al.*, 2015). This suggests that the *Sphingobium* CMET-H metaldehyde-degrading genes could be disseminated further to other bacterial strains via conjugation.

1.6 Metazachlor

Metazachlor (C14H16ClN3O), a synthetic chloroacetamide herbicide, finds widespread application across the globe to safeguard oil seed crops, which includes Brassicaceae family members like rape, broccoli, cabbage, cauliflowers, Brussels sprouts among others, and extends to ornamental trees and shrubs (Karier, Kraus and Kolber, 2017; Lewis *et al.*, 2016). Its chemical structure is displayed in figure 2-1. Within the agricultural sector, it serves to control broad-leaved weeds, winter grasses, and annual grasses, with applications being made pre-emergence and during early post-emergence for the control of broad-leaved weeds, winter grasses and annual grasses (FAO, 1999). Categorized under the chloroacetamide herbicide class, its compounds act by inhibiting the biosynthesis of very long chain fatty acids (VLCFAs) in plant cell plasma membranes, leading to lack of membrane rigidity, cell leakage, impaired cell division and eventual plant death (Böger, 2003). In line with international water stewardship initiatives, mid-October is set as the recommended cut-off for application, with no restriction for non-drained land, and no more than 750g/ha should be applied (BASF, 2020). Frequently available in the form of a liquid suspension concentrate, its application is executed via spraying (EFSA, 2008).

Figure 1-2 Chemical structure of metazachlor made using PubChem chemical structure sketcher V2.4 (Ihlenfeldt, Bolton and Bryant, 2009).
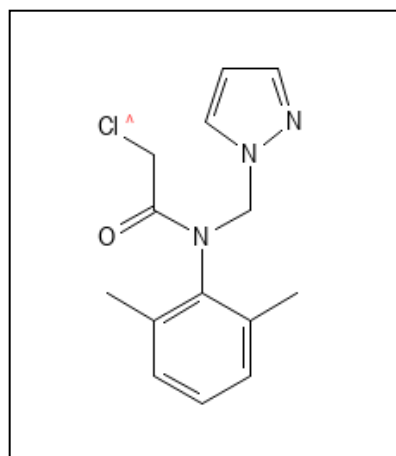
In terms of its physicochemical properties, metazachlor displays moderate solubility in water (450mg/L at 20°C) and it is classified as mobile in soil (K_{oc} = 54.0) (Lewis et al., 2016). Owing to these properties, after its application, metazachlor can be transferred to non-target areas via mechanisms such as run-off, field drainage and spray-drift and has been detected previously in both surface waters and groundwater, posing threats to drinking water resource quality (Kreuger, J., 1998; Ulrich et al., 2021; Huber, Bach and Frede, 2000; Karier, Kraus and Kolber, 2017). It is classified by WHO as a slightly hazardous pesticide (class III) with low toxicity to mammals (LD_{50} 2150mg/kg) (WHO, 2019). There are concerns, however, regarding metazachlor's carcinogenicity, leading to its classification as 'suspected of causing cancer' under the CLP Regulation (CE) No 1272/2008 (CLP category 2, H351) based on rat and mouse studies (EFSA, 2018).

The pollution caused by Metazachlor presents a substantial risk to aquatic wildlife and has the classification of being both acutely (H400) and chronically toxic (H410) to aquatic life forms (Lewis et al., 2016). Past research focusing on aquatic communities within freshwater microcosm and mesocosm environments have shown that exposure to varying concentrations of metazachlor at a range of concentrations initiating from 0.5 µg/L can instigate alterations in community composition due to reductions in numerous species of macrophytes, plankton and phytoplankton, indicating potential long-term impacts on ecosystems (Mohr et al., 2007; Mohr et al., 2008; Wijewardene et al., 2021). Plant species containing a high number of VLCFAs are reportedly the most vulnerable. Further research on species of fish and crustaceans have underscored potential negative impacts due to exposure to metazachlor, for example, when zebrafish are exposed to 5 µg/L, an increase in vitellogenin production is noted, indicating potential negative consequences on reproduction, and in the early life stages of marbled crayfish, detrimental effects such as elevated mortality, inhibited growth, developmental delays, and restricted locomotion have been reported at environmentally significant concentrations of 0.0115 µmol/L and 0.0790 µmol/L (Velisek et al., 2020; Jurčíková et al., 2007).

The main metabolites of metazachlor, metazachlor oxalic acid ($C_{14}H_{15}N_3O_3$) (also known as 479M04) and metazachlor ethane sulfonic acid ($C_{14}H_{17}N_3SO_4$) (also known as 479M08), have also been detected in surface waters, drinking water and groundwater (Ulrich et al., 2018; Ulrich et al., 2021; Karier, Kraus and Kolber, 2017). These metabolites are mobile, with K_{oc} values of 18.9mL/g and 8.8mL/g for 479M04 and 479M08, respectively (Lewis et al., 2016). They are classed as persistent and moderately persistent with DT_{50} (the time required

for a 50% reduction in concentration, or half-life) values of 96.3 and 115 days for 479M04 and 479M08 in the field, respectively, and they are classed as highly leachable, properties which allow their pollution of water sources. In the study mentioned in the previous paragraph by Vesilek *et al.* (2020), 479M04-induced adverse effects in early-life-stage marbled crayfish highlight the threats posed to aquatic organisms by the pollution of aquatic environments with major metazachlor metabolites. Other metazachlor metabolites also exist, which include 479M06, 479M09, 479M11, 479M16 and 479M12 (EFSA, 2017; Lewis *et al.*, 2016). In experiments described by the European Food Safety Authority (EFSA, 2017; EFSA 2008), Metabolites 479M04, 479M08, 479M09, 479M011 and 479M012 each demonstrated higher persistence in soil than metazachlor at DT_{50} values of 22.4 – 578d, 60.2-375d, 13.8–39.0d, 21.0–52.4 and 63–148d, respectively, compared to 3.1–25.3d for metazachlor. Additionally, 479M04, 479M08, 479M09, 479M011 and 479M012 have demonstrated higher mobilities in soil than metazachlor at K_{foc} (organic-carbon normalized Freundlich distribution coefficient) values of 1–94 mL/g, 4–78.5 mL/g, 4.9–6.8 mL/g and 18.1–23.5 mL/g, respectively, compared to 53.8–220 mL/g for metazachlor. These metabolites, therefore, have the potential to contaminate groundwater. 479M09 and 479M11 were deemed as toxicologically relevant in terms of human health if found to contaminate water sources as it could not be excluded that they share the suspected carcinogenicity of the parent compound metazachlor. Metabolites 479M04, 479M08 and 479M12, however, were deemed as less toxic than their parent compound metazachlor and not toxicologically relevant to human health based on acceptable daily intake values of 0.33 mg/kg, 0.2 mg/kg and 0.38mg/kg compared to metazachlor at 0.08mg/kg from studies with mice and rats (EFSA, 2018).

Although it has not been responsible for many UK drinking water compliance failures in the past, detections of metazachlor and its metabolites in water sources and the low K_{oc} values and soil mobilities of each of these compounds pose future risks to water quality, wildlife and human health (Cosgrove *et al.,* 2019). Furthermore, if stewardship and agricultural practice guidelines are not followed and metazachlor and metazachlor metabolite pollution levels are not maintained below regulatory limits, restrictions or bans on metazachlor use could be imposed in future, which could lead to losses of important crops.

1.6.1 Environmental degradation of metazachlor

Metazachlor exhibits stability against direct photodegradation, aqueous photolysis, and aqueous hydrolysis, and it's considered not to biodegrade readily in surface water and sediment (EFSA, 2008). The degradation of the compound in water, however, is evident

from the aforementioned detection of its metabolites in water sources. In addition, past research that quantified metazachlor degradation in freshwater mesocosms provided evidence of degradation merely a day post metazachlor application, evidenced by the detection of metazachlor metabolites (Mohr *et al.*, 2007). Metazachlor concentrations in the mesocosms decreased to a range of 7.3 – 15.7% from the initial concentration in pond mesocosms, and 4.2 – 11.8% in stream mesocosms. There have been suggestions about the involvement of microorganisms in metazachlor's degradation in water. One such example is a study which investigated the effect of biofilms on aqueous metazachlor degradation within microcosms, which demonstrated large metazachlor losses which could not be explained by mechanisms of photolysis, biofilm sorption or volatilization, leading to the suggestion that microorganisms were using metazachlor as a carbon source and in turn carrying out its degradation (Bighiu and Goedkoop, 2021). No microbial determinants of metazachlor degradation in water were identified previously.

Metazachlor is degraded quickly in soil, with typical, lab and field DT_{50} values of 8.6, 10.8 and 6.8 days, respectively, leading to the production of the main metabolites 479M04, 479M08 along with 479M06, 479M09, 479M11 and 479M12 (EFSA, 2017). Knowledge of the metazachlor biodegradation pathways is limited, however, and no microbial species to date have been identified that are responsible. Rates of degradation have been shown to depend on different factors including soil organic carbon content, soil sorption coefficients and temperature (Wang *et al.*, 2018; Beulke and Malkomes, 2001).

Bacterial species have been discovered previously which can degrade other members of the chloroacetamide class of herbicides to which metazachlor belongs, including strains of the *Paracoccus sp.*, *Proteiniclasticum sediminis, Sphingobium baderi, Rhodococcus* sp., *Sphingobium quisquiliarum, Pseudomonas oleovorans, Xanthomonas axonopodis and Sphingobium wittichii* species (Chen *et al.*, 2014; Cheng *et al.*, 2017; Hou *et al.*, 2014; Liu *et al.*, 2012; Xu *et al.*, 2006; Zhang *et al.*, 2011; Ahmad, 2020). Laue, Field and Cook (1996), however, discovered a soil-derived bacterial strain capable of degrading the metazachlor sulfonic acid metabolite.

It has been suggested that glutathione transferases may be the enzyme class responsible for metazachlor degradation in soil, which carry out glutathione conjugation of chloroacetamide compounds (Laue, Field and Cook, 1996). The main route of degradation of metazachlor in plants is also via glutathione conjugation, and the process was also observed in rats (EFSA, 2008). Both evidence of glutathione conjugation and utilisation of

glutathione transferases have been found in bacterial strains previously which can initiate degradation of chloroacetamides, including strains belonging to the *Enterobacteriaceae* and *Pdeudomonaceae* families (Zablotowicz *et al.*,1995; Zablotowicz *et al.*, 2001). It is likely, therefore, that a microorganism that could initiate metazachlor degradation in soil via glutathione transferase activity.

1.7 Methods for studying xenobiotic-degrading organisms, genes and pathways, and their horizontal gene transfer

Traditional microbial culture-based methods have been invaluable for the isolation of xenobiotic-degrading organisms through time and have allowed the isolation and study of many different pesticide degraders derived from the environment (Vijaya and Venkateswarlu, 2008; Ishag *et al.*, 2016; Chaudhry, Ali and Wheeler, 1988). This can be achieved through selective enrichment cultures of environmental samples such as soil supplemented with pesticide compounds as a sole source of carbon, thereby selecting organisms which can utilise the compound as a growth substrate (Abraham and Silambarasan, 2013). While culture-based methods continue to be a worthwhile approach, only a small fraction of the total microbial species on earth can be cultured in laboratory conditions and culturing techniques are, therefore, limited by the growth requirements of the organisms (Vartoukian, Palmer and Wade, 2010). These unculturable microbes are referred to as viable but non-culturable organisms and led to the origination of the great plate count anomaly concept, based on observations of considerably lower viable microbial cell counts produced from environmental microbial samples on culture media compared to direct microscopic cell counts (Staley and Konopka,1985; Jin *et al.*, 2017). There are likely, therefore, xenobiotic-degrading organisms which are undiscovered due to our inability to isolate them using microbial culture methods.

It is also possible to study horizontal gene transfer within microbial communities in the laboratory. Previous experiments have utilised soil microcosms to study and induce horizontal gene transfer between indigenous organisms and exogenous organisms which are added to the soil environments, leading to increases in microbial degradation capabilities and detections of horizontal gene transfers (French, Zhou and Terry, 2020; Hill and Top, 1998). The results of these studies could help in the design of bioremediation approaches because of the value of horizontal gene transfer in the dissemination of xenobiotic-degrading capabilities for bioremediation approaches such as the bioaugmentation of polluted sites ().

Molecular approaches have enabled a greater depth of understanding of xenobiotic-degrading organisms. These techniques are collectively referred to as the 'Omics approach and include metabolomics, transcriptomics, proteomics, metagenomics and genomics techniques which allow us to gain insights into xenobiotic-degrading microorganisms' metabolic pathways, messenger RNA expression levels, proteins and genes (Mishra *et al.*, 2021). These techniques can overcome culturability problems as the materials of interest for study such as genomic DNA can be directly extracted from environmental samples containing the organisms (Brockman, 1995). Whole genome sequencing has shown itself to be a beneficial molecular technique in studying xenobiotic-degrading genes and over time , it has become increasingly affordable and more accessible. Whole genome sequencing, in combination with bioinformatics strategies, facilitates the annotation of entire gene sets of xenobiotic-degrading organisms, predicts their functions and determines their genomic organisation (Ravintheran *et al.*, 2019). Comparative genomics analyses, utilizing the genomes of xenobiotic-degrading organisms and non-degrading reference organisms, assists in identifying candidate genes for degradation (Verma *et al.*, 2014). Whole genome sequencing, supplemented by bioinformatic strategies, can further enable detection of horizontal gene transfer events linked to xenobiotic-degrading genes, by examining the structure of the genome and the genetic context of degrading genes, which leads to the identification of mobile genetic elements like plasmids bearing xenobiotic-degrading genes and insertion sequences (Kaminski *et al.,* 2019).

Bioinformatics approaches used to annotate and predict the functions of genes and their metabolic pathways are often based on sequence similarity. There are prokaryotic genome annotation tools such as Prokka which have been designed to identify and annotate whole genome sequences in a matter of minutes (Seemann, 2014). Gene functions are predicted based on their sequence similarities to the sequences of known proteins within databases. There are limits, however, to these sequence similarity-based predictions as in many cases genes cannot be assigned a predicted function and are left unannotated or annotated as hypothetical proteins due to a lack of significantly similar known proteins within the databases searched, especially in the case of more novel genomes (Ruiz-Perez, Conrad and Konstantinidis, 2021). Another common approach is the identification of conserved domains using databases such as the Conserved Domain Database (CDD) and associated tools such as the NCBI Conserved Domain Search tool (CD-Search) (Marchler-Bauer *et al.*, 2015; Marchler-Bauer and Bryant, 2004). The identification of domains within a gene of interest with high similarity to domains in the databases allow for a confident functional inference. This is not always possible, however, if there are no domains that can be found with sufficient similarity to those that already exist.

1.8 Aims of the project

There is a wealth of evidence that demonstrates the importance of horizontal gene transfer to microbial xenobiotic degradation, and how these processes can be utilised for the design of bioremediation approaches, such as bioaugmentation of polluted sites. There is evidence to suggest that metaldehyde degradation genes can be disseminated between bacterial strains in soil environments, however, there is yet to be experimental evidence provided of this horizontal gene transfer process occurring in real-time. Additionally, novel pathways for the microbial degradation of metaldehyde and metazachlor are likely to exist in nature, however, their identification has not been possible in the past, perhaps due to the challenges associated with traditional culturing methods used for the isolation of degrading microorganisms. For bioremediation approaches to be designed for tackling metaldehyde and metazachlor pollution, an increased understanding of the degradation processes and their horizontal gene transfer capabilities are required. Whole genome sequencing and bioinformatics analyses for novel metaldehyde and metazachlor-degrading strains could identify novel genes responsible for degradation and identify novel metabolic pathways that could be harnessed for bioremediation of contaminated environments.

The following project aims were proposed:

- To test a microcosm-based facilitated horizontal gene transfer approach for the transfer of metaldehyde and metazachlor-degrading genes between donor microbial strains indigenous to soil microcosms and chosen exogenous culturable recipient strains
- To isolate novel metaldehyde and metazachlor-degrading organisms from soil microcosm microbial communities using selective enrichment procedures coupled with the facilitated horizontal gene transfer approach
- To identify the genetic determinants conferring metaldehyde and metazachlor degradation capabilities in novel metaldehyde and metazachlor-degrading organisms
- To further knowledge of the microbial biodegradation pathways for metaldehyde and metazachlor
- To investigate the horizontal transmissibility of metaldehyde and metazachlor degradation genes through the facilitated horizontal gene transfer approach and a simple *in vitro* conjugation assay

Chapter 2: Materials and methods

2.1 Growth media

2.1.1 MSM and MSM agarose

MSM was made by dissolving 55mM Na_2HPO_4, 11mM KH_2PO_4, 6mM NH_4Cl and 4mM $MgSO_4.7H_20$ in pure water. All MSM was supplemented with 4mL/L trace elements solution and autoclaved before use in experiments. For MSM agarose, agarose was added to MSM at 0.75% (w/v) and autoclaved. Agarose plates were ~25mL.

For metaldehyde-supplemented MSM, metaldehyde (99%, Acros Organics) was dissolved in MSM overnight on a heated magnetic stirrer at 65°C to produce the desired concentration of metaldehyde, followed by filter sterilisation (0.2µM). In order to prepare metaldehyde-supplemented MSM agarose, solutions of filter-sterilised metaldehyde-supplemented MSM and autoclaved MSM agarose were prepared separately, bought to 65°C in a water bath and combined to produce the desired MSM agarose volume and metaldehyde concentration.

For metazachlor-supplemented MSM, metazachlor (≥ 98.0 %, Sigma-Aldrich) was first dissolved in a small amount of acetone to make a 1000x solution relative to the desired final metazachlor concentration, followed by the addition of pure water to further dissolve at 65°C at 10x concentration before being added to MSM for the final desired concentration and filter sterilised (0.2µM) (see 2.3.3 and 2.4 for relevant concentrations). In order to prepare metazachlor-supplemented MSM agarose, solutions of filter-sterilised metazachlor-supplemented MSM and autoclaved MSM agarose were prepared separately, bought to 65°C in a water bath and combined to produce the desired MSM agarose volume and metazachlor concentration (see 2.3.3 and 2.4 for relevant concentrations).

2.1.2 Trace elements solution

Two slightly different trace element solutions were used throughout the study. The first was prepared previously at the University of York according to the method described by Vishniac and Santer (1957). The second was prepared following the depletion of the first solution and

it contained 0.14M Na_2EDTA, 0.0076M $ZnSO_4.7H_2O$, 0.037M $CaSO_4.2H_2O$, 0.025M $MnCl_2.4H_2O$, 0.018M $FeSO_4.7H_2O$, 0.0009M $(NH_4)6MO_7O_{24}.4H_2O$, 0.0064M $CuSO_4.5H_2O$ and 0.0067M $CoCl_2.6H_2O$ dissolved in pure water and adjusted to pH 6 using 2M KOH. This solution was then filter sterilised ($0.2\mu M$).

2.1.3 Lysogeny broth (LB) and LB agar

Lysogeny broth (LB) was prepared by dissolving 10g Tryptone, 5g yeast extract and 10g NaCl in 1L pure water, followed by autoclaving. For LB agar, 15g agar was added before autoclaving at 1.5% (w/v). Agar plates were ~25mL.

2.1.4 Nutrient agar

Nutrient agar was prepared by dissolving 5g Peptone-D protease peptone (Bio Basic), 3g meat extract (Sigma-aldrich) and 15g agar at 1.5% (w/v) in 1L pure water, followed by autoclaving.

2.1.5 Phosphate-buffered saline

Phosphate-buffered saline (PBS) was made by dissolving one Oxoid™ PBS tablet (Sigma-Aldrich) per 100mL pure water to give 0.137M NaCl, 0.003M KCl, 0.008M Na_2HPO_4 and 0.0015M KH_2PO4 (Thermo Scientific). All PBS solutions were autoclaved before use.

2.2 Bacterial strains and glycerol stock revival

Bacterial strains *A. calcoaceticus* RUH 2202 and *Sphingobium chlorophenolicum* DSM 7098 were purchased previously from the Belgian Coordinated Collection of Microorganisms and the Leibniz Institute Deutsche Sammlung von Mikroorganismen und Zellkulturen culture collection, respectively. Strains *A. calcoaceticus* E1 and *Sphingobium* CMET-H were isolated in previous work (Thomas *et al.*, 2017; Castro-Gutiérrez *et al.*, 2020). Glycerol stocks of each strain were also prepared previously at the University of York. For use in experiments, frozen stocks were revived by streaking frozen stocks onto solid LB agar or nutrient agar plates incubated at 25-30°C in the dark until single colonies were formed. For *Acinetobacter* strains, incubation was up to 48h whereas for *Sphingobium* strains the

incubation period was at least 48h. All subsequent growth conditions for the growth of the strains on solid LB or nutrient agar were the same throughout the study.

2.3 Facilitated horizontal gene transfer and selective enrichment for the isolation of metaldehyde and metazachlor-degrading organisms from soil microcosms

2.3.1 Soil sampling

For the experiments related to metaldehyde, two soil samples were collected in October 2021 including a 530g sample of domestic garden compost from a domestic garden in the Dringhouses area of York, and a 600g sample of allotment soil from Hob Moor allotments, York. Samples were kept in plastic boxes, tied loosely in a plastic bag and kept at 4°C in the dark until use. The sample sites were chosen to represent typical domestic compost and allotment soils in North Yorksire.

For the experiments relating to metazachlor, agricultural soil samples were collected during previous work isolating metazachlor-degrading organisms at the University of York in 2021 from Applegarth Farm, Welbury, Northallerton, North Yorkshire DL6 2SF by kind permission of Mr J Sadler, 54.41650350929117, -1.3861764608169669 (sample coordinates) (See the next section for treatment history). The sample sites were chosen to represent typical agriculturally managed land in North Yorkshire.

2.3.2 Facilitated horizontal gene transfer in soil microcosms

All soil microcosms were constructed using zip-lock bags measuring 32cm x 22.5cm. For metaldehyde-related experiments, the soil samples added to microcosms were 40g and for metazachlor-related experiments, soil samples added to the microcosms used in this study were 30g. Numbering systems were devised for the soil microcosms according to their individual treatments which are displayed in tables 2-1 (metaldehyde experiments) and 2-2 (metazachlor experiments) and are referred to throughout the thesis.

Soil microcosm no.	Soil type	Treatment	Strain type	Set
1	Allotment	Metaldehyde	DSM 7098	2
2	Allotment	No metaldehyde	DSM 7098	2
3	Domestic	Metaldehyde	DSM 7098	2
4	Domestic	No metaldehyde	DSM 7098	2
5	Allotment	Metaldehyde	RUH 2202	1
6	Allotment	No metaldehyde	RUH 2202	1
7	Domestic	Metaldehyde	RUH 2202	1
8	Domestic	No metaldehyde	RUH 2202	1
9	Allotment	Metaldehyde	None	1
10	Allotment	No metaldehyde	None	1
11	Domestic	Metaldehyde	None	1
12	Domestic	No metaldehyde	None	1
13	Allotment	Metaldehyde	None	2
14	Allotment	No metaldehyde	None	2
15	Domestic	Metaldehyde	None	2
16	Domestic	No metaldehyde	None	2

Table 2-1 Numbering scheme for the soil microcosms constructed for the facilitated horizontal gene transfer of metaldehyde-degrading genes. Soil microcosms consisted of zip lock bags containing samples of allotment (Allotment) and domestic compost (Domestic) soil samples, which were treated (Metaldehyde) or untreated (No metaldehyde) with metaldehyde at 15mg/kg soil and inoculated with 400µl overnight LB cultures of *S. chlorophenolicum* DSM 7098 (DSM 7098) or *A. calcoaceticus* RUH 2202 (RUH 2202) or an uninoculated LB control (None). Set 1 microcosms were constructed on 24th November 2021 and set 2 were constructed on 13th December 2021.

Liquid LB cultures of the *A. calcoaceticus* strain RUH 2202 and E1 and the *Sphingobium* strains CMET-H and DSM 7098 used for the inoculation of soil microcosms were prepared by transferring single colonies of each of these strains grown on solid LB agar into 5mL liquid LB media in 50mL plastic falcon tubes (Thermo Fisher Scientific) by pipette tip transference and incubated at 30°C and 200rpm until turbid (overnight for *Acinetobacter* strains and between 24h and 48h for *Sphingobium* strains).

Metaldehyde treatments consisted of the addition of 400µL of a filter-sterilised (0.2µM) 1.5mg/mL solution of metaldehyde dissolved in methanol distributed via pipette on top of the soil to give a final concentration of 15mg/kg soil. 400µL pure methanol was used as a control for non-metaldehyde-treated soils. For the metazachlor treatments that were carried out

previously, 10mL of a 75mg/L filter-sterilised solution of metazachlor (Sigma-Aldrich) was prepared and added to soils, to give a final concentration of 7.5mg/kg soil. 7.5mg metazachlor was dissolved in 100μL acetone to make a 1000x stock solution (7,500mg/L). This solution was then diluted in sterile water to make the final 10mL 75mg/L solution and filter sterilised (0.2μM). 10mL sterile water only was used for the non-treated control soil treatment.

Soil microcosm no.	Soil type	Treatment	Strain type
1	Agricultural	Metazachlor	E1
2	Agricultural	No metazachlor	E1
3	Agricultural	Metazachlor	CMET-H
4	Agricultural	No metazachlor	CMET-H
5	Agricultural	Metazachlor	None
6	Agricultural	No metazachlor	None

Table 2-2 Numbering scheme for the soil microcosms constructed for the facilitated horizontal gene transfer of metazachlor-degrading genes. Soil microcosms consisted of zip lock bags containing samples of agricultural soil (Agricultural), which were either treated (Metazachlor) or untreated (No metazachlor) on two separate occasions with metazachlor at 7.5mg/kg soil and inoculated with 300μl overnight LB cultures of *A. calcoaceticus* E1 (E1) or *Sphingobium* CMET-H (CMET-H) or an uninoculated LB control (None).

Two sets of 8 metaldehyde-related microcosm experiments were set up labelled 1-16. Set 1 involved facilitated horizontal gene transfer using the recipient *A. calcoaceticus* RUH 2202 strain and set 2 involved facilitated horizontal gene transfer using the recipient *S. chlorophenolicum* DSM 7098 strain. Soil samples for set 1 were added to zip-lock bags on 24th November 2021. The following day, these 8 microcosms were either treated with metaldehyde solution or methanol controls. The solutions were massaged into the soils, and the zip-lock bags were left open for at least 2h to allow for methanol evaporation and then re-sealed and kept at room temperature under a laboratory bench covered with blue roll. The following day, the microcosms were treated with either 400μl *A. calcoaceticus* RUH 2202 culture or 400μl liquid LB culture control. The soils were massaged once more to distribute the culture. The microcosms were re-sealed and left at room temperature under the laboratory bench concealed with blue roll for a period of 50 days to allow horizontal gene transfer. Soil samples for set 2 of the metaldehyde-related experiments were added to microcosms on 13th December. The same procedure was used for the addition of the metaldehyde and methanol control treatments as was described for set 1. After methanol evaporation, microcosms were either treated with 400μl *S. chlorophenolicum* DSM 7098

culture or 400µl liquid LB control. These microcosms were re-sealed and kept in the same conditions as set 1 for 31 days to facilitate horizontal gene transfer.

For metazachlor-related experiments, metazachlor treatment solutions were prepared and added to the agricultural soils prior to this study in 2021. Two 100g samples of the agricultural soil collected previously were each added to zip locks measuring 32cm x 22.5cm in July 2021 to form 2 microcosms. Each microcosm was treated twice with either the metazachlor solution or the control water treatment. The first treatment was added to the relevant microcosm on 30th June 2021. These microcosms were then incubated at room temperature in a cupboard for 12 days. On the 12th day, the relevant metazachlor and control treatments were repeated once more, and the soil microcosms were then left in a cupboard at room temperature until their use in this study. For this study, on 9th December 2021, 30g soil samples were taken from the two 100g microcosms and added to fresh zip-lock bags. Six microcosms were constructed and labelled 1-6, three containing metazachlor-treated soil and three containing non-treated soil. The treated and untreated microcosms were then each inoculated with 200µl cultures of either *Sphingobium* CMET-H or *A. calcoaceticus* E1 or an uninoculated LB only control. These microcosms were then re-sealed and left in the same conditions as described for the metaldehyde sets of microcosms, for 32 days to facilitate horizontal gene transfer.

2.3.3 Selective enrichment for the isolation of metaldehyde and metazachlor-degrading strains from soil microcosms

Three liquid culture selective enrichment steps for soil microcosm samples were performed (P1-P3) followed by culture on solid media. Soil samples of 1g were taken from each soil microcosm and each was added to a 50mL falcon tube containing 10mL MSM supplemented with 4mL/L trace elements and either 75mg/L metazachlor or 100mg/L metaldehyde as the sole source of carbon (P1). The samples were then incubated for 24h at 30°C and 200rpm. For subsequent P2 and P3 passages, 100µl of the previous passage culture was added to 10mL fresh supplemented MSM and incubated in the same conditions as P1. 100µl aliquots of the P3 cultures were transferred to solid MSM agarose plates supplemented with trace elements (4mL/L) and either metaldehyde (80mg/L) or metazachlor (75mg/L) as sole sources of carbon and incubated at 25-30°C for 72h in the dark.

Six areas of each supplemented MSM agarose plate containing colonies were further subcultured onto solid pesticide-supplemented MSM agarose plates until single colonies of pure cultures were obtained, followed by further subculturing onto both pesticide-supplemented and non-supplemented MSM agarose plates. The growth of bacterial strains subcultured on supplemented and non-supplemented plates was compared to identify strains which showed stronger growth on pesticide-supplemented plates compared to non-supplemented plates, which were identified as candidate pesticide-degrading strains. Degrading strains were subcultured once more onto supplemented plates before re-streaking onto solid LB agar plates and grown until single colonies were produced (up to 72h). These colonies were re-streaked ~monthly onto further LB plates for their maintenance throughout the study until they were utilised for liquid growth assays (section 2.4).

2.4 Liquid growth assays in MSM supplemented with 150mg/L metaldehyde or metazachlor

Single colonies of the candidate pesticide degrading strains that were grown on solid LB agar plates were transferred via pipette tip transference to 5mL liquid LB in 50mL falcon tubes. Strains were grown overnight at 30°C and 200rpm in the dark.

Liquid growth assays for the candidate pesticide-degrading strains were performed in sets within a 96-well plate format. 2µl of the overnight culture of each strain was grown in triplicate in 198µl MSM supplemented with trace elements and either metaldehyde (150mg/L) or metazachlor (150mg/L) as sole sources of carbon or non-supplemented MSM. Triplicate wells containing 200µl uninoculated pesticide-supplemented MSM served as bacteria-free controls. For the metazachlor experiments, the non-supplemented MSM media contained 0.001% (v/v) acetone to account for acetone present in metazachlor-supplemented MSM serving as a potential alternative carbon source. 96-well plates were incubated in a Tecan Sunrise^{TM} absorbance microplate reader (Tecan Group) at 25-30°C for 72h, with optical density (OD) readings taken every 30 min at 620nm (OD_{620nm}) for each well following 10-second shaking periods and 5-second settle times. One set of 10 strains was grown for 53.5h due to an error incurred by the plate reader which halted the progress of the assay. The plate reader was paired with Tecan Magellan^{TM} software (Tecan Group).

Simple growth curves were produced for the OD_{620nm} readings of individual triplicated cultures using RStudio (RStudio Team, 2016). Strains whose triplicated growth curves

demonstrated increased growth (in terms of higher optical densities at 620nm) in pesticide-supplemented MSM on average compared to non-supplemented MSM were selected for further analysis. Further growth curves for these selected candidate strains were plotted using hourly average OD_{620nm} readings. Average peak OD_{620nm} values were calculated for the growth of strains in pesticide-supplemented and non-supplemented MSM and two-sample t-tests were performed using the triplicated data for the two conditions.

2.5 16S rRNA gene amplicon sequencing and analyses

Single colonies of the candidate pesticide-degrading strains that were grown on solid LB agar plates were transferred to 5mL liquid LB in 50mL flacon tubes and grown overnight at 30°C and 200rpm in the dark.

For the crude cell lysate preparation for strain 5.1, 500µL overnight culture was added to a 1.5mL Eppendorf tube and centrifuged at 3000 rpm for 5 minutes in a Sigma 1-13 Benchtop Centrifuge (Sigma Laboratory Centrifuges). The supernatant was discarded, and the pellet was resuspended in 100µL nuclease-free water in a 200µL PCR tube. The tube was then incubated in a BIO-RAD T100 Thermal Cycler (BIO-RAD) at 99°C block temperature and 105°C lid temperature for 10 min. For strains 6.6, 4.2.2, 4.5.2 and 6.4.2, 100µL overnight cultures were used instead, pelleted by centrifugation, resuspended in 100µL sterile nuclease-free water and incubated in the same conditions as strain 5.1. Crude cell lysates were stored at -20°C until use.

16S ribosomal RNA (rRNA) genes were amplified for each strain using universal 27F and 1492R primers. PCR reaction mixtures (50µL volume for strain 5.1 and 20µL for strains 4.2.2, 4.5.2 and 6.4.2) contained the following components and final concentrations: Promega 5X colourless GoTaq buffer at 1.5mM (Promega), dNTPs at 0.2mM, 27F primer at 0.2µM, 1492R primer at 0.2µM, Promega GoTaq G2 polymerase (5U/µL) at 1.25U (Promega), template DNA at ~50ng (for the 50µL reaction volume, 5µL template DNA was used and for 20µL reaction volumes, 2µL DNA was used) and sterile nuclease-free water to make up to the final reaction volumes. DNA positive control consisted of "soil bacteria" DNA at 25ng/µL concentration to ensure that the PCR process was working correctly to amplify the 16S rRNA gene, and the negative control was nuclease-free water which served to identify any possible contamination of PCR reagents or equipment. PCR amplification was performed using the BIO-RAD T100 Thermal Cycler (BIO-RAD) and the thermocycling

program was 95°C/90s, 95°C/30s, 58°C/30s and 72°C/60s for 29 cycles, followed by 72°C/10min.

PCR products were visualised using electrophoresis and a Safe Imager blue-light transilluminator (Invitrogen). Electrophoresis gels at 1% (w/v) agarose were used along with DNA gel loading dye (6X) (Thermo Scientific), the GeneRuler 1kb plus ready-to-use DNA ladder (Thermo Scinetific) and SYBR™ Safe DNA Gel Stain (10,000x) (Thermo scientific). For strain 5.1, this was done using the purified PCR product DNA.

PCR products were then purified using the QIAquick PCR purification kit (Qiagen). The concentration and purity of the resulting purified DNA was quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific). The purified 16S rRNA gene amplicons were then sequenced using the Source Bioscience sanger sequencing service (Cambridge, UK).

The Sanger sequence traces obtained from by Source Bioscience were trimmed using SnapGene to remove primer and low-quality sequences at the beginnings and ends of the sequences. Nucleotide Basic Local Alignment Search Tool (BLAST, BLASTN) searches of the partial 16S rRNA genes against the prokaryotic 16S rRNA sequence database were used to assign species to the candidate pesticide-degrading strains. Strains were assigned at the species level based on the top BLASTN hit and a minimum percent identity threshold of 98.65% (Kim *et al.*, 2014).

2.6 Ethanol utilisation assays for *A. calcoaceticus* strains 5.1, 6.6, E1 and RUH 2202

Ethanol utilisation assays were performed to test the presence and lack of ethanol utilisation traits in *A. calcoaceticus* strains RUH 2202 and E1, respectively. These assays were also performed for the candidate metaldehyde-degrading strains 5.1 and 6.6 to facilitate phenotypic comparisons between *A. calcoaceticus* strains 5.1, 6.6, E1 and RUH 2202.

Single colonies of strains *A. calcoaceticus* strains E1, RUH 2202, 5.1 and 6.6 that were grown on solid LB agar plates were transferred by pipette tip transference to 50mL falcon tubes containing either 5mL MSM supplemented with trace elements (4mL/L) or 5mL MSM supplemented with 0.2% (v/v) ethanol. Each strain in each treatment condition was grown in triplicate. Cultures were incubated in the dark at 30°C and 200rpm for 48h. 1mL aliquots of

each culture were used to measure culture readings at 600nm (OD_{600nm}) using a Jenway 6300 spectrophotometer (Jenway) at 0h, 24h and 48h. Average OD_{600nm} values were calculated for each time point and used to produce bar graphs in RStudio (Rstudio Team, 2016).

2.7 Mercury resistance assays for *A. calcoaceticus* strains E1, RUH 2202, 5.1 and 6.6

Mercury resistance assays were performed to experimentally verify the hypothesis that plasmid pAME76 in strain *A. calcoaceticus* E1 confers mercury resistance, and to investigate whether this trait is present in *A. calcoaceticus* RUH 2202. The assays were also performed for strains 5.1 and 6.6 for phenotypic comparisons with *A. calcoaceticus* strains E1 and RUH 2202 and to determine whether they may have acquired pAME76 via horizontal gene transfer.

Mercury (II) chloride ($HgCl_2$) (pure, Scientific Laboratory Supplies Ltd.) solutions were made by dissolving the compound in sterile water at room temperature, followed by filter sterilisation (0.2 µm). Single colonies of *A. calcoaceticus* strains E1, RUH 2202, 5.1 and 6.6 that were grown on solid LB agar plates were suspended in 100µL sterile PBS in 1.5mL Eppendorf tubes. 50µl of these suspensions were re-streaked onto half-portions of solid LB agar plates supplemented with HgCl2 at 0mg/L (0M), 10mg/L (0.037mM) and 15mg/L (0.055 mM), with the LB-only plates serving as controls. The plates were incubated at 25-30°C in the dark for 72h.

2.8 Antibiotic resistance assays

Nalidixic acid and rifampicin resistance assays were performed for *A. calcoaceticus* strains E1 and RUH 2202 and *Sphingobium* strains CMET-H and DSM 7098 to identify possible selection mechanisms for conjugation assays.

Rifampicin stock solutions were made by dissolving rifampicin (LKT Laboratories) in methanol in 50mL plastic falcon tubes at room temperature. For nalidixic acid stock solutions, nalidixic acid sodium salt (Santa Cruz Biotechnology) was added to ethanol in 50mL flacon tubes and warmed using a water bath until dissolved (up to 75°C). Appropriate amounts of these stock solutions were added to cooled molten LB agar (65°C) to produce

solid LB agar plates containing 20µg/mL and 50µg/mL of rifampicin and 20µg/mL and 30µg/mL nalidixic acid. Single colonies of the *A. calcoaceticus* strains E1, RUH 2202, and the *Sphingobium* strains DSM 7098 and CMET-H that were grown on solid LB agar plates were each re-streaked onto antibiotic-supplemented plates and grown for up to 72h at 25-30°C in the dark.

2.9 Mating assays between *A. calcoaceticus strains* E1 and RUH 2202

Single colonies of the *A. calcoaceticus* strains RUH 2202 and E1 that were grown on solid LB agar plates were each added to 5mL liquid LB in 50mL falcon tubes and grown overnight. 100µL of these cultures were each diluted in 900µL fresh LB and OD_{600nm} readings were taken to ensure cell numbers were approximately equal for each strain. The cultures were then centrifuged at 4,000rpm for 10 min at 20°C in a Beckman Coulter Allegra X-22R centrifuge. The pellets were then resuspended in 5mL sterile LB in fresh 50mL falcon tubes. Three mating cultures were then produced, each consisting of 1mL of each of the *A. calcoaceticus* RUH 2202 and E1 strains' resuspended pellets combined in fresh 50mL falcon tubes at an approximate 1:1 ratio. The remaining suspended pellets were kept at 4°C for later use. The mating cultures were incubated for 24h at 30°C in the dark to allow conjugation to occur. Mating cultures were serially diluted and 100µL aliquots of the dilutions at 10^4, 10^5 and 10^6 were spread onto solid MSM agarose plates supplemented with 15mg/L $HgCl_2$ and 0.2% (v/v) ethanol. $HgCl_2$ was added directly to the LB agar when molten at 65°C, whereas 100µL aliquots of a 50.2% (v/v) solution of ethanol were spread over the top of the plates, resulting in the final 0.2% ethanol (v/v) concentration.

2.10 Whole genome sequencing for *A. calcoaceticus* strains 5.1, 6.6, RUH 2202, 4.2.2, 4.5.2 and 6.4.2

A single colony of 5.1, 6.6, RUH 2202, 4.2.2, 4.5.2 and 6.4.2 that were grown on solid LB agar plates were each suspended in 200µL sterile PBS in 1.5mL Eppendorf tubes. 100µL of these solutions were then each added to 10mL sterile LB in a 50mL falcon tube and incubated overnight at 30°C and 200rpm in the dark. 100µL of these cultures were diluted with 900µL sterile LB and OD_{600nm} readings were taken using the Jenway 6300 spectrophotometer and multiplied by 10 to give the undiluted culture volumes. Cell numbers between the equivalent of 8 OD_{600nm} ($4x10^9$ cells) and the equivalent of 12 OD_{600nm} ($6x10^9$ cells) of each of the cultures were harvested. The appropriate volumes of each culture

required to produce the numbers of cells within the correct range were added to sterile 50mL falcon tubes and centrifuged for 15 min at 4500rpm in a Beckman Coulter Allegra X-22R centrifuge (Beckman Coulter). The pellets were then resuspended in 1mL PBS in 1.5mL Eppendorf tubes and centrifuged for a further 15 min at 4500rpm in a Sigma 1-13 Benchtop Centrifuge (Sigma Laboratory Centrifuges). The pellets were then resuspended in 0.5 mL of 1x DNA/RNA Shield reagent (Zymo Research) in 2mL screw cap tubes and sent for sequencing at MicrobesNG (Birmingham, UK).

Both Illumina and Oxford Nanopore whole genome sequencing, annotation and assemblies were provided by MicrobesNG using their Standard and Enhanced services. The Standard service consisted of Illumina short-read sequencing, annotation and *de novo* assembly only. The Enhanced service consisted of both Illumina short-read and Oxford Nanopore long-read sequencing, annotation and *de novo* assembly. Assembly for the enhanced service incorporated the Oxford Nanopore long-reads as scaffolds for the Illumina short reads, a technique which is able to produce high quality, fully assembled, complete genomes. A full description of their sequencing protocols and their analysis pipeline are both detailed on the MicrobesNG website. (http://www.microbesng.com). Illumina sequencing reads were trimmed by MicrobesNG using Trimmomatic 0.30 and their quality was assessed using Samtools, BedTools and BWA-MEM (Bolger, Lohse and Usadel, 2014; li *et al.*, 2009; Quinlan and Hall, 2010; Li, 2013). Genomes were then assembled using Unicycler v0.4.0 and the contigs were annotated using Prokka 1.11 (Wick *et al.*, 2017; Seeman, 2014). Assembly metrics were calculated using QUAST and the taxonomic distributions of read-mapping were calculated using Kraken (Wood and Salzberg, 2014). Taxonomic distributions were to be used for verification of the taxonomic assignments provided by 16S rRNA gene amplicon sequencing in case of error. Strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 were sequenced using the Enhanced Service. Strains 4.2.2, 4.5.2 and 6.4.2 were sequenced using the Standard Service only due to financial constraints.

The assembly graphs for strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 obtained from MicrobesNG were then examined and edited using Bandage version 0.8.1 (Wick *et al.*, 2015). Calculations of coding and non-coding sequences were performed in Microsoft Excel using the files containing annotated predicted genes produced by whole genome sequencing. Genome maps for each strain were made using Artemis 18.2.0 and DNAPlotter (Carver *et al.*, 2012; Carver *et al.*, 2009). Average nucleotide identity (ANI) calculations were performed between genomes using an online ANI calculation server, with default parameters (http://enve-omics.ce.gatech.edu/ani/). The reference genomes which were not produced in

these studies were retrieved from the NCBI genome database (Wheeler *et al.*, 2007). These included the complete reference genome assemblies of *Pseudomonas aeruginosa* PAO1 (ASM676v1), *Pseudomonas oryzihabitans* (ASM151881v1), *Pseudomonas putida* (ASM41267v1), *Pseudomonas plecoglossicida* (ASM339125v1) and *Pseudomonas entomophila* L48 (ASM2610v1). A threshold of >95% ANI was chosen for the delineation of species, meaning that any two strains sharing an ANI of at least 95% were assigned to the same species (Rodriguez-R & Konstantinidis, 2014).

2.11 Bioinformatic analysis of the whole genome sequences of strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202

Pairwise whole genome sequence alignments were performed using the progressiveMauve algorithm in Mauve 20150226 build 10 (Darling *et al.*, 2004). The genomes of 5.1 and 6.6 were each used as the reference sequences compared against RUH 2202 using the progressiveMauve default parameters. Files containing single nucleotide polymorphisms (SNPs), gaps and annotated 1-1 orthologs were exported using the relevant interface tools. The parameters used for exporting the orthologs were 70% minimum identity and coverage and 100% maximum identity and coverage.

Global alignments of predicted amino acid sequences were performed using the EMBOSS Needle pairwise sequence alignment tool using default parameters (Madeira *et al.*, 2022). Amino acid sequence global alignments were visualised using the Clustal Omega multiple sequence alignment tool and visualised and edited using Jalview 2.11.2.5 (Madeira *et al.*, 2022; Waterhouse *et al.*, 2009). Individual sequence similarity searches for predicted amino acid sequences were performed using the Protein Basic Local Alignment Search Tool (BLASTP) with default parameters. The exception to this was the search for a gene 15 homolog, for which the low complexity region filter was removed and the organism filtered for Bacteria (taxid:2). The top BLASTP hits were selected as the most closely-related protein homologs. NCBI's CD-Search tool was used to identify conserved domains within predicted amino acid sequences (Marchler-Bauer *et al.*, 2015). Default parameters were used for the analysis of gene 2947, and for gene 15 the composition-based statistics adjustment was removed. PSORTb 3.0.3 was used to predict the subcellular localisations of predicted proteins using default parameters (Yu *et al.*, 2010). Artemis 18.2.0 was used for the exploration and visualisation of the genetic context of predicted proteins and the calculation of GC% content across genome sequences and for specific genes (Carver *et al.*, 2012).

Chapter 3: Facilitated horizontal gene transfer approaches for the isolation of novel metaldehyde and metazachlor-degrading organisms

3.1 Introduction

It has been shown previously that metaldehyde undergoes microbial biodegradation in soils, and several soil-derived bacterial strains capable of this trait were identified previously (Castro-Gutiérrez *et al.*, 2020; Thomas *et al.*, 2017). The genomes of two model degrading strains, *A. calcoaceticus* E1 and *Sphingobium sp.* CMET-H were sequenced and their metaldehyde-degrading genes were revealed. A gene cluster consisting of *mahX*, *mahY* and *aldH* was identified in *A. calcoaceticus* E1, present on a plasmid named pAME76. This plasmid also displayed the mercury resistance genes *merD, merA, merC and merR* with the potential to confer mercury resistance, however, this had not been proven experimentally. The genome sequence of *A. calcoaceticus* E1 was able to be used as a reference to search for metaldehyde-degrading genes in other species, including *A. bohemicus* JMET-C, *A. lwoffii* SMET-C, *P. vancouverensis* SMET-B and *C. jiangsuensis* SNO-D. It was revealed that the other strains that were compared possessed highly similar gene clusters to that found in *A. calcoaceticus* E1.

mahS, a gene homolog of *mahX* was later identified as a metaldehyde-degrading gene in *Sphingobium* CMET-H, present on a plasmid named pSM1 (Castro-Gutiérrez *et al.*, 2022). It was hypothesized that acetoin-degrading genes in proximity to *mahS* are also involved in degradation in *Sphingobium* CMET-H, rather than genes resembling *mahY* and *aldH*. In the case of *A. calcoaceticus* E1, the degrading gene cluster showed evidence of horizontal gene transfer-mediated acquisition, in the form of insertion sequences flanking the gene cluster, a higher GC% content surrounding this locus compared to the genomic average for the strain and the fact that the gene cluster is highly conserved among the other degrading species which were compared. Plasmid pAME76 is hypothesized to lack conjugative abilities, however, this had not been tested experimentally. On the other hand, because strains of the *Acinetobacter* species, including *Acinetobacter calcoaceticus*, are well known for their competence for natural transformation, it was previously hypothesised that the *Acinetobacter*

calcoaceticus metaldehyde-degrading genes and plasmid could be transferred via transformation to other bacterial strains (Palmen and Hellingwerf, 1997; Johnsborg, Eldholm, and Håvarstein, 2007).

The metaldehyde-degrading plasmid pSM1 in the model metaldehyde-degrading strain *Sphingobium* CMET-H displays the genetic components required for conjugation, suggesting it is also able to be further disseminated to other bacterial strains by horizontal gene transfer. This had not been proven experimentally.

There is evidence to suggest that metazachlor is degraded in the environment, including detections of its metabolites in water sources, soil degradation studies and experiments which have tracked its degradation in aquatic microcosm and mesocosm settings (Mohr *et al.*, 2007; EFSA, 2017; Bighiu and Goedkoop, 2021). Much less is known, however, about its microbial degradation in comparison to other pesticides and no microbial species responsible for the process was identified previously. Bacterial species which can degrade other chloroacetamide compounds, however, have been discovered previously (Chen *et al.*, 2014; Cheng *et al.*, 2017; Hou *et al.*, 2014; Liu *et al.*, 2012; Xu *et al.*, 2006; Zhang *et al.*, 2011; Ahmad, 2020). Furthermore, glutathione transferases have been shown to provide the main route of degradation in rats, plants and chloroacetamide-degrading bacteria, and these enzymes are predicted to be responsible for the first step of metazachlor degradation in soil (Zablotowicz *et al.*,1995; Zablotowicz *et al.*, 2001; Laue, Field and Cook, 1996; EFSA, 2008). Identification of a metazachlor-degrading microorganism could provide an avenue for the design of bioremediation approaches.

The microbial communities that reside within soils are some of the largest and most diverse on the planet, and one gram of soil, depending on its type, can contain between 10^6 - 2×10^9 prokaryotic cells, comprising up to 5×10^4 different species (Whitman, Coleman and Wiebe,1998; Roesch *et al.*, 2007). These organisms present a wide range of metabolic pathways which can be identified and exploited for biotechnological research purposes, including those which facilitate xenobiotic degradation. It is not always simple to study xenobiotic-degrading organisms, however, as traditional methods for their isolation from the environment are problematic because about only 1% of the total estimated bacterial species present in soils can be cultured using existing laboratory culture media (Pham and Kim, 2012). Alternative methods, therefore, which do not rely on culturing the xenobiotic-

degrading organisms themselves can be advantageous to the study of novel xenobiotic-degrading genes and pathways.

It has been shown previously that horizontal gene transfer can be facilitated and studied within soil microcosms. The process can be induced between donor strains indigenous to the soil microcosms and chosen, recipient strains used to inoculate them, leading to the uptake of xenobiotic-degrading genes and increases in degrading organisms (French, Zhou and Terry, 2020; Hill and Top,1998). These approaches could help to provide evidence of the horizontal transmissibility of xenobiotic-degrading genes and provide avenues for the design of bioremediation approaches.

3.1.1 Experimental rationale and aims of the chapter

The work described in this chapter aimed to exploit the natural horizontal gene transfer process within soil microbial communities to isolate novel metaldehyde and metazachlor-degrading strains. This would rely on- and provide evidence for the horizontal transmissibility of the genetic determinants of metaldehyde and metazachlor degradation, thus, demonstrating their horizontal transmissibility in a soil microbial community.

Soil microcosms were constructed to represent soil microbial communities and to provide the donors for horizontally transferred metaldehyde and metazachlor degradation genes. Additions of metaldehyde or metazachlor to microcosms were intended to create a selective pressure favouring the utilisation of microbial degradation pathways targeting these xenobiotics. Culturable bacterial strains were utilised as the intended recipients of horizontally transferred metaldehyde and metazachlor degradation genes donated by the microbes indigenous to the soil microcosms, and microcosms were allocated a time period for horizontal gene transfer to occur. By facilitating the uptake of degrading genes into chosen culturable strains, culturability problems which may have prevented the isolation of novel degrading organisms in the past would not apply.

The non-degrading, ethanol-utilising *Acinetobacter calcoaceticus* strain, RUH 2202, was selected as a suitable recipient of horizontally transferred metaldehyde-degrading genes based on previous work which demonstrated its lack of metaldehyde degradation capabilities (Thomas *et al.*, 2017). To provide a *Sphingobium* species recipient strain, experimental testing to ensure a lack of metaldehyde degradation capability in *S. chlorophenolicum* DSM 7098, a closely-related suspected non-degrader, was carried out. For the experiments investigating horizontal gene transfer of metazachlor-degrading genes, the previously

identified model metaldehyde-degrading strains *A. calcoaceticus* E1 and *Sphingobium* CMET-H were selected as the intended recipients of horizontally transferred metazachlor-degrading genes. Thus, in theory, metaldehyde-degrading variants of *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098, and metazachlor-degrading variants of *A. calcoaceticus* E1 and *Sphingobium* CMET-H could be produced by the facilitated horizontal transfer approach within soil microcosms, which could be cultured in a laboratory and taken forward for further analyses.

A simple selective enrichment method (Abraham and Silambarasan, 2013) was coupled with the facilitated horizontal gene transfer approach for the re-isolation of the metaldehyde-degrading variants of *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098, the metazachlor-degrading variants of *A. calcoaceticus* E1 and *Sphingobium* CMET-H and other possible degrading organisms from soil microcosm samples. This method was inspired by previous studies which utilised MSM containing metaldehyde as the sole source of carbon for the selection of metaldehyde-degrading organisms from soil samples (Thomas *et al*, 2017; Castro-Gutiérrez *et al.*, 2020).

A simple *in vitro* mating assay testing the conjugative capabilities of the *A. calcoaceticus* E1 plasmid, pAME76, was performed to verify whether pAME76 can be transferred to the non-degrading *A. calcoaceticus* strain, RUH 2202, via conjugation. To do this, a suitable selection mechanism was required. Given that previous work demonstrated that *A. calcoaceticus* RUH 2202, but not E1, can utilise ethanol as a carbon source, ethanol utilisation assays were performed to verify these traits in the E1 and *A. calcoaceticus* RUH 2202 strains used in this study. Additionally, based on the presence of the mercury resistance genes *merD, merA, merC and merR* demonstrated on plasmid pAME76 in *A. calcoaceticus* E1, mercury resistance assays were performed to investigate the mercury resistance capabilities of both *A. calcoaceticus* strains E1 and RUH 2202. These assays aimed to aid the design of a selection mechanism for isolating metaldehyde-degrading RUH 2202 variants from mating cultures containing both *A. calcoaceticus* strains E1 and RUH 2202 by selecting for a combination of ethanol utilisation and mercury resistance traits. Hypothetically, successful conjugation of plasmid pAME76 would produce ethanol-utilising RUH 2202 variants which demonstrate mercury resistance capabilities, conferred by the mercury resistance genes present on pAME76, thereby providing experimental evidence for the horizontal transmissibility of pAME76. Dependent on the results of these experiments, these traits could be used to perform phenotypic comparisons between RUH 2202, E1 and potential metaldehyde-degrading variants of RUH 2202 and to infer the presence of the pAME76 plasmid in metaldehyde-degrading RUH 2202 variants.

Antibiotic resistance assays were performed to identify a potential antibiotic-based selection mechanism for the design of conjugation assays demonstrating the conjugative transfer of plasmid pSM1 between the metaldehyde-degrading strain *Sphingobium sp.* CMET-H and the non-degrading strain *S. chlorophenolicum* DSM 7098 and the conjugative transfer of plasmid pAME76 between the metaldehyde-degrading strain *A. calcoaceticus* E1 and the non-degrading strain *A. calcoaceticus* RUH 2202. The identification of an antibiotic resistance trait that was present in *S. chlorophenolicum* DSM 7098 and *A. calcoaceticus* RUH 2202 but absent from *Sphingobium* CMET-H and *A. calcoaceticus* E1 could, in combination with a selection mechanism for metaldehyde-degrading capabilities, facilitate the selection of metaldehyde-degrading *S. chlorophenolicum* DSM 7098 and *A. calcoaceticus* RUH 2202 variants from a mating cultures containing combinations of the *Sphingobium* CMET-H and DSM 7098 strains and combinations of the *A. calcoaceticus* E1 and RUH 2202 strains, and provide experimental verification of the conjugative capabilities of plasmids pSM1 and pAME76.

The aims of this chapter are:

- To isolate metaldehyde-degrading variants of strains *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098 and metazachlor-degrading variants of *A. calcoaceticus* E1 and *Sphingobium* CMET-H from soil microcosms following a period allocated to facilitated horizontal gene transfer
- To determine the success of the facilitated horizontal gene transfer approach for inducing the uptake of metaldehyde and metazachlor-degrading genes in *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098
- To investigate the *in vitro* horizontal gene transfer of the previously identified metaldehyde-degrading genes and plasmids from strains *A. calcoaceticus* E1 and *Sphingobium* CMET-H to non-degrading strains *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098 in a simple mating assay

3.2 Results

3.2.1 Isolation of metaldehyde-degrading bacterial strains from soil microcosms

3.2.1.1 Verification of *S. chlorophenolicum* DSM 7098 as a non-degrading strain

An initial test was performed to verify whether *S. chlorophenolicum* DSM 7098 could be used as a non-degrading recipient strain in horizontal gene transfer experiments. *S. chlorophenolicum* DSM 7098 and *Sphinbogium CMET-H* were revived from glycerol stocks and grown on nutrient agar until single colonies were produced (data not shown). A single colony of each strain was re-streaked onto metaldehyde-supplemented minimal salts media (MSM) at 150mg/L, alongside the metaldehyde-degrading strain *Sphingobium* CMET-H. No colonies of *S. chlorophenolicum* DSM 7098 were visible after 72h growth, whereas *Sphingobium* CMET-H produced a prominent streak of colonies (figure 3-1). Based on this it was evident that minimal degradation would occur.
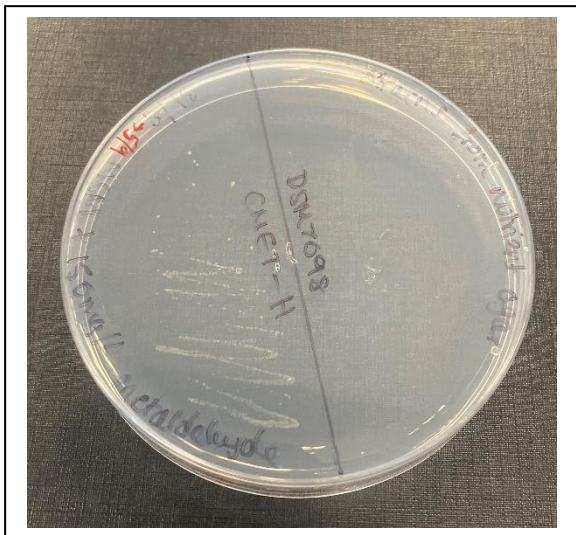
Figure 3-1 MSM agarose plate supplemented with 150mg/L metaldehyde and inoculated with a single colony of strains *Sphingobium* CMET-H and *S. chlorophenolicum* DSM 7098, incubated for 72h at 30°C in the dark.

3.2.1.2 Selective enrichment for the re-isolation of metaldehyde-degrading bacterial strains from soil microcosm environments

To investigate the horizontal transmissibility of metaldehyde-degrading genes, horizontal gene transfer was facilitated *in situ* within soil microcosms between culturable, non-degrading "recipient strains", *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098, and strains within the indigenous soil microcosm bacterial community. Coupled with the facilitated horizontal gene transfer approach, a selective enrichment method was developed for the re-isolation of metaldehyde-degrading variants of *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098 from the soil microcosm environments. The soil microcosm treatment conditions are displayed in table 2-1.

The selective enrichment method tested for the re-isolation of *A. calcoaceticus* RUH 2202 and *S. chlorophenolicum* DSM 7098 metaldehyde-degrading variants from the soil microcosms utilised MSM supplemented with metaldehyde as the sole source of carbon. Soil microcosm samples were passaged three times (P1-P3) in liquid MSM supplemented with metazachlor (75mg/L) and trace elements (4mL/L). For each passage, 1g microcosm material was added to 10mL supplemented MSM in 50mL falcon tubes and grown for 24h at 30°C and 200rpm. 100µl aliquots of these cultures were transferred to 10mL fresh supplemented MSM for each subsequent passage. 100µL aliquots of the P3 cultures were then spread onto solid metaldehyde-supplemented MSM agarose plates at 85mg/L and incubated at 25-30°C for 72h in the dark, leading to the growth of distinguishable bacterial colonies (data not shown).

Six areas containing colonies which demonstrated different morphologies (data not shown) were sampled and subcultured from each metaldehyde-supplemented MSM agarose plate until single colonies of pure cultures were obtained on both further metaldehyde-supplemented (80µg/mL) plates and MSM-only plates. This allowed a comparison of the strains' growth in the presence and absence of metaldehyde, with the aim that possible oligotrophs or agarose degraders were identified and eliminated.

Seventy candidate metaldehyde-degrading strains which grew noticeably better on metaldehyde-supplemented plates vs MSM-only controls were further subcultured onto solid LB agar. The candidate degrading isolates were isolated from microcosms numbered 3, 4, 5,

6, 8, 11, 12, 13, 14 and 16 whereas no candidate strains were isolated from microcosms numbered 1, 2, 7, 9, 10 or 15 (table 3-1) (see table 2-1 for the microcosm numbering system). Degraders were isolated from microcosms containing both allotment and domestic compost soils, as well as from microcosms both treated and untreated with metaldehyde, suggesting that netiher the soil type nor the addition of metaldehyde largely impacted the isolation of suspected degrading organisms. It was often difficult, however, to confidently identify genuine metaldehyde degraders based on their growth on the solid supplemented MSM agarose media alone, due to the background growth of many of the strains on the non-supplemented control plates. Further analysis was deemed necessary to determine whether the strains isolated at this point in the study were actually true metaldehyde-degrading organisms.

Soil microcosm no.	No. metaldehyde degrader isolates	Soil microcosm no.	No. degrader isolates
1	0	9	0
2	0	10	0
3	2	11	15
4	8	12	8
5	9	13	12
6	8	14	5
7	0	15	0
8	1	16	2

Table 3-1 Numbers of candidate metaldehyde degraders isolated via selective enrichment of soil microcosm samples and the soil microcosms from which they were isolated, based on the numbering system described in table 2-1.

3.2.1.3 Liquid growth assays for the verification of the metaldehyde-degrading capabilities of candidate degrading bacterial isolates identified by selective enrichment of soil microcosm samples

To further verify the inferred metaldehyde-degrading capabilities of the candidate metaldehyde-degrading strains isolated by selective enrichment, liquid growth assays were carried out to compare their growth in liquid minimal salts media (MSM) supplemented with metaldehyde at 150mg/L and MSM only. Sets of candidate strains were cultured at 30°C for 72 hours in 96-well plate format, with triplicate cultures of each strain grown in metaldehyde-

supplemented MSM and MSM only, with uninoculated metaldehyde MSM and non-supplemented MSM controls. Optical density readings were taken every 0.5 hours at 620nm. One set of 10 strains was grown for 53.5 hours only due to an error incurred by the plate reader used which halted the progress of the assay (as indicated in appendix 6.1).

Two candidate metaldehyde-degrading bacterial strains were identified, named 5.1 and 6.6, which were selected for further analysis based on their demonstration of improved growth in metaldehyde-supplemented MSM compared to non-supplemented MSM. This was based on a comparison of initial growth curves which were produced for the individual triplicate cultures of the candidate strains grown in supplemented MSM and MSM only, from which the most promising candidate strains were chosen for further analysis. An example set of initial growth curves is shown in figure 3-2 (the remaining growth curves can be found in appendix 6.1). Strain 5.1 was isolated from soil microcosm 5, which contained a sample of allotment soil treated with metaldehyde and inoculated with *Acinetobacter calcoaceticus* RUH 2202 overnight culture. Strain 6.6 was isolated from soil microcosm 6, which contained a sample of allotment soil which was inoculated with RUH 2202 culture, with no addition of metaldehyde. 5.1 and 6.6 produced glossy, round, white colonies when grown on solid LB agar (appendix 6.2). None of the other candidate degrading isolates demonstrated improved growth in MSM supplemented with metaldehyde compared to MSM only, based on the initial growth curve data, and these strains were excluded from any further analysis. This means that no metaldehyde-degrading variants of *S. chlorophenolicum* DSM 7098 were isolated. It is important to note that for some of the isolated strains, there was considerable variation between individual triplicate cultures grown in the same conditions, which meant that assigning metaldehyde-degrading traits to many of the strains was difficult.

Average OD values were calculated for the triplicated cultures of 5.1 and 6.6 to produce further growth curves which better visualised their growth (figure 3-3). Both strains demonstrated improved growth in MSM supplemented with metaldehyde compared to their growth in MSM only. 5.1 and 6.6 showed increased average peak OD values of 0.67 at 31.5 hours and 0.40 at 50 hours, respectively when grown in metaldehyde-supplemented MSM compared to their average peak OD values of 0.40 at 45.5 hours and 0.27 at 48 hours for 5.1 and 6.6, respectively, when grown in MSM only (table 3-2). For strain 6.6, however, there were large standard deviations of the triplicated OD readings between the growth curves of 6.6 grown in supplemented MSM and MSM only, indicating large variation in the optical density readings obtained for individuate triplicate cultures. It is also important to note that

there was some background growth of both strains in non-supplemented MSM cultures, which suggests that despite there being no carbon source added during preparation of the media, the strains were able to scavenge organic carbon either due to carryover during the preparation of the liquid growth assays or from trace amounts present in the MSM.
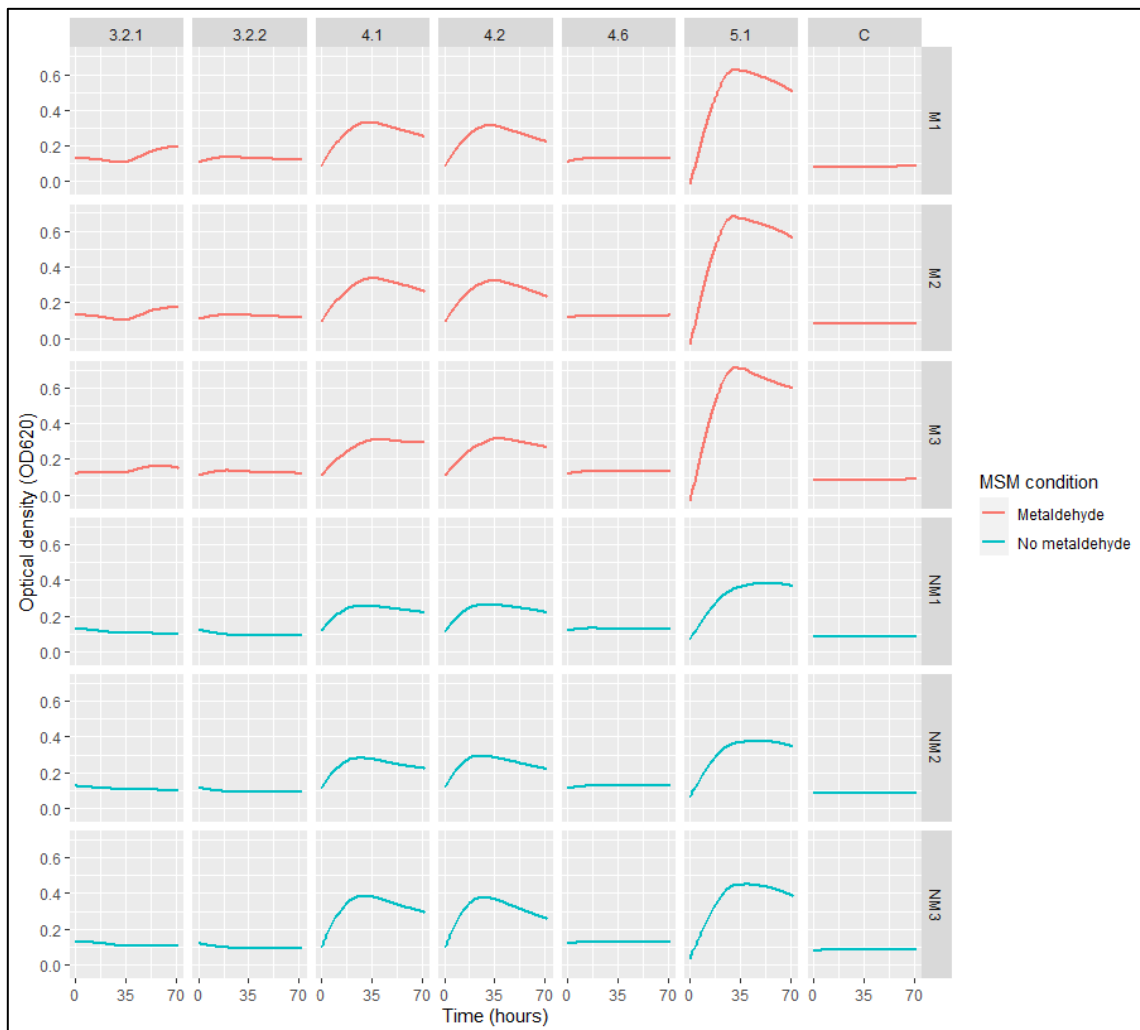
Figure 3-2 Hourly optical density readings at 620nm (OD) for individual triplicate cultures of metaldehyde-degrading candidate isolates 3.2.1, 3.2.2, 4.1, 4.2, 4.6, 5.1 and uninoculated controls (C) (labelled above the graphs) grown in supplemented MSM (150mg/L) (shown in red, with individual cultures labelled M1-M3 and non-supplemented MSM (shown in blue, with individual cultures labelled NM1-NM3).

To further test the statistical significance of the differences in average peak OD values observed between the growth of 5.1 and 6.6 in supplemented and non-supplemented MSM, two-sample T-tests were performed using the triplicated growth curve data. The difference in peak OD values for strain 5.1 when grown in supplemented MSM and MSM only was

statistically significant (t = 6.4932, df = 4, p-value = 0.002901). For strain 6.6, however, the difference in peak OD values was not found to be statistically significant (t = 1.7032, df = 4, p-value = 0.1637), which further demonstrates the lack of statistical significance of the improved growth of 6.6 in supplemented MSM compared to growth in MSM only.
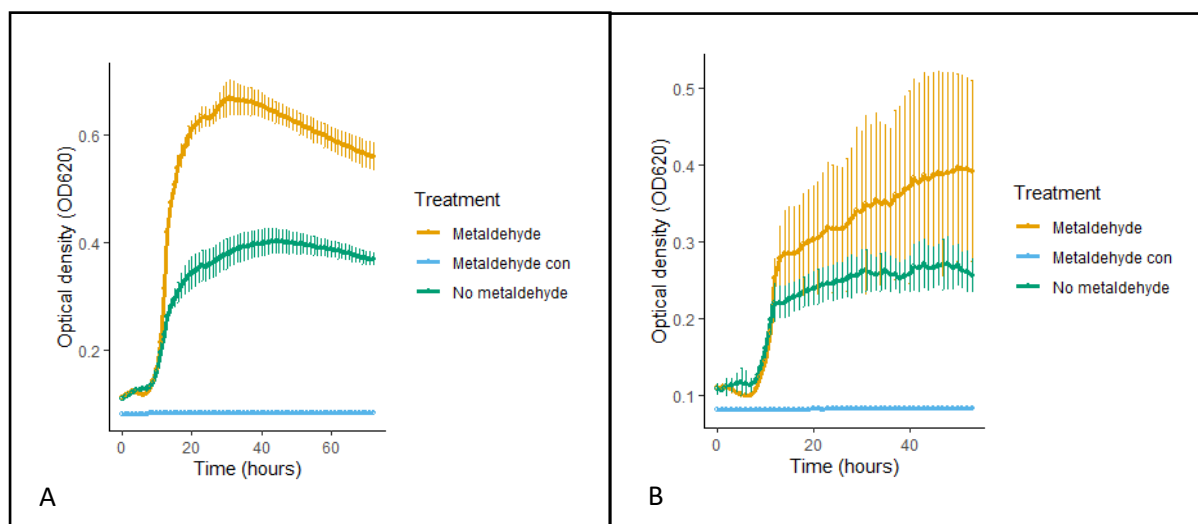
Figure 3-3. Average hourly optical density readings taken at 620nm (OD620) during growth of triplicate cultures of 5.1 (A) and 6.6 (B) in MSM supplemented with 150mg/L metaldehyde (labelled Metaldehyde), represented by the orange line and non-supplemented MSM (labelled No metaldehyde), represented by the green line. Data for uninoculated wells containing metaldehyde MSM only (labelled Metaldehyde con) are represented by the blue line (Metaldehyde con). Error bars represent standard deviation of the triplicate cultures.

Strain	Met MSM peak OD	Time (hours)	MSM only peak OD	Time (hours)
5.1	0.67	31.5	0.40	45.5
6.6	0.40	50	0.27	48

Table 3-2. Average peak OD values calculated from triplicate liquid cultures of 5.1 and 6.6 grown in metaldehyde supplemented MSM at 150mg/L (Met MSM peak OD) and non-supplemented MSM (MSM only peak OD) and the time points at which these readings were taken.

After re-streaking colonies of 5.1, 6.6, *Acinetobacter calcoaceticus* E1 and *Acinetobacter calcoaceticus* RUH 2202 onto solid metaldehyde-supplemented and non-supplemented MSM agarose, it was demonstrated that the previously identified non-metaldehyde-degrading strain RUH 2202, as well as 5.1 and 6.6, appeared to be able to grow on non-supplemented MSM agarose, as well as on metaldehyde-supplemented MSM agarose

(figure 3-4). This did not seem to be the case for the metaldehyde degraders *A. calcoaceticus* E1 or *Sphingobium* CMET-H (figure 3-1) which could not grow on MSM only. Again, this suggests that 5.1, 6.6 and *A. calcoaceticus* RUH 2202 were able to utilise organic carbon in the media due to carryover or presence of trace amounts of organic carbon already present in the MSM.
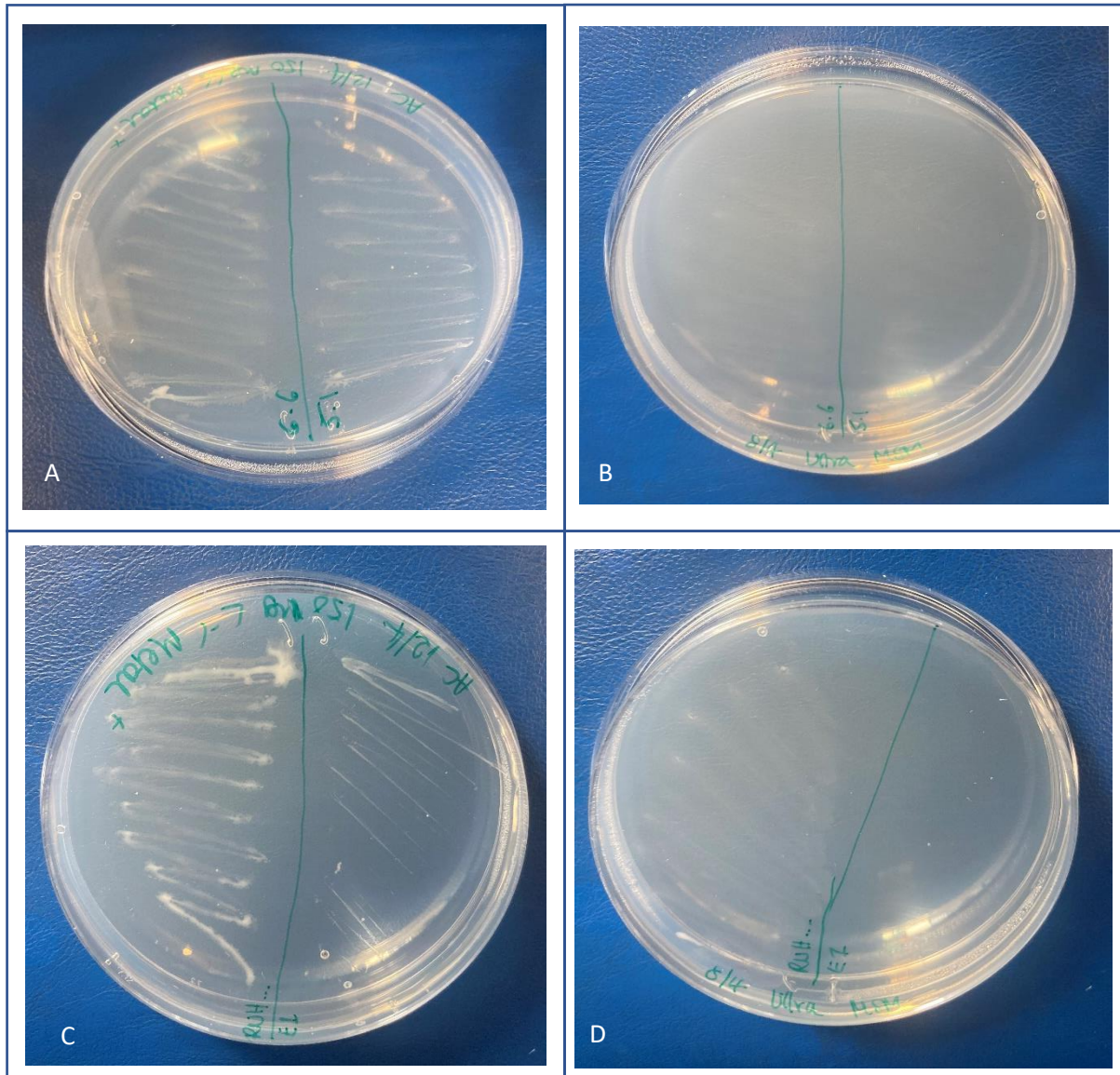
Figure 3-4 Growth of strains 5.1, 6.6, *A. calcoaceticus* RUH 2202 (RUH 2202) and *A. calcoaceticus* E1 (E1) on metaldehyde-supplemented MSM (150mg/L) (panels A and C) and MSM only (panels B and D). Strains are labelled as appropriate on plates.

3.2.1.4 16S rRNA gene amplicon sequencing for the taxonomic classifications of strains 5.1 and 6.6

16S rRNA gene amplicon sequencing was carried out to obtain full 16S rRNA gene sequences for strains 5.1 and 6.6. Agarose gel electrophoresis visualising the 16S rRNA amplicons can be viewed in Appendix 6.3. Partial 16S rRNA gene sequences were obtained and trimmed to remove low-quality and primer sequence regions, resulting in 1190 and 1240 base pairs (bp) amplicons for 5.1 and 6.6, respectively. The resulting sequences are displayed in appendix 6.4. BLASTN analysis of the 16S rRNA partial gene sequences produced top hits for both strains of partial 16S sequences from *Acinetobacter calcoaceticus*, which shared over 99% identity with 99-100% query cover (table 3-3). Both 5.1 and 6.6, therefore, were assigned to this species.

Strain	Amplicon length (bp)	Top hit	% Identity	% Query cover	Hit accession no.	Species assigned
5.1	1190	*Acinetobacter calcoaceticus* strain ATCC 23055	100	99%	NR_117619.1	*Acinetobacter calcoaceticus*
6.6	1240	*Acinetobacter calcoaceticus* strain ATCC 23055	99.68%	100	NR_117619.1	*Acinetobacter calcoaceticus*

Table 3-3 BLASTN hits for the 16S rRNA sequences of strains 5.1 and 6.6.

3.2.1.5 Liquid growth assays investigating the ethanol utilisation capabilities of *A. calcoaceticus* strains 5.1, 6.6, RUH 2202 and E1

It was previously demonstrated that the non-degrading strain *A. calcoaceticus* RUH 2202 can use ethanol as a carbon source, whereas the degrading strain E1 can not (Thomas *et al.*, 2017; Thomas, 2016). To learn more about the phenotypic traits of strains 5.1 and 6.6 compared to RUH 2202 and E1, liquid growth assays testing their ethanol utilisation were carried out in MSM supplemented and non-supplemented with 0.2% (v/v) ethanol. Cultures of each strain along with uninoculated controls were grown over 48 hours, with optical density readings taken at 0, 24 and 48 hours at OD_{600nm}.

Liquid cultures for strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 became significantly turbid after 24 hours of growth in MSM supplemented with ethanol, whereas E1 cultures and uninoculated controls of ethanol-supplemented MSM and MSM only did not (data not shown). Average OD values for 5.1, 6.6 and *A. calcoaceticus* RUH 2202 increased after 24 hours when media was supplemented with ethanol compared to MSM-only cultures, whereas for strain E1, the addition of ethanol to the cultures caused little increase in growth (figure 3-5). Peak OD values for 5.1, 6.6 and RUH 2202 for ethanol-supplemented MSM were 0.96 at 24 hours, 0.93 at 48 hours and 1.39 at 24 hours, respectively and in MSM only were 0.014 at 24 hours, 0.022 at 48 hours and 0.015 at 48 hours for 5.1, 6.6 and RUH 2202, respectively (table 3-4). For strain E1, the peak OD value for its growth in ethanol-
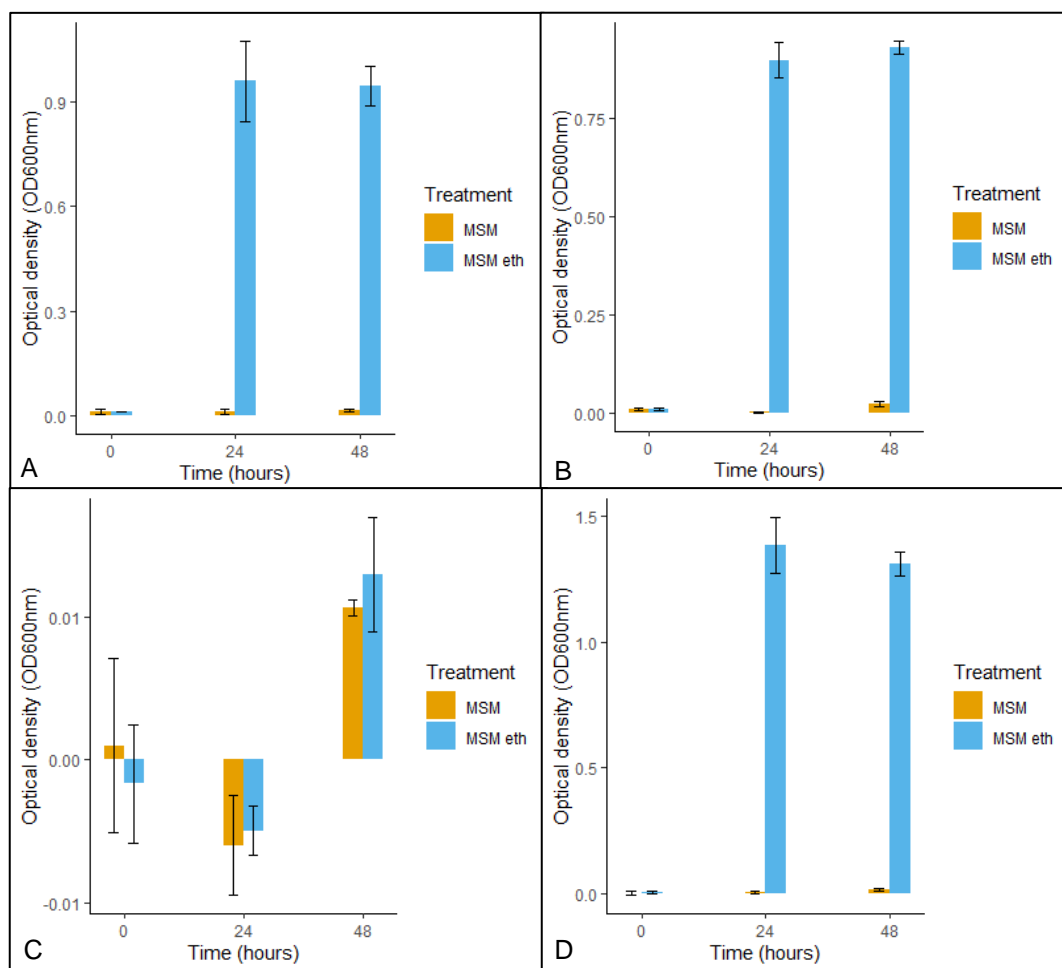
Figure 3-5 Average Optical density readings at 600nm for *A. calcoaceticus* strains 5.1 (panel A), 6.6 (panel B), E1 (panel C) and RUH 2202 (panel D) grown in MSM only (MSM) and MSM supplemented with 0.2% ethanol (MSM eth) over 48 hours. Error bars represent standard deviation of 3 biological replicates.

supplemented MSM cultures was 0.013 at 48 hours, which showed little increase compared to the peak OD value of 0.011 at 48h when this strain was grown in MSM only. These results suggest that 5.1 and 6.6, unlike *A. calcoaceticus* E1, can use ethanol as a carbon source for growth (as well as RUH 2202) and that this trait could be used in future experiments to select ethanol-utilising strains over *A. calcoaceticus* E1, in mating experiments, for example, between *A. calcoaceticus* strains E1 and RUH 2202.

Strain	Eth MSM peak OD	Time (hours)	MSM only peak OD	Time (hours)
5.1	0.96	24	0.014	24
6.6	0.93	48	0.022	48
RUH 2202	1.39	24	0.015	48
E1	0.013	48	0.011	48

Table 3-4. Peak average OD values for *A. calcoaceticus* strains 5.1, 6.6 and *A. calcoaceticus* strains RUH 2202 and E1 from 48h liquid growth assay in 0.2% ethanol supplemented MSM (Eth MSM) and MSM only (MSM only). Peak OD values represent highest average OD value.

3.2.1.6 Assays investigating the mercury resistance capabilities of *A. calcoaceticus* strains 5.1, 6.6, RUH 2202 and E1

Mercury resistance assays were carried out to verify whether *A. calcoaceticus* E1 demonstrates mercury resistance, which was hypothesised due to the presence of a mercury resistance operon present on its metaldehyde-degrading plasmid pAME76 (Castro-Gutiérrez *et al.*, 2022). The assays also tested strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 for this trait, for phenotypic comparison purposes. Each strain was grown on solid LB agar plates containing mercury (II) chloride ($HgCl_2$) at 0, 10mg/L and 15mg/L concentrations, with the LB-only plates serving as controls. The mercury resistance trait was confirmed for strain *A. calcoaceticus* E1, demonstrated by the lawns of colonies visible on LB plates containing both 10mg/L and 15mg/L $HgCl_2$ concentrations (figure 3-6). *A. calcoaceticus* RUH 2202, 5.1 and 6.6, however, demonstrated a comparative lack of mercury resistance, showing no visible colonies present at 15mg/L and a few visible colonies at 10mg/L $HgCl_2$, the latter likely representing spontaneous mutants. These results suggest that the inferred metaldehyde-degrading trait observed in 5.1 and 6.6 is not conferred by the metaldehyde-degrading plasmid pAME76. These results also demonstrate that mercury resistance could be used in future repeated experiments to select *A. calcoaceticus* E1 over 5.1, 6.6 and *A. calcoaceticus* RUH 2202 and potentially "track" the horizontal transfer of plasmid pAME76

between bacterial strains. It could also aid the identification of new degrading strains containing the pAME76 plasmid, perhaps, if incorporated into selective enrichment procedures along with selection for the mercury degradation trait.
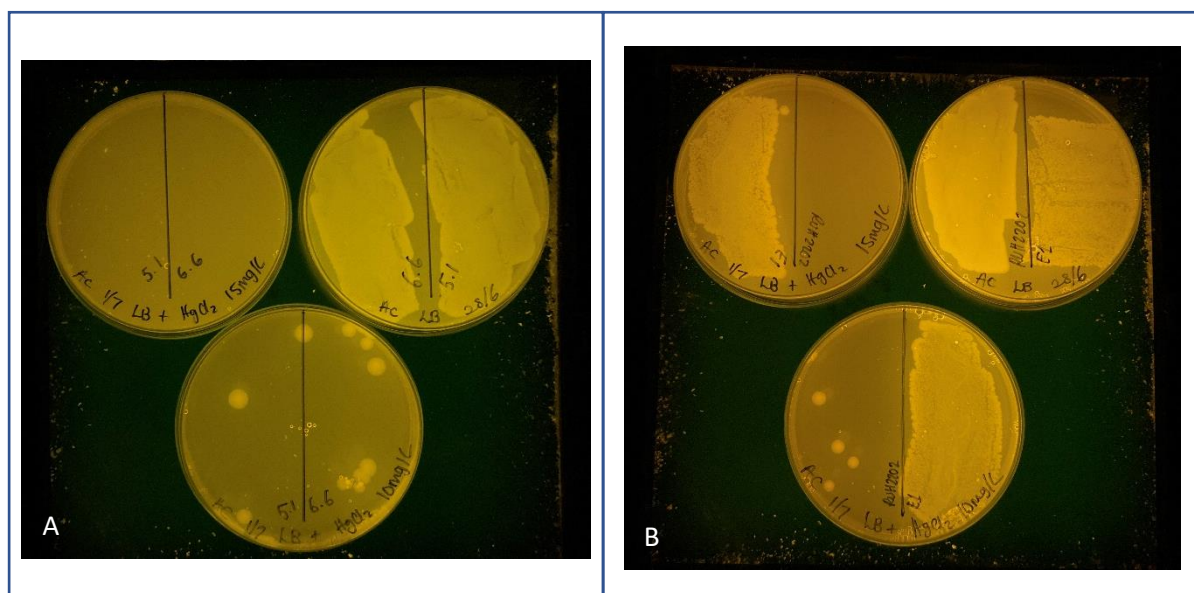
Figure 3-6 Growth of strains 5.1 and 6.6 (A) and *A. calcoaceticus* strains RUH 2202 and E1 (B) on solid LB agar plates supplemented or non-supplemented with 10mg/L or 15mg/L $HgCl_2$ visualised using a blue-light transilluminator. Plates are labelled with strain names and mercury concentrations accordingly. Note that on the image in panel A, the presence of some air bubbles and some light marks on the transilluminator resemble colonies on the plate inoculated with 5.1 and 6.6 , however, no colonies were present on this plate.

Due to the similarity of strains 5.1 and 6.6 to *A. calcoaceticus* RUH 2202 in terms of their species assignment and their ethanol utilisation and mercury resistance traits, it was inferred that 5.1 and 6.6 may be possible *A. calcoaceticus* RUH 2202 variants which demonstrate a metaldehyde-degrading trait. These strains may also, therefore, demonstrate the success of the facilitated horizontal transfer and selective enrichment approaches in inducing the uptake of metaldehyde-degrading genes in a chosen culturable non-degrading host, donated by an indigenous soil microcosm-derived organism via horizontal gene transfer. Ultimately, further experimental assays which can more accurately track bacterial metaldehyde degradation over time and confirm whether 5.1 and 6.6 belong to the *A. calcoaceticus* RUH 2202 strain could provide more robust conclusions as to whether or not they are true metaldehyde-degrading *A. calcoaceticus* RUH 2202 variants and whether the facilitated horizontal gene transfer approach was successful in inducing the acquisition of metaldehyde-degrading genes in *A. calcoaceticus* RUH 2202.

3.2.1.7 Identifying alternative selection mechanisms for the design of conjugation assays between *A. calcoaceticus* strains E1 and RUH 2202, and *Sphingobium* strains CMET-H and DSM 7098

Previous work suggested that the metaldehyde-degrading trait in *Sphingobium* CMET-H is conferred by a conjugative plasmid named pSM1 (Castro-Gutiérrez, *et al.*, 2022). To identify a potential selection mechanism for the design of mating assays testing the conjugative capability of pSM1, antibiotic resistance assays were carried out involving both *Sphingobium* CMET-H and the non-degrading organism *S. chlorophenolicum* DSM 7098. The assays were also performed with *A. calcoaceticus* strains RUH 2202 and E1 to identify possible alternative selection mechanisms other than those based on ethanol utilisation and mercury resistance. Strains were grown for up to 72h at 25-30°C on solid LB agar plates containing rifampicin at both 20µg/mL and 50µg/mL and plates containing nalidixic acid at concentrations of 20µg/mL and 30µg/mL. The *Acinetobacter* strains were able to grow on media containing both the rifampicin concentrations, demonstrating resistance, whereas both of the *Sphingobium* strains were only able to produce colonies at 20µg/mL (data not shown). No visible colonies of any of the strains tested were identified at either nalidixic acid concentration. An appropriate antibiotic resistance selection mechanism based on the selection of antibiotic resistant *S. chlorophenolicum* and *A. calcoaceticus RUH 2202* could not be identified by these experiments, therefore, no mating assay could be designed to investigate the conjugation of pSM1 or pAME76.

3.2.1.8 Mating assays between *A. calcoaceticus* strains E1 and RUH 2202 to test the conjugative capabilities of the metaldehyde-degrading plasmid pAME76

Mating experiments were designed to test whether the pAME76 metaldehyde-degrading plasmid in *A. calcoaceticus* E1 can be horizontally transferred to the non-degrading strain *A. calcoaceticus* RUH 2202 via conjugation. Previous work suggested that pAME76 lacks the genetic components required for conjugation, however, this had not previously been tested experimentally (Castro-Gutiérrez *et al.*, 2022a). Based on the success of the mercury resistance and ethanol utilisation assays, a selective approach involving a combination of ethanol and mercury-supplemented solid MSM agarose media was used to select potential pAME76 transconjugants. Cells from overnight liquid LB cultures of *A. calcoaceticus* strains E1 and RUH 2202 were centrifuged and resuspended in fresh liquid LB to produce three

2mL 50:50 mating cultures in falcon tubes. Mating cultures were incubated for 24 hours to allow conjugation to occur, followed by serial dilution, with 10-4, 10-5 and 10-6 dilutions selected for growth on solid MSM agarose plates containing 0.2% ethanol as a carbon source and 15mg/L HgCl$_2$. No successful transconjugant colonies were identified on plates containing any of the diluted mating cultures. These results confirm earlier observations that pAME67 lacks conjugative capability due to the absence of genetic components that are required for this process (Castro-Gutiérrez et al, 2022a).

3.2.2 Isolation of metazachlor-degrading bacterial strains from soil microcosms

3.2.2.1 Selective enrichment for the re-isolation of metazachlor-degrading bacterial strains from soil microcosm environments

An *in situ* facilitated horizontal gene transfer approach, similar to that used for the facilitated horizontal gene transfer of metaldehyde-degrading genes, was used to investigate the horizontal transmissibility of metazachlor-degrading genes. This was facilitated in soil microcosms between culturable metaldehyde-degrading "recipient strains" *A. calcoaceticus* E1 and *Sphingobium* CMET-H and donor bacterial strains indigenous to the soil microcosm environments. A selective enrichment method, similar to the method described for isolating metaldehyde degraders, was combined with this approach for the re-isolation of metazachlor-degrading variants of *A. calcoaceticus* E1 and *Sphingobium* CMET-H and possible alternative degrading organisms from the soil microcosms. Summaries of the microcosm treatments are displayed in table 2-2.

The selective enrichment method designed for the re-isolation of metazachlor-degrading *A. calcoaceticus* E1 and *Sphingobium* CMET-H variants utilised MSM supplemented with metazachlor as a sole source of carbon. Soil microcosm samples were passaged three times (P1-P3) in liquid MSM supplemented with metazachlor (75mg/L) and trace elements (4mL/L). For each passage, 1g microcosm material was added to 10mL supplemented MSM in 50mL falcon tubes and grown for 24h at 30°C and 200rpm. 100µl aliquots of these cultures were transferred to 10mL fresh supplemented MSM for each subsequent passage. 100µL aliquots of the P3 cultures were then spread onto solid metazachlor-supplemented MSM agarose plates at 75mg/L and incubated at 25-30°C for 72h in the dark, leading to the growth of distinguishable bacterial colonies (data not shown).

Six areas containing colonies which demonstrated different morphologies were sampled and subcultured from each metazachlor-supplemented MSM agarose plate until single colonies of pure cultures were obtained on both further metazachlor-supplemented (75µg/mL) plates and MSM-only plates. This allowed a comparison of the strains' growth in the presence and absence of metazachlor, with the aim that possible oligotrophs or agarose degraders were identified and eliminated. Forty three candidate metazachlor-degrading strains which grew noticeably better on metazachlor-supplemented plates vs MSM-only controls were subcultured onto solid LB agar. Candidate degrading strains were isolated from the microcosms numbered 1,2,3,4 and 6, whereas no candidate strains were identified from soil microcosm 5 (table 3-5) (see table 2-2 for the microcosm numbering system). Suspected degraders were isolated from both metazachlor-treated and untreated microcosms, suggesting that the addition of metazachlor did not determine the isolation of metazachlor degraders from the microcosms. As was observed for the metaldehyde degrader selective enrichment results, however, it was often difficult to confidently identify genuine metazachlor degraders based on their growth on the solid supplemented MSM agarose media alone, due to the background growth of many of the strains on the non-supplemented control plates. Further analysis was deemed necessary to determine whether the strains isolated at this point in the study were true metazachlor-degrading organisms.

Soil microcosm no.	No. of metazachlor degrader isolates
1	9
2	6
3	13
4	7
5	0
6	8

Table 3-5. Numbers of candidate metazachlor degraders isolated via selective enrichment of soil microcosm samples and the soil microcosms from which they were isolated, based on the numbering system described in table 2-2.

3.2.2.2 Liquid growth assays for the verification of the metazachlor-degrading capabilities of candidate degrading bacterial isolates, identified by selective enrichment of soil microcosm samples

To verify the metazachlor-degrading capabilities of the candidate strains isolated from soil microcosm selective enrichments, liquid growth assays were performed comparing their growth in liquid MSM supplemented with metazachlor (150 mg/L) and non-supplemented MSM with acetone (0.001%v/v). Acetone was added to non-supplemented MSM control media at this stage because of the acetone present in the metazachlor stock solutions which were used to supplement MSM throughout the study. This aimed to allow the detection of strains which may be using acetone as a carbon source in the metazachlor-supplemented media rather than metazachlor itself. These assays were performed similarly to those described for the metaldehyde-degrading candidate strains, with sets of strains grown in triplicate cultures in both supplemented MSM and MSM only at 30°C for 72h in 96-well plate format. OD readings were taken every 0.5 hours at 620nm.

Three candidate metazachlor-degrading bacterial strains, 4.2.2, 4.5.2 and 6.4.2, were isolated from soil microcosms and selected for further analysis based on their demonstration of improved growth in metazachlor-supplemented MSM compared to non-supplemented MSM. This was based on a comparison of initial growth curves which were produced for the individual triplicate cultures of the candidate strains grown in supplemented MSM and MSM only, from which the most promising candidate strains were chosen for further analysis. An example set of initial growth curves is shown in figure 3-8 and the remaining growth curves are displayed in appendix 6.5. Strains 4.2.2 and 4.5.2 were isolated from an agricultural soil microcosm inoculated with the metaldehyde-degrading bacterium *Sphingobium sp.* CMET-H with no metazachlor treatment. 6.4.2 was isolated from untreated agricultural soil which lacked both metazachlor and bacterial inoculation. The colonies produced by each of these two strains on LB agar were pale yellow and round (appendix 6.7). None of the other candidate metazachlor-degrading isolates demonstrated improved growth in MSM supplemented with metazachlor compared to MSM only, based on the initial growth curve data, and these strains were excluded from any further analysis. This means that no metazachlor-degrading variants of *A. calcoaceticus* E1 were isolated. For some of the candidate degrading strains, however, there was considerable variation between individual triplicate cultures grown in the same conditions. Assigning metazachlor-degrading traits to many of the strains, therefore, was difficult.

Average OD values were calculated for triplicated cultures of 4.2.2, 4.5.2 and 6.4.2 to produce further growth curves which better visualised their growth (figure 3-9). All three strains demonstrated improved growth in MSM supplemented with metazachlor compared to non-supplemented MSM. Average peak OD values for triplicated cultures of strains 4.2.2, 4.5.2 and 6.4.2 in metazachlor MSM were 0.58 at 31.5 hours, 0.74 at 52 hours and 0.17 at 25.5 hours, respectively (table 3-6). Average peak OD values for growth in MSM only were 0.38 at 70 hours, 0.50 at 19.5 hours and 0.15 at 9.5 hours for strains 4.2.2, 4.5.2 and 6.4.2, respectively, demonstrating lower OD values compared to those obtained in supplemented MSM. Strain 6.4.2 showed only a very small increase in peak OD of 0.02. It is also important to note that there was some background growth of all strains in liquid MSM only.
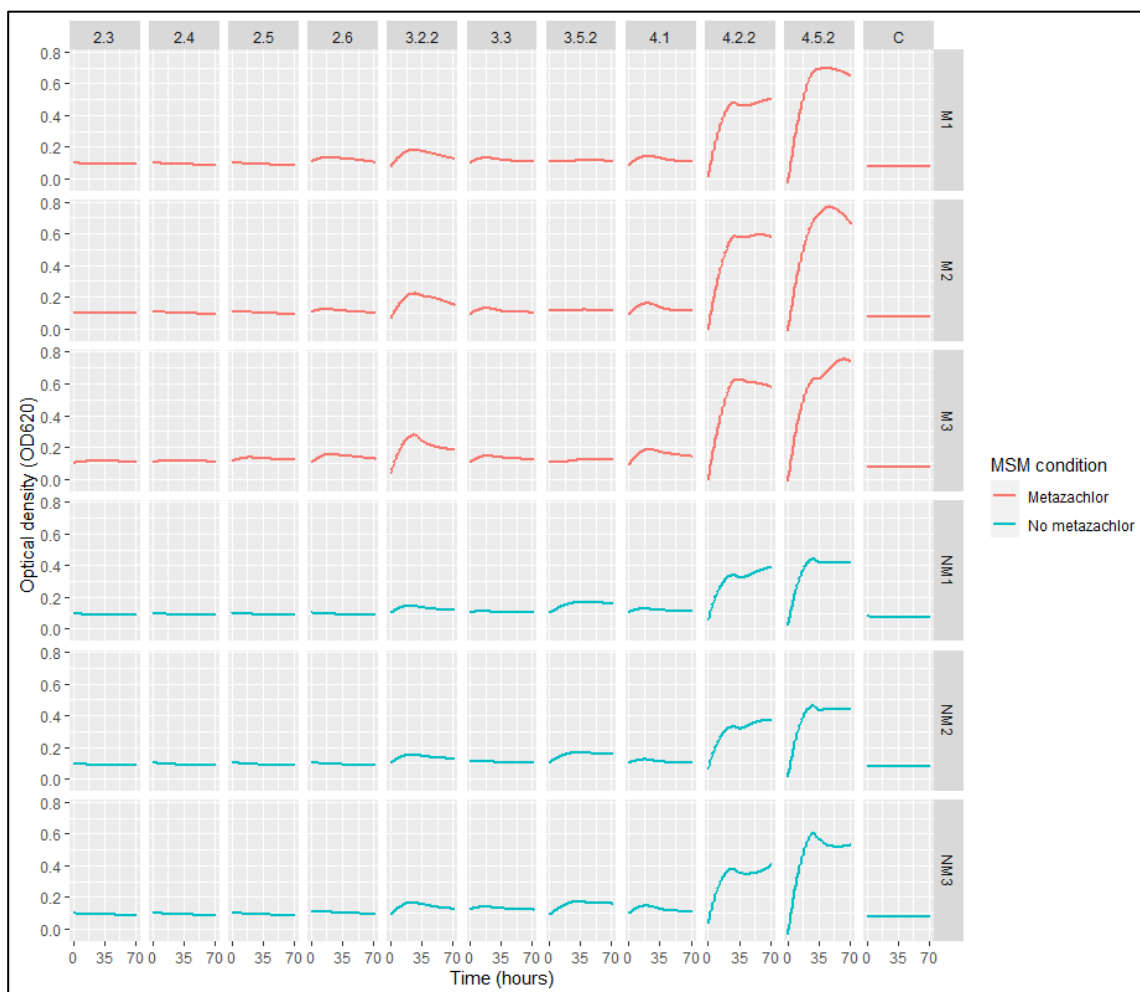
Figure 3-7 Hourly optical density readings at 620nm (OD) for individual triplicate cultures of metazachlor-degrading candidate isolates 2.3, 2.4, 2.5, 2.6, 3.2.2, 3.3, 3.5.2, 4.1, 4.2.2, 4.5.2 and uninoculated controls (C) (labelled above the graphs) grown in supplemented MSM (150mg/L) (shown in red, with individual cultures labelled M1-M3) and non-supplemented MSM (shown in blue, with individual cultures labelled NM1-NM3).
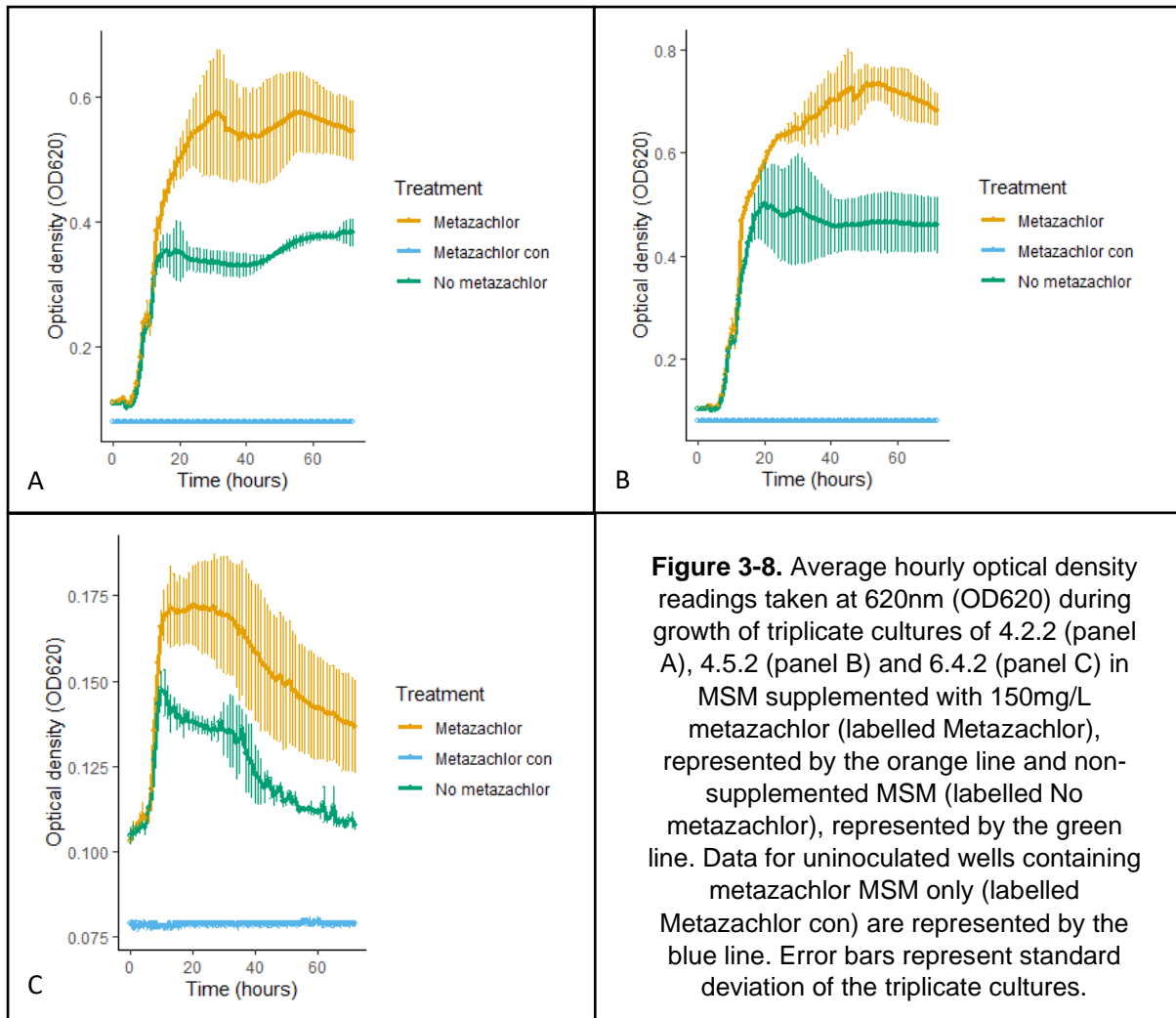
Figure 3-8. Average hourly optical density readings taken at 620nm (OD620) during growth of triplicate cultures of 4.2.2 (panel A), 4.5.2 (panel B) and 6.4.2 (panel C) in MSM supplemented with 150mg/L metazachlor (labelled Metazachlor), represented by the orange line and non-supplemented MSM (labelled No metazachlor), represented by the green line. Data for uninoculated wells containing metazachlor MSM only (labelled Metazachlor con) are represented by the blue line. Error bars represent standard deviation of the triplicate cultures.

Strain	Met MSM peak OD	Time (hours)	MSM only peak OD	Time (hours)
4.2.2	0.58	31.5	0.38	70
4.5.2	0.74	52	0.50	19.5
6.4.2	0.17	25.5	0.15	9.5

Table 3-6 Average peak OD values calculated from triplicate liquid cultures of 4.2.2, 4.5.2 and 6.4.2 grown in metazachlor supplemented MSM at 150mg/L (Met MSM peak OD) and non-supplemented MSM (MSM only peak OD) and the time points at which these readings were taken.

Two-sample t-tests were carried out to further test the statistical significance of the differences in average peak OD values observed between the growth of 4.2.2, 4.5.2 and 6.4.2 in supplemented and non-supplemented MSM, using the triplicated growth curve data. Only the difference in average peak OD values for strain 4.5.2 grown in metazachlor-

supplemented MSM compared to MSM only was statistically significant (t = 4.71, df = 4, p-value = 0.00924) and the improved growth seen when 4.2.2 and 6.4.2 were grown in metazachlor MSM compared to non-supplemented MSM were non-significant (t = 3.0823, df = 4, p-value = 0.03685 for strain 4.2.2 and t = 3.0414, df = 4, p-value = 0.03835 for strain 6.4.2). The lack of statistical significance in the growth of strains 4.2.2 and 6.4.2, along with the background growth observed in non-supplemented MSM suggest their metazachlor-degrading capabilities are ambiguous. To properly verify the metazachlor-degrading trait, improved assays which can more sensitively verify and "track" metazachlor degradation over time are required to determine whether 4.2.2 and 6.4.2 are true metazachlor degraders and would more accurately verify the same trait in 4.5.2.

3.2.2.3 16S rRNA gene amplicon sequencing for the taxonomic classification of strains 4.2.2, 4.5.2 and 6.4.2

16S rRNA gene amplicon sequencing was carried out to obtain full 16S rRNA gene sequences for strains 4.2.2, 4.5.2 and 6.4.2. Agarose gel electrophoresis visualising the 16S rRNA amplicons as well as the trimmed gene sequences are displayed in appendix 6.3 and appendix 6.7. Partial 16S rRNA gene sequences were obtained and trimmed to remove low-quality and primer sequence regions, resulting in amplicons of 1265bp, 1205bp and 1201bp for 4.2.2, 4.5.2 and 6.4.2, respectively.

BLASTN analysis produced top hits of partial 16S rRNA sequences from *Pseudomonas oryzihabitans* for strains 4.2.2 and 6.4.2, sharing over 99% identity with 99% query cover leading to these strains both being assigned to the *P. oryzihabitans* species (table 3-7). For strain 4.5.2, the top hit was a partial 16S rRNA gene sequence from *Pseudomonas putida*, however, the shared percent identity was 98.53%, (with low query cover, 75%) which is below the chosen threshold of 98.65% identity for species assignment (Kim *et al.*, 2014). 4.5.2, therefore, could only be assigned to the *Pseudomonas* genus. It is important to note that the sequence obtained for strain 4.5.2 in particular was of low quality, with the sequence trace demonstrating 63 uncalled bases throughout parts of the trimmed sequence, (represented by "N" in the final trimmed sequence), which may explain the lack of a BLASTN hit with a high enough shared percent identity to meet the threshold for species assignment.

Since strain 6.4.2 was isolated from soil microcosm 6, which lacked any addition of metazachlor treatment or inoculation with strains *A. calcoaceticus* E1 or *Sphingobium* CMET-H, no evidence for the success of the facilitated horizontal gene transfer approach for inducing uptake of metazachlor-degrading genes by the chosen non-degrading bacterial intended recipients *Acinetobacter calcoaceticus* E1 and *Sphingobium* CMET-H could be provided. Furthermore, because 4.2.2 and 4.5.2 (along with 6.4.2), belong to the *Pseudomonas* genus, it can be concluded that neither of these strains represents variants of the *Sphingobium* CMET-H strain which was added to soil microcosm 4.

Strain	Amplicon length	Top hit	% Identity	% Query cover	Hit accession no.	Species assigned
4.2.2	1265	*Pseudomonas oryzihabitans* strain L-1	99.37	99	NR_025881.1	*Pseudomonas oryzihabitans*
4.5.2	1205	*Pseudomonas putida* strain ICMP 2758	98.53	75	NR_114794.1	*Pseudomonas sp.*
6.4.2	1201	*Pseudomonas oryzihabitans* strain L-1	99.42	99	NR_025881.1	*Pseudomonas oryzihabitans*

Table 3-7 BLASTN hits for the 16S rRNA sequences for strains 4.2.2, 4.5.2 and 6.4.2.

3.3 Discussion

3.3.1 Isolation of metaldehyde-degrading bacterial strains from soil microcosms

Previous work indicated that the genes responsible for the microbial degradation of metaldehyde in soil were acquired via horizontal gene transfer and have the potential to be disseminated further throughout soil microbial communities by additional horizontal gene transfer processes (Castro-Gutiérrez *et al.*, 2022a). Evidence that additional undiscovered metaldehyde degradation pathways exist in nature was also demonstrated previously. Here, a facilitated horizontal gene transfer approach coupled with selective enrichment was utilised to investigate the horizontal transmissibility of metaldehyde-degrading genes within soil microcosm microbial communities and to explore this method as a potential avenue for the isolation of novel metaldehyde-degrading organisms and genes.

The selective enrichment approach utilised for the isolation of metaldehyde-degrading strains from soil microcosms led to the identification of 70 suspected metaldehyde-degrading strains grown on solid-metaldehyde-supplemented MSM agarose. There were issues with distinguishing confidently between genuine metaldehyde degraders and non-degrading strains that could grow on non-supplemented MSM agarose. Following the liquid growth assays, most of these candidate strains were discarded because there was little difference observed between the growth of their triplicated cultures in metaldehyde-supplemented MSM and MSM only. These results suggested that the enrichment method used was insufficient for the selection of metaldehyde-degrading strains only. Furthermore, only 6 areas of each initial metaldehyde-supplemented MSM agarose plate produced following selective enrichment in liquid cultures were further subcultured onto both supplemented and non-supplemented plates for additional analysis, meaning that the numbers of metaldehyde-degrading organisms isolated from the soil microcosms may not have been wholly representative of the soil microbial communities within and that other metaldehyde-degrading strains could have been identified. Future experiments should aim to subculture all colonies grown on metaldehyde-supplemented agarose plates to ensure potential degraders are not lost.

The selective enrichment method used in this study differed slightly from the enrichment methods used in previous studies, which may explain the challenges experienced when identifying metaldehyde-degrading strains. Both studies by Thomas *et al.* (2017) and Castro-Gutiérrez *et al.* (2020) utilised up to four 3-day passages of 1g soil samples in 100mL liquid metaldehyde-supplemented MSM, whereas in this study 1g soil samples were cultured in 3 24-hour passages in 10mL metaldehyde-supplemented MSM. Longer liquid culture passages could facilitate the selection of metaldehyde-degrading strains with slower growth rates, a factor which may have led to the identification of further metaldehyde-degrading strains in this study. Additional liquid culture passages would also increase the selective enrichment for metazachlor-degrading strains and could, therefore, improve the isolation of metazachlor degraders and reduce the number of non-degrading isolates. It is also possible that some of the isolated strains could utilise break-down products of metaldehyde produced due to the degrading capability of other organisms, or unrelated microbial by-products produced by these strains (Castro-Gutiérrez, 2020). Ultimately, it suggests that in these liquid culture passages, metaldehyde was not serving as the only source of carbon. In future, selective enrichment procedures modelled on those used by the previous studies should be used to obtain more accurate identifications of metaldehyde-degrading organisms so that true metaldehyde degraders within soil microcosms are isolated rather than non-degraders.

The short liquid culture passage durations used in the selective enrichment procedure in this study may have factored into the lack of metaldehyde-degrading *S. chlorophenolicum* DSM 7098 variants isolated from soil microcosms. In the study by Castro-Gutiérrez *et al.* (2020), two metaldehyde-degrading *Sphingobium* strains (CMET-H and HMET-G) were slower growing and had longer lag phases when grown in metaldehyde-supplemented MSM compared to some of the other metaldehyde-degrading strains they identified, including each of the metaldehyde-degrading *Acinetobacter* species. The 24-hour liquid culture passage periods utilised in this study for liquid culture passages, therefore, may not have supported and maintained the growth of any metaldehyde-degrading *Sphingobium* strains. This might explain why the two *Acinetobacter* strains 5.1 and 6.6 were isolated from soil microcosms, but *S. chlorophenolicum* DSM 7098 variants were not. The lack of *S. chlorophenolicum* DSM 7098 metaldehyde-degrading variants able to be isolated meant that no evidence of the success of the facilitated horizontal gene transfer method was able to be provided using this strain as a recipient of metaldehyde-degrading genes. This reinforces the need for longer liquid culture passage durations of 72 hours or above in future experiments so that slower-growing metaldehyde-degrading strains such as the potential *S. chlorophenolicum* DSM 7098 variants are not excluded from soil microcosm isolations.

Thomas *et al.*, (2017) were able to isolate metaldehyde-degrading-strains from domestic soil, but none from agricultural soil, whereas Castro-Gutiérrez *et al.*, 2020 were able to isolate metaldehyde-degrading strains from agricultural soils as well as allotment soils. It has been shown previously that many different factors can affect the efficiency of gene transfer within soils, including soil type, moisture content, organic matter content, pH, and incubation temperature (Richaume, Angle and Sadowsky, 1989). These are perhaps factors which may have contributed to the suspected metaldehyde-degrading *A. calcoaceticus* RUH 2202 variants 5.1 and 6.6 being isolated from soil microcosms 5 and 6 containing allotment soil but not from microcosms 7 and 8 which contained domestic compost, as differences between the two soil types may have affected horizontal gene transfer efficiency. No specific analyses of these conditions were performed, however, and could be beneficial to investigate in future experiments to provide evidence for the successful facilitated horizontal gene transfer of metaldehyde-degrading genes into chosen recipient strains such as *S. chlorophenolicum* DSM 7098 in soil microcosms and demonstrate the horizontal transmissibility of the previously-identified metaldehyde-degrading plasmids pAME76 and pSM1, none of which could be shown in this study.

On account of the fact that the soil microcosms used in this study were sealed during the time period allocated to horizontal gene transfer, an anaerobic environment may have been created. It has been shown previously that microbial xenobiotic degradation can occur in both aerobic and anaerobic conditions, therefore, it may be beneficial to perform future experiments with aerated microcosms, such as the method used by Gautam *et al.* (2003), to investigate whether aerobic conditions may lead to the identification of further novel metaldehyde and metazachlor-degrading organisms via facilitated horizontal gene transfer in microcosms (Neilson, 1990).

Following liquid growth assays in metaldehyde-supplemented and non-supplemented MSM, the metaldehyde-degrading traits of 5.1 and 6.6 were suspected. There was some background growth of strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 in liquid MSM and on solid MSM agarose media which had no added carbon source, however, suggesting that these strains were scavenging organic carbon, either due to carryover or traces present in the growth media.

The method used in this study for verifying metaldehyde degradation in liquid media was much more straightforward than those used in previous studies. Approaches used in the past for verifying the metaldehyde degradation capabilities of soil-derived strains combined analytical techniques including liquid chromatography-mass spectrometry (Thomas *et al.*, 2017) and gas chromatography (Castro-Gutiérrez *et al.*, 2020) with liquid growth assays similar to those used in this study, which allowed the authors to statistically correlate decreases in metaldehyde concentration in liquid MSM cultures with the increases in growth of the degrading strains, providing robust evidence of microbial metaldehyde degradation. These techniques would also be useful for verifying the concentrations of metaldehyde in bacterial growth media. It is also important to note that the method with which candidate degrading strains were initially chosen in this work was based on a simple comparison of the growth curves produced for individual triplicate cultures absent of any statistical analysis being performed. Had all of the strains' data been analysed in the same way that the data for the most promising candidates 5.1 and 6.6 were, further metaldehyde-degrading strains may have been identified. Strains 5.1 and 6.6 may not wholly represent the entire metaldehyde-degrading population in the soils, therefore. Ultimately, the method used could not fully determine the metaldehyde-degrading capabilities of the isolated soil microbial strains, including those of 5.1 and 6.6. Future experiments should incorporate statistical analyses for every candidate metaldehyde-degrading strain produced from selective enrichments for more robust conclusions to be made regarding their degradation capabilities and should incorporate analytical techniques such as gas chromatography to verify the degradation

capabilities of 5.1, 6.6 and any other candidate degrading strains. Better evidence for the degradation capabilities for 5.1 and 6.6 could also be attained in further work by identifying metaldehyde-degrading genes or plasmids resembling those found in previous metaldehyde-degrading strains.

In previous studies on the microbial degradation of metaldehyde, the growth and degradation rates of degrading strains differed between the different strains (Castro-Gutiérrez *et al.*, 2020; Castro-Gutiérrez *et al.,* 2022b). This has also been observed for the degradation of other pesticides in which different species differed in the extents to which they could grow in the presence of and degrade the compounds in question (Fuentes *et al.*, 2010). On account of the fact that only the strains demonstrating the largest, significant growth rate enhancements in the presence of metaldehyde were selected as candidate degraders, and because the identification of degraders was based solely on this rather than tracking metaldehyde degradation, degrading strains demonstrating smaller improvements in growth in response to metaldehyde presence would not have been detected as candidate degraders. This may also have been the case for the metazachlor degradation experiments. In future work, the use of the analytical techniques used to track metaldehyde degradation previously, as discussed above, along with tracking growth rates could potentially aid in the identification of further degrading strains whose growth rates are not enhanced significantly as a result of metaldehyde degradation, but still possess the degradation trait.

It was inferred that strains 5.1 and 6.6 were possible metaldehyde-degrading variants of the *A. calcoaceticus* RUH 2202 strain which was added to the soil microcosms from which they were isolated. They were both assigned to the *A. calcoaceticus* species based on 16S rRNA amplicon sequencing, to which *A. calcoaceticus* RUH 2202 belongs. 16S rRNA gene amplicon sequencing is not robust enough, however, to distinguish between bacterial strains of the same species because the 16S rRNA gene is too highly conserved (Li, Raoult, and Fournier, 2009). It was necessary, therefore, to perform further analysis to determine whether 5.1 and 6.6 belong to the *A. calcoaceticus* RUH 2202 strain.

Ethanol utilisation assays performed for *A. calcoaceticus* strains 5.1, 6.6, RUH 2202 and E1 demonstrated that the former three strains can utilise ethanol as a carbon source, whereas *A. calcoaceticus* E1 could not. These results align with previous studies which demonstrated that *A. calcoaceticus* RUH 2202, but not *A. calcoaceticus* E1, can utilise ethanol as a carbon source (Thomas *et al.*, 2017). Ethanol utilisation is a trait common for many *Acinetobacter calcoaceticus* strains, however, and a study conducted by Nemec *et al.* (2011) indicated that

91% of *Acinetobacter calcoaceticus* strains known at that time demonstrated ethanol utilisation.

Mercury resistance studies confirmed the former hypothesis formed by Castro-Gutiérrez *et al.* (2022a) that *Acinetobacter calcoaceticus* E1 is mercury resistant due to the presence of a mercury resistance operon located on its metaldehyde-degrading plasmid pAME76. The lack of mercury resistance in 5.1, 6.6 and *A. calcoaceticus* RUH 2202 further added evidence that these strains all belong to the same type. The mercury resistance assay also demonstrated that 5.1 and 6.6 had most likely not acquired their metaldehyde-degrading plasmid pAME67 due to their lack of mercury resistance and that these strains could not represent members of the *A. calcoaceticus* E1 strain. This needed to be verified more accurately, however, as well as the possibility that 5.1 and 6.6 possessed the pAME76 plasmid. It is important to note that other *Acinetobacter* strains are also susceptible to mercury according to previous work (Dhakephalkar and Chopade, 1994). Whether or not strains 5.1 and 6.6 were *A. calcoaceticus* RUH 2202 type strains was ambiguous because other *Acinetobacter calcoaceticus* strains have demonstrated the same ethanol utilisation and mercury susceptibility characteristics in previous studies and 16S rRNA gene amplicon sequencing can not facilitate bacterial strain typing. Further work was needed to address these uncertainties and to form conclusions about the success of the facilitated horizontal gene transfer approach.

The in vitro mating assay between *A. calcoaceticus* strains E1 and RUH 2202 was unable to demonstrate the horizontal transmissibility of plasmid pAME76, as no transconjugants could be selected following mating in liquid culture. This result aligns with the hypothesis formed by Castro-Gutiérrez *et al.* (2022b) that pAME76 can not be transferred to other strains via conjugation due to its lack of the genes required for this process. At this point in the study, it could be concluded that no evidence could be provided for the horizontal gene transfer of plasmid pAME76 within *in vitro* mating cultures. No assay could be performed to investigate the conjugation of pSM1 *in vitro,* therefore, it was also concluded that no evidence for horizontal gene transfer of pSM1 could be provided either. The selection mechanism designed, however, could be used alongside metaldehyde degradation assays in future studies which aim to demonstrate the uptake of the pAME76 plasmid by *A. calcoaceticus* RUH 2202 via natural transformation.

Despite no successful antibiotic-based selection mechanisms being identified for *S. chlorophenolicum* DSM 7098 in this study, other potential mechanisms may work for future

mating experiments. *S. chlorophenolicum* DSM 7098 was originally isolated from pentachlorophenol-contaminated soil and can degrade this pesticide through a pentachlorophenol-dehalogenase (Nohynek *et al.*, 1995; Takeuchi, Hamana and Hiraishi, 2001; Schenk, T., Müller, R. and Lingens, F., 1990). In future, it could be beneficial to verify whether *Sphingobium* CMET-H shares pentachlorophenol degradation capabilities. If *Sphingobium* CMET-H lacks this trait, conjugation assays between *Sphingobium* strains DSM 7098 and CMET-H could be designed incorporating selection mechanisms targeting both metaldehyde and pentachlorophenol degradation traits for the selection of *S. chlorophenolicum* DSM 7098 variants with a pSM1 plasmid donated by *Sphingobium* CMET-H via conjugation.

3.3.2 Isolation of metazachlor-degrading bacterial strains from soil microcosms

Previous studies indicated that metazachlor undergoes microbial biodegradation in soils, however, no microorganism able to facilitate the process had been isolated. Additional studies predicted that the enzymes responsible for this process were glutathione transferases, and it was hypothesized that an organism possessing these enzymes may be competent for metazachlor degradation. Here, a facilitated horizontal gene transfer approach coupled with selective enrichment was utilised to investigate the horizontal transmissibility of metazachlor-degrading genes within soil microcosm microbial communities and to explore this method as a potential avenue for the isolation of novel metazachlor-degrading organisms and genes.

The selective enrichment method used for isolating metazachlor-degrading organisms yielded similar results and challenges as were discussed in the previous section (see 3.3.1), most likely because a similar selective enrichment procedure was used for both sets of experiments. Distinguishing between genuine candidate metazachlor-degrading organisms and organisms growing on non-supplemented MSM agarose was challenging due to the background growth of many of the strains on non-supplemented MSM agarose. From 30 candidate strains isolated on solid metazachlor-supplemented MSM agarose following selective enrichment in metazachlor-supplemented liquid MSM media, only 3 isolates were chosen as candidate metazachlor-degrading organisms after liquid growth assays were performed, due to most strains displaying similar growth in metazachlor-supplemented and non-supplemented MSM. Again, similarly to the results discussed in section 3.3.1, these

results suggest that the selective enrichment method utilised for the isolation of metazachlor-degrading bacteria was insufficient because many non-degrading organisms were isolated, meaning that additional degraders present in the soil microcosms may not have been isolated. Only 6 areas containing colonies on the initial metazachlor-supplemented plates were subcultured further onto metazachlor-supplemented and non-supplemented MSM agarose plates for further analysis, therefore, the number of candidate metazachlor-degrading isolates identified by subculturing onto further solid media may not have been wholly representative of the microbial metazachlor degrader populations within the soil microcosms. and some candidate strains may have been missed, including possible metazachlor-degrading *A. calcoaceticus* E1 and *Sphingobium* CMET-H variants which could not be identified in this study. Future studies should ensure to sample and subculture all of the colonies grown on solid metazachlor-supplemented media.

A recent attempt was made to isolate metazachlor-degrading strains in a study by Guillaume (2021). In this study a selective enrichment procedure was utilised which involved 4-week-long liquid culture passages of 10g soil samples in MSM supplemented with metazachlor, followed by serial dilutions and plating onto solid MSM agarose supplemented with metazachlor. Two notable differences in the method used in this study compared to this one included the longer duration of liquid culture passages of 7 days compared to 24 hours and 4 liquid culture passages rather than three. As discussed in the section relating to metaldehyde, liquid culture passages of a longer duration could allow for the selection of metazachlor-degrading strains with slower growth rates, a factor which may have led to the identification of further metazachlor-degrading strains in this study. The addition of further liquid culture passages would increase the selective enrichment for metazachlor-degrading strains and could, therefore, improve the isolation of metazachlor degraders and reduce the number of non-degrading isolates. When tested in liquid growth assays measuring the growth of the strains with metazachlor as a carbon and nitrogen source, strains grew poorly. Out of 14 candidate strains, three were taken forward for degradation assays. The selection of two of these strains was based on their phenol oxidase activity rather than their ability to effectively grow with metazachlor as a carbon source. This study demonstrated similar results in that after selective enrichment, most of the candidate strains that were identified were discarded from further analysis as they did not demonstrate significant growth in MSM supplemented with metazachlor, indicating that the selection for degrading organisms was poor. Microbial metazachlor degradation assays were performed using liquid chromatography, which led to the identification of one potential metazachlor-degrading strain named MZ 3.

The species identity of the candidate metazachlor-degrading strain MZ 3 from Guillaume's study (2021) was *Pseudomonas sp.* based on 16S rDNA sequencing. Interestingly, strains 4.2.4, 4.5.2 and 6.4.2 isolated from this study were also members of this genus, which may be significant if the strains from both studies are proven to be true metazachlor degraders in future experiments. *Pseudomonas* strains have also been isolated previously which can degrade other chloroacetamide compounds (Xu *et al.*, 2006; Zablotowicz *et al.*, 2001). Strain 4.5.2 could only be assigned at the genus level in the work described in this chapter. A possible reason for this is the reduced quality of the 16S rRNA sequence produced for this strain due to the presence of miscalled bases, which most likely contributed to the lack of a BLASTN match significant enough to allow species demarcation above the 95% identity threshold chosen for this study (Rodriguez-R and Konstantinidis, 2014). 16S rRNA gene amplicon sequencing could be repeated for this strain to address issues which may have affected sequence quality. It has also been shown previously that the resolution of species assignments based on 16S rRNA amplicon sequencing alone is inadequate for classifying some of the species within the *Pseudomonas* genus (Yonezuka *et al.*, 2017). Further work, therefore, was required to verify the species identifications for 4.2.2, 4.5.2 and 6.4.2 more accurately.

The metazachlor-degrading traits of 4.2.2, 4.5.2 and 6.4.2 were slightly ambiguous due to the lack of statistical significance for the growth of 4.2.2 and 6.4.2 in metazachlor-supplemented MSM compared to MSM only and the background growth of all three strains in liquid metazachlor-supplemented MSM. This could be due to oligotrophic traits in these strains or contaminating carbon sources in the media other than that provided by the metazachlor, possibly provided by the acetone added to the control media. As mentioned in the previous discussion section, analytical techniques used to track the degradation of compounds including pesticides over time such as gas chromatography or liquid chromatography could more accurately verify the metazachlor-degrading trait in 4.2.2, 4.5.2 and 6.4.2 in future work by tracking the disappearance of metazachlor in media containing the strains as well as liquid growth assays in metazachlor added as sole carbon source. Similar approaches have been used to study microbial chloroacetamide degradation previously (Zhang *et al.,* 2011; Xu *et al.,* 2006).

Due to their assignment to the *Pseudomonas* genus and to the fact that strain 6.4.2 was isolated from a soil microcosm which was not inoculated with either of the recipient strains *A. calcoaceticus* E1 or *Sphingobium* CMET-H, these strains can not provide evidence for the

success of the horizontal gene transfer approach for inducing the uptake of metazachlor-degrading genes by either *A. calcoaceticus* E1 or *Sphingobium* CMET-H. The conditions within the soil microcosms may not have favoured horizontal gene transfer as previous studies highlighted the impact that soil type, moisture content, organic matter content, pH, and temperature have on gene transfer success in soil microcosms (Richaume, Angle and Sadowsky, 1989). Experimenting with these conditions in future experiments could lead to the success of the facilitated horizontal gene transfer method in producing metazachlor-degrading *A. calcoaceticus* E1 and *Sphingobium* CMET-H variants.

3.3.3 Conclusions

The facilitated horizontal gene transfer approach coupled with selective enrichment was unable to produce metaldehyde-degrading variants of *S. chlorophenolicum* DSM 7098, and, therefore, provided no evidence of the horizontal transmissibility of metaldehyde-degrading genes. Strains 5.1 and 6.6, however, were identified as possible *A. calcoaceticus* RUH 2202 metaldehyde-degrading variants, based on their assignment to the *Acinetobacter calcoaceticus* species and their phenotypic characteristics similar to *A. calcoaceticus* RUH 2202. They present, therefore, potential novel metaldehyde-degrading bacterial strains. It was concluded that further work was needed, however, to disambiguate the metaldehyde-degrading capabilities of 5.1 and 6.6, identify their strain type and provide evidence of the success of the facilitated horizontal gene transfer approach. No direct evidence of the horizontal transmissibility of pAME76 or pSM1 could be provided at this stage in the study, however, further work could verify this more confidently.

As for the facilitated horizontal transfer approach for studying metazachlor degradation, no metazachlor-degrading variants of *A. calcoaceticus* E1 or *Sphingobium* CMET-H were isolated. No evidence, therefore, was provided for the success of this method in inducing the uptake of metazachlor-degrading genes into *A. calcoaceticus* E1 and *Sphingobium* CMET-H. Strains 4.2.2, 4.5.2 and 6.4.2, however, were identified as possible novel metazachlor-degrading *Sphingobium* strains. Their metazachlor-degrading trait could not confidently be confirmed, and further work was deemed necessary to verify this. The experiments discussed in chapter 4 aimed to address some of the issues that were discussed here.

Chapter 4: Whole genome sequencing and bioinformatics for the identification of candidate metaldehyde and metazachlor-degrading genes

4.1 Introduction

The work described in chapter 2 led to the identification of two candidate novel metaldehyde-degrading strains, 5.1 and 6.6. These strains were isolated from selective enrichments of allotment soil microcosm samples treated with metaldehyde and inoculated with the non-degrading strain *A. calcoaceticus* RUH 2202. These strains demonstrated improved growth in metaldehyde-supplemented MSM compared to MSM only. 5.1 and 6.6 resemble the non-degrading strain *A. calcoaceticus* RUH 2202, which suggests the possibility that 5.1 and 6.6 are metaldehyde-degrading variants of *A. calcoaceticus* RUH 2202, providing possible evidence for successful facilitated horizontal gene transfer of metaldehyde-degrading genes into *A. calcoaceticus* RUH 2202. The resemblance was based on the fact that 5.1, 6.6 and RUH 2202 are all *A. calcoaceticus* strains and can each utilise ethanol as a source of carbon, unlike *A. calcoaceticus* E1. It was possible, however, that 5.1 and 6.6 belong to another strain because other *Acinetobacter calcoaceticus* strains share these characteristics, necessitating further verification of the strain types of 5.1 and 6.6. In addition, the possibility that 5.1 and 6.6 possessed the metaldehyde-degrading plasmid pAME76 which is found in *A. calcoaceticus* E1 was deemed unlikely due to a lack of mercury resistance traits in these strains, however, more robust evidence to verify this was needed.

Three candidate novel metazachlor-degrading strains, 4.2.2, 4.5.2 and 6.4.2 were also isolated from selective enrichments of agricultural soil microcosm samples which followed the facilitated horizontal gene transfer approach. The microcosms were inoculated with *Sphingobium* CMET-H in the cases of 4.2.2 and 4.5.2 and uninoculated for 6.4.2. These strains showed improved growth in metazachlor-supplemented MSM, however, only the improved growth of 4.5.2 was statistically significant. Additionally, background growth of all of the strains in non-supplemented MSM meant that the metazachlor-degrading trait in each of these three strains is ambiguous. The strains each belong to the *Pseudomonas* genus, therefore, they can not represent the success of the facilitated horizontal gene transfer method for inducing the uptake of metaldehyde degradation genes in *A. calcoaceticus* E1 and *Sphingobium* CMET-H. They do, however, present candidates for the first metazachlor-degrading strains that have been isolated to date.

Whole genome sequencing is a valuable tool for the study of the genetic determinants of xenobiotic biodegradation. Used in combination with bioinformatics approaches including comparative genomics methods, it has allowed the identification of degrading genes in xenobiotic-degrading organisms (Ravintheran *et al.*, 2019). Furthermore, bioinformatics approaches can help to identify regions of the genome which demonstrate evidence of horizontal acquisition, for example, MGEs (Kaminski *et al.*, 2019). Combined sequencing approaches, which utilise both short-read sequencing and long-read sequencing have greatly improved the accuracy of genome sequencing, by using long reads as scaffolds onto which short reads can be mapped, meaning that de novo genome assemblies are of higher quality, even in the cases of complex bacterial genomes (De Maio, *et al.*, 2019).

4.1.1 Aims of this chapter

The work described in this chapter sought to provide candidates for the genes conferring the metaldehyde degradation traits in 5.1 and 6.6 by using a combination of whole genome sequencing and bioinformatics approaches. A hybrid whole genome sequencing approach utilising both Oxford Nanopore long-read sequencing and Illumina short-read sequencing was used to obtain complete genome sequences of the candidate metaldehyde-degrading strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202. Comparative genomics approaches were then utilised to compare the genomes to identify genetic differences between strains 5.1 and 6.6 and *A. calcoaceticus* RUH 2202, thereby providing potential gene candidates for the metaldehyde-degrading trait of 5.1 and 6.6. Further bioinformatic approaches were incorporated to further verify the possibility that 5.1 and 6.6 belong to the *A. calcoaceticus* RUH 2202 strain, to examine the genetic context of their candidate degrading genes and to predict their functions. Furthermore, the genomes were searched for evidence of horizontal gene transfer in the form of deviations in GC% content from their genomic averages and MGEs, which, if associated with novel genes in 5.1 and 6.6 that are absent from *A. calcoaceticus* RUH 2202 could indicate horizontal acquisition (Ravenhall *et al.*, 2015). This could demonstrate whether the facilitated horizontal gene transfer approach described -in chapter 3 was successful in inducing the acquisition of metaldehyde-degrading genes in *A. calcoaceticus* RUH 2202.

Illumina whole genome sequencing aimed to provide contig-level genome sequence assemblies for the metazachlor-degrading strains 4.2.2, 4.5.2 and 6.4.2. Genome sequencing sought to provide more accurate species assignments for the candidate metazachlor-degrading strains, due to the unreliability of the 16S rRNA gene amplicon

sequencing performed in the previous chapter. The genomes of 4.2.2, 4.5.2 and 6.4.2 were then searched for the presence of glutathione transferase enzymes to identify candidate metazachlor degradation genes, based on previous studies which have suggested their involvement in the first step of microbial metazachlor biodegradation (Laue, Field and Cook, 1996; EFSA, 2008; Zablotowicz *et al.*,1995; Zablotowicz *et al.*, 2001).

The aims of this chapter are:

- To identify candidate metaldehyde-degrading genes in 5.1 and 6.6 and candidate metazachlor-degrading genes in 4.2.2, 4.5.2 and 6.4.2
- To determine whether 5.1 and 6.6 belong to the *A. calcoaceticus* RUH 2202 strain type
- To provide more accurate species assignments for 4.2.2, 4.5.2 and 6.4.2
- To investigate whether the genes conferring metaldehyde degradation were conferred by horizontal gene transfer
- To determine whether the facilitated horizontal gene transfer method succeeded in producing metaldehyde-degrading variants of *A. calcoaceticus* RUH 2202

4.2 Results

4.2.1 Combined Oxford Nanopore and Illumina whole genome sequencing and bioinformatics for *A. calcoaceticus* strains 5.1, 6.6 and RUH 2202

4.2.1.1 Combined Oxford Nanopore and Illumina whole genome sequencing for strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202

Combined Oxford nanopore long-read and Illumina short-read sequencing approaches were performed by MicrobesNG (Birmingham, UK) to obtain the sequenced genomes of candidate metaldehyde-degrading strains 5.1 and 6.6 and the RUH 2202 strain. This would allow comparative genomics analyses to be performed to identify possible genetic determinants of metaldehyde degradation in strains 5.1 and 6.6 and detect evidence of horizontal gene transfer. Quality statistics for the initial Illumina short-read sequencing and assemblies performed for each strain are provided in table 4-1.

Strain	Median insert size (bp)	Mean coverage	No. reads	Number of contigs	Largest contig (bp)	Total length (bp)	GC (%)	N50 (bp)	#N's
5.1	713	95.9	782612	21	1031881	3864988	38.62	463657	0
6.6	721	126.9	1068876	24	1031881	3866744	38.63	463652	0
RUH 2202	704	86.3	700517	21	1031873	3866390	38.63	499316	0

Table 4-1 Quality statistics for Illumina short-read whole genome sequencing and initial assemblies for strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202.

The short reads from the Illumina sequencing were used in combination with the long reads from oxford nanopore sequencing which allowed complete, fully assembled genomes for each of the bacterial strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 to be obtained from MicrobesNG. The genomes are assembled into single contigs comprising 3,915,206 bp for both strains 5.1 and 6.6 and 3,915,122 bp for *A. calcoaceticus* RUH 2202 (table 4-2). After examination of the genome assembly graphs was performed in Bandage, circular genomic topologies were confirmed for all three genomes, visualised as large single nodes connected by a single edge (Wick *et al.*, 2015) (appendix 6.8). The genomes of 5.1, 6.6 and *A. calcoaceticus* RUH 2202 are, therefore, organised into single chromosomes without the indication of any plasmids being present. This information suggests that the metaldehyde-degrading trait in strains 5.1 and 6.6 is not conferred by a plasmid acquired through horizontal gene transfer.

Strain	Contig length (bp)	Topology	GC%
5.1	3,915,206	Circular	38.73
6.6	3,915,206	Circular	38.73
RUH 2202	3,915,122	Circular	38.73

Table 4-2 Length of the contigs produced by hybrid short-read and long-read whole genome sequencing and assemblies for strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 (RUH 2202), their topology and their percentage GC content (GC%).

Automated annotation of the genome assemblies by Prokka, provided by MicrobesNG identified the same numbers of 3586 coding sequences, 74 transfer RNA genes (tRNA) and one transfer messenger RNA (tmRNA) gene in each of the genomes of 5.1, 6.6 and *A. calcoaceticus* RUH 2202. This suggests that neither the genome of 5.1 nor that of 6.6 have encountered large numbers of gene losses or gains compared to the genome of RUH 2202.

Chromosome maps for the whole genome sequences of 5.1, 6.6 and *A. calcoaceticus* RUH 2202 are displayed in figure 4-1 and were made using Artemis and DNAPlotter (Carver *et al.*, 2012; Carver *et al.*, 2009). There is a noticeable spike in GC content present in all three genomes, which, upon closer inspection in Artemis, is localised to genes with the locus tags 235307E_51_00777, 235308E_66_00777 and 235309E_RUH2202_00777. In 5.1, 6.6 and

Figure 4-1 Chromosome maps of 5.1, 6.6 and *A. calcoaceticus* RUH 2202 (RUH) created using Artemis and DNAPlotter. Tracks represent (from outside in): major tick marks : 200,000bp steps, minor tick marks : 40,000bp steps, coding strands on the forward strand in dark green, coding strands on the reverse strand in orange, tRNA genes in dark blue (outer tRNA gene track: forward strand, inner tRNA track: reverse strand), tmRNA genes in light green, GC content (gold = above average, purple = below average) and GC skew (gold = above average, purple = below average).

A. calcoaceticus RUH 2202 the GC contents for these genes are 50.71%, 50.65% and 50.7% for 5.1, 6.6 and *A. calcoaceticus* RUH 2202, respectively, deviating from their chromosomal average of 38.73%. They are each assigned hypothetical protein annotations by Prokka. BLASTP analysis of the amino acid sequences of these genes revealed shared percent identities of 88.75%, 88.31% and 88.75% with an immunoglobulin (Ig) -like domain-containing protein from *Acinetobacter calcoaceticus* for 5.1, 6.6 and *A. calcoaceticus* RUH 2202, respectively (accession no. WP_205468625.1).

The most frequent species to which genome sequencing reads were mapped, calculated in Kraken and provided by MicrobesNG, was *Acinetobacter calcoaceticus* for each of the three strains (appendix 6.9). To quantitatively confirm close evolutionary relationships between 5.1, 6.6 and *A. calcoaceticus* RUH 2202 (in addition to experiments carried out in chapter 3 testing ethanol utilisation and mercury resistance), pairwise average nucleotide identity (ANI) calculations were performed to compare the nucleotide sequences of the genomes of 5.1, 6.6, *A. calcoaceticus* RUH 2202 and previously identified metaldehyde-degrading strain *A. calcoaceticus* E1. ANI values between 5.1, 6.6 and *A. calcoaceticus* RUH 2202 are 100%, whereas ANI values between 5.1 and 6.6 and *A. calcoaceticus* E1 are lower, at just under 96% (table 4-3). These results, along with those gathered in chapter 3, suggest that strains 5.1 and 6.6 are, indeed, more closely related to *A. calcoaceticus* RUH 2202 than *A. calcoaceticus* E1, and are likely to represent variants of the *A. calcoaceticus* RUH 2202 strain with which the soil microcosms 5 and 6 were inoculated (see chapter 3).

Comparison	ANI (%)	Mean ANI (%)	Median ANI (%)
5.1 vs RUH 2202	100	100	100
6.6 vs RUH 2202	100	100	100
5.1 vs 6.6	100	100	100
5.1 vs E1	95.85	95.85	96.42
6.6 vs E1	95.85	95.85	96.42
RUH 2202 vs E1	95.80%	95.8	96.4

Table 4-3 Pairwise average nucleotide identity calculations performed between strains 5.1, 6.6 and *A. calcoaceticus strains* RUH 2202 and E1 using the average nucleotide identity calculator from http://enve-omics.ce.gatech.edu/ani/.

4.2.1.2 Bioinformatic analysis of the whole genome sequences of 5.1, 6.6 and *A. calcoaceticus* RUH 2202

To investigate and visualise the genetic differences between the candidate metaldehyde-degrading RUH 2202 variants 5.1 and 6.6 and the non-degrading RUH 2202 strain, pairwise and multiple whole genome sequence alignments were carried out in progressiveMauve using the metaldehyde-degrading strains as reference genomes (figure 4-2) (Darling *et al.*, 2004). The genome sequences are aligned along their entire lengths as single locally collinear blocks (LCBs) connected by a single line, with high similarity profiles along most of their lengths indicating high levels of conservation. There are, therefore, no large-scale structural rearrangements such as translocations, recombinations or inversions that are identifiable in the genomes of 5.1 and 6.6 relative to RUH 2202, which would be demonstrated by multiple LCBs with connections between different genomic loci, or LCBs positioned in the reverse orientation below the centre line on which LCBs are positioned. This was further verified by viewing the annotated 1-1 orthologs files produced by the genome alignments, which verified that all of the genes within 5.1 and 6.6 are positionally homologous to the genes within RUH 2202. There is also no evidence of any large insertions or deletions, which would be present as large gaps in the sequence alignments, demonstrating no large insertions or deletions of genes or genomic islands. There are, however, a small number of loci within the sequence alignments where the LCB similarity profiles decrease slightly, indicating decreases in sequence conservation and, therefore, genetic differences between the strains.
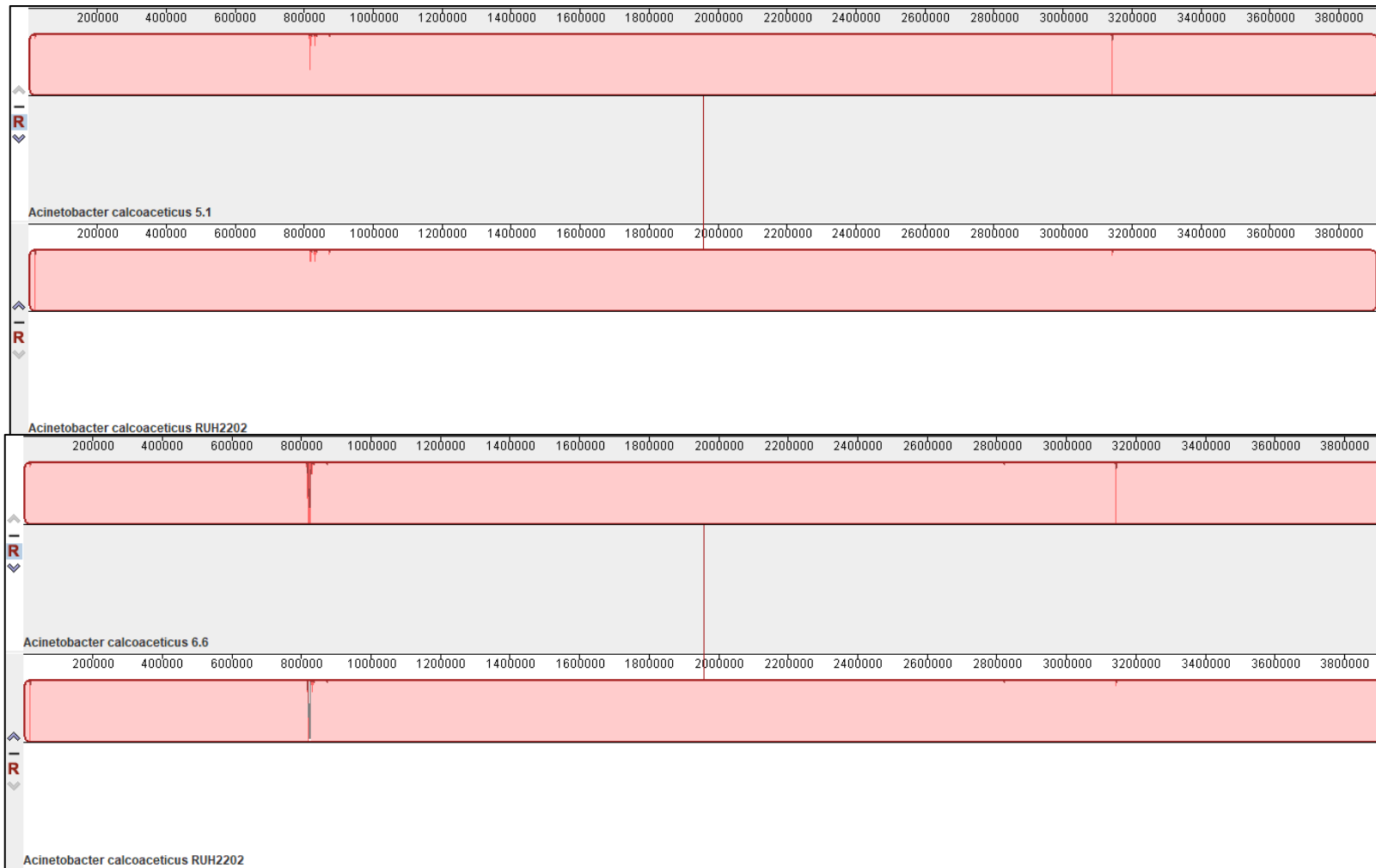
Figure 4-2 ProgressiveMauve pairwise sequence alignment of strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202. Areas where decreased similarity profiles are seen are labelled with arrows. Red blocks represent LCBs, connected by a single line to indicate synteny. Colouring inside the blocks represents similarity profiles which are correlated with the levels of sequence conservation between the genomes. Scales above the genomes indicate base pair numbers. Labels below the genomes indicate strain type.

The genome alignments were browsed manually in conjunction with the exported alignment gaps and SNPs files to determine the precise locations of the genetic differences observed in 5.1 and 6.6 relative to *A. calcoaceticus* RUH 2202 (figure 4-3, figure 4-4 and table 4-4). These differences are localised to 4 different genes in 5.1 including hypothetical proteins 15 and 777, 235307E_51_00808 (hypothetical protein 808) and 235307E_51_02947 (hypothetical protein 2947). Alignment gaps between 5.1 and *A. calcoaceticus* RUH 2202 are localised to proteins 15 and 2947 and SNPs are found within 777 and 808. In strain 6.6, the genetic differences are localised to within genes encoding 5 different predicted hypothetical proteins including 15, 777, 235308E_66_00808 (hypothetical protein 808), 35308E_66_02947 (hypothetical protein 2947) and adenylate kinase 2654. Alignment gaps between 6.6 and *A. calcoaceticus* RUH 2202 are localised to 15, 777 and 2947 and SNPs are found within 777, 808 and 2654.
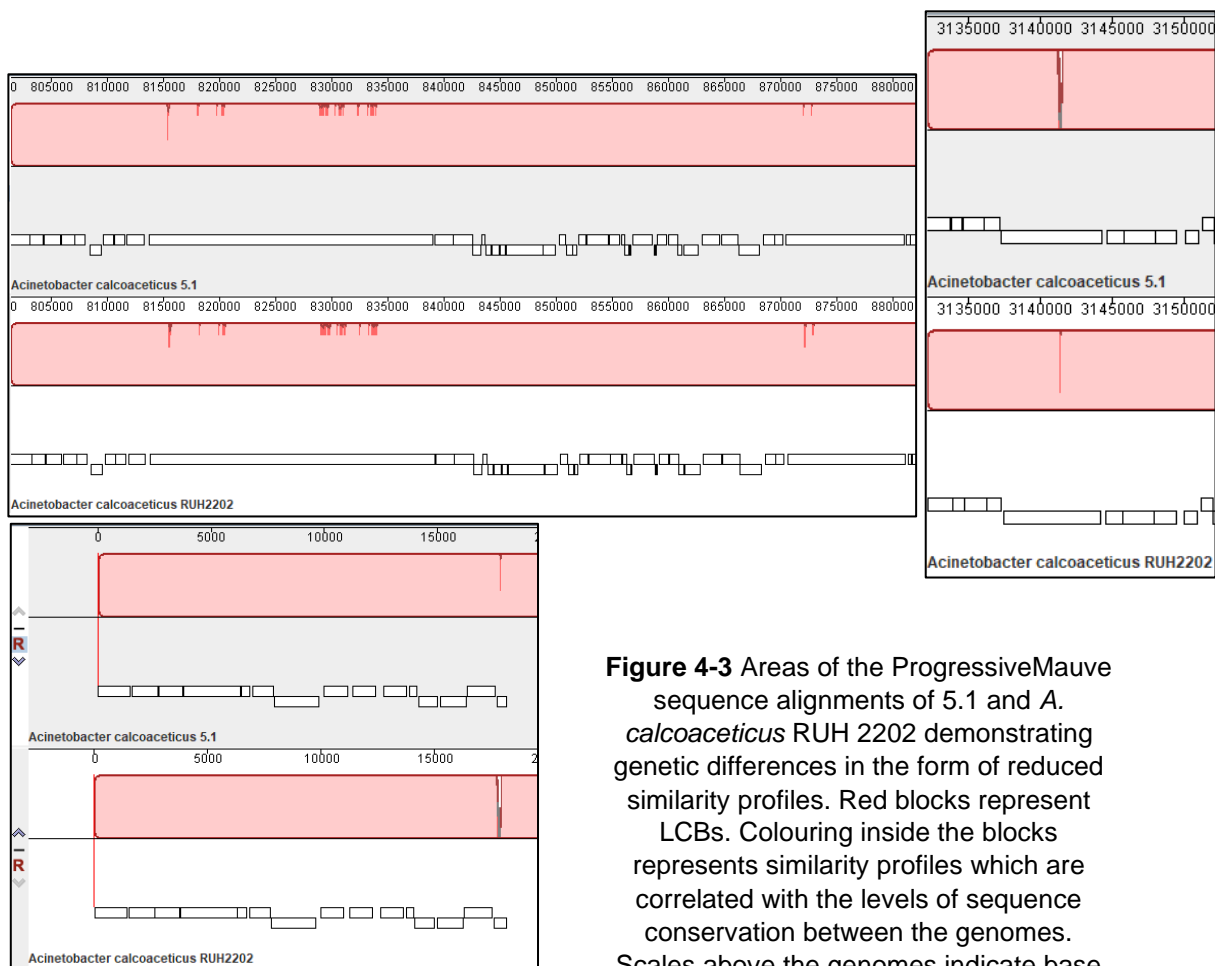
Figure 4-3 Areas of the ProgressiveMauve sequence alignments of 5.1 and *A. calcoaceticus* RUH 2202 demonstrating genetic differences in the form of reduced similarity profiles. Red blocks represent LCBs. Colouring inside the blocks represents similarity profiles which are correlated with the levels of sequence conservation between the genomes. Scales above the genomes indicate base pair numbers. Labels below the genomes indicate strain type.
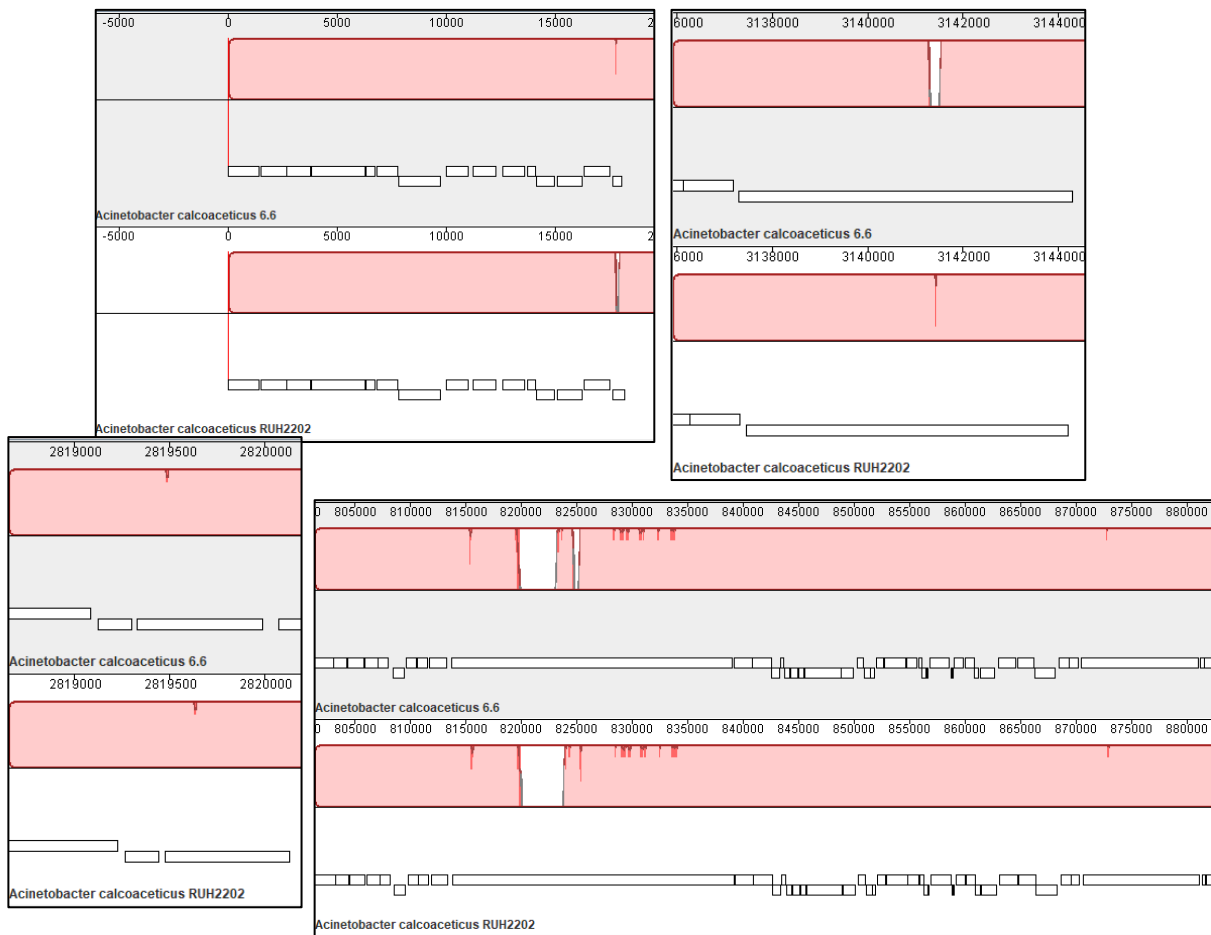
Figure 4-4 Areas of the ProgressiveMauve sequence alignments of 6.6 and *A. calcoaceticus* RUH 2202 demonstrating genetic differences in the form of reduced similarity profiles. Red blocks represent LCBs. Colouring inside the blocks represents similarity profiles which are correlated with the levels of sequence conservation between the genomes. Scales above the genomes indicate base pair numbers. Labels below the genomes indicate strain type.

Strain	Gene	File source	Number of SNPs
5.1	15	Alignment gaps	NA
5.1	777	SNPs	74
5.1	808	SNPs	4
5.1	2947	Alignment gaps	NA
6.6	15	Alignment gaps	NA
6.6	777	Alignment gaps	79
6.6	808	SNPs	1
6.6	2654	SNPs	1
6.6	2947	Alignment gaps	NA

Table 4-4 Genes containing genetic differences and the file sources from which these differences were identified. For genes containing SNPs, number of SNPs are displayed, and N/A is displayed for genes which alignment gaps correspond to.

To understand whether the nucleotide sequence differences identified in progressiveMauve were reflected in their encoded predicted proteins, the amino acid sequences in 5.1, 6.6 and *A. calcoaceticus* RUH 2202 were compared using the EMBOSS Needle pairwise sequence alignment tool (Madeira *et al.*, 2022) (tables 4-5 and 4-6). The sequence alignments demonstrate amino acid differences in the predicted proteins of genes 15 and 2947 only in 5.1 compared to *A. calcoaceticus* RUH 2202 equivalents. Hypothetical protein 15 in 5.1 is 73.9% identical to hypothetical protein 15 in *A. calcoaceticus* RUH 2202, with gaps of 48 missing Amino acids relative to *A. calcoaceticus* RUH 2202. As for 2947, its sequence is 96.7% identical to 2947 in *A. calcoaceticus* RUH 2202, with 76 amino acid gaps. Although nucleotide sequence differences were highlighted in progressiveMauve between the sequences of 777 and 808 in 5.1 compared to *A. calcoaceticus* RUH 2202, the resulting amino acid sequences predicted to be produced by these genes are 100% identical in 5.1 and *A. calcoaceticus* RUH 2202.

Strain	Locus tag	Identity (%)	Similarity (%)	Gaps	Mismatches	Alignment length	Score
5.1	235307E_51_00015	73.9	73.9	48 (26.1%)	0	184	657.5
5.1	235307E_51_00777	100.0	100.0	0 (0.0%)	2	8438	43420.0
5.1	235307E_51_00808	100.0	100.0	0 (0.0%)	0	3509	17958.0
5.1	235307E_51_2654	100.0	100.0	0 (0.0%)	0	217	1118.0
5.1	235307E_51_02947	96.7	96.7	76 (3.3%)	0	2322	11304.5
6.6	235308E_66_00015	73.9	73.9	48 (26.1%)	0	184	657.5
6.6	235308E_66_00777	99.2	99.6	0 (0.0%)	66	8438	43137.0
6.6	235308E_66_00808	100.0	100.0	0 (0.0%)	0	3509	17958.0
6.6	235308E_66_02654	99.5	99.5	0 (0.0)	1	217	1112.0
6.6	235308E_66_02947	96.7	96.7	76 (3.3%)	0	2322	11304.5

Table 4-5. Global amino acid sequence alignments performed in emboss needle between genes in strains 5.1 and 6.6 and *A. calcoaceticus* RUH 2202. Gaps column: first number indicates number of gaps, number in brackets indicates gap percentage.

Gene number	Identity (%)	Similarity (%)	Gaps	Mismatches	Alignment length	Score
15	100.0	100.0	0.0	0	136	691.0
777	99.2	99.6	0.0	64	8438	43144.0
2654	99.5	99.5	0.0	1	217	1112.0
2947	100.0	100.0	0.0	0	2322	11732.0

Table 4-6 Global amino acid sequence alignments performed in emboss needle for genes 15 and 2947 in strains 5.1 and 6.6

In strain 6.6, the predicted proteins 15, 777, 2654 and 2947 demonstrated amino acid sequence differences. The sequences of hypothetical proteins 15 and 2947 in 5.1 were 100% identical to 15 and 2947 in 6.6 and share the same sequence identities with the *A. calcoaceticus* RUH 2202 variants. Hypothetical protein 777 shared 99.2% sequence identity with hypothetical protein 777 in *A. calcoaceticus* RUH 2202 with 66 mismatches. The adenylate kinase 2654 in 6.6 shares 99.5% sequence identity with the adenylate kinases in both 5.1 and *A. calcoaceticus* RUH 2202, with one mismatch. 2654 in 5.1 and *A. calcoaceticus* RUH 2202, however, share 100% identity, meaning they are identical proteins in these two strains. 808 in 6.6, in addition to strain 5.1, is 100% identical to 808 in *A. calcoaceticus* RUH 2202, indicating that this protein is identical in all 3 strains. Due to 100% identities shared by the predicted proteins 777, 808 and 2654 with their *A. calcoaceticus* RUH 2202 equivalents in either one or both strains 5.1 and 6.6, these proteins were excluded as candidates for metaldehyde degradation. These instances where genes demonstrate genetic differences between their nucleotide sequences but not their predicted protein sequences can be explained by the redundancy of the genetic code, in that multiple DNA codons (synonymous codons) can encode one single amino acid residue once transcribed and translated, meaning that nucleotide sequence differences including SNPs between sequences may not necessarily result in different proteins being produced (Spencer and Barral, 2012).

Global alignments of the amino acid sequences of the hypothetical protein 15 and 2947 variants were visualised using Clustal Omega and Jalview (Madeira *et al.*, 2022; Waterhouse *et al.*, 2009) (figure 4-5 and figure 4-6). Due to the large lengths of the amino acid sequences of the 2947 variants, only the section of the alignment which demonstrates the genetic differences is shown.

Figure 4-5 Clustal Omega amino acid sequence alignment for gene 15 in strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202

Figure 4-6 Section of the Clustal Omega amino acid sequence alignment demonstrating the amino acid differences in 2947 between 51, 6.6 and *A. calcoaceticus* RUH 2202

The gaps found in the alignment of hypothetical protein 15 in 5.1, 6.6 and *A. calcoaceticus* RUH 2202 form a large deletion of 46 consecutive amino acids in 5.1 and 6.6 relative to *A. calcoaceticus* RUH 2202 (residues 65-112), within a region of repeating serine, lysine and aspartic acid residues (SKD). For the 2947 variants, the amino acid gaps in the alignments are found in *A. calcoaceticus* RUH 2202 relative to 5.1 and 6.6 and are interspaced at varying intervals within a large region of alternating serine and aspartic acid residue repeats (SD), which comprises residues 150-1327 in *A. calcoaceticus* RUH 2202. This demonstrates that there are insertions of 76 pairs of serine and aspartic acid residues in 5.1 and 6.6 compared to *A. calcoaceticus* RUH 2202.

BLASTP analyses were performed using the predicted amino acid sequences of hypothetical proteins 15 and 2947 to search for homologous proteins which may provide suggestions for shared biological functions (table 4-7 and 4-8). Default parameters were unable to produce any hits of potential homologues for gene 15. This was rectified by removing the low complexity region filter and setting the organism to "Bacteria (taxid:2)". Without the organism filter being set to bacteria, there were top hits involving proteins from distant phylogenetic domains including hits to eukaryotic proteins in fungi and beetles, hence the need to filter the organisms more thoroughly to identify homologous bacterial proteins. The top hit for gene 15 in 5.1 and 6.6 was a hypothetical protein from *Acinetobacter* sp. AHP123, sharing 76.52% identity and 84% query cover. On the other hand, for gene 15 in *A. calcoaceticus* RUH 2202, the top hit for gene 15 was a tetratricopeptide repeat protein from *Borrelia turcica*, sharing 91.93% identity and 85% query cover, suggesting possible different functions for gene 15 variants in 5.1 and 6.6 compared to *A. calcoaceticus* RUH 2202. For gene 2947, the closest relative for 5.1, 6.6 and *A. calcoaceticus* RUH 2202 was a protein containing a biofilm-associated protein prefix-like domain from *Acinetobacter pittii,* sharing 98.34% identity with 100% query cover, suggesting similar biofilm-related functions for the gene variants in all 3 strains.

NCBI's CD-Search tool was also used to search for conserved domains within the hypothetical proteins in the three strains as a further attempt to assign them a potential biological function and to investigate whether any of the genetic differences between the proteins in 5.1 and 6.6 are localised to functional domains. For gene 15, no domain hits were produced using default parameters. To rectify this, the composition-based statistics adjustment was removed. This produced non-specific hits only to amidase domain-containing proteins of the PRK08581 superfamily which comprised, mostly, the section of the

Gene	Hit name	Percent identity	Query cover	E-value	Total score	Accession length	Accession no.
00015	Hypothetical protein [*Acinetobacter sp. AHP123*]	76.52%	84%	1e-30	116	103	WP_2520607 41.1
2947	BapA prefix-like domain-containing protein [*Acinetobacter pittii*]	98.34%	100%	0.0	3048	2077	QHQ31359.1

Table 4-7 BLASTP results for genes 15 and 2947 which are identical in strains 5.1 and 6.6.

Gene	Hit name	Percent identity	Query cover	E-value	Total score	Accession length	Accession no.
00015	Tetratricopeptide repeat protein [*Borrelia turcica*]	91.93%	85%	8e-54	2596	442	WP_23861 4506.1
2947	BapA prefix-like domain-containing protein [*Acinetobacter pittii*]	98.34%	100%	0.0	4397	2077	QHQ31359 .1

Table 4-8 BLASTP results for genes 15 and 2947 in strain RUH 2202.

amino acid sequence which contained the SD repeat region. When the same analysis was performed for gene 15 in *A. calcoaceticus* RUH 2202, no hits were produced. This is interesting because the differences observed between the amino acid sequences of 15 in 5.1 and 6.6 and *A. calcoaceticus* RUH 2202 were concentrated in this same SD repeat region. It is unclear from this analysis alone, however, whether the deletion of amino acids in the SD repeat region of gene 15 in 5.1 and 6.6 may have an actual effect on the function of the protein compared to *A. calcoaceticus* RUH 2202, as no specific domain hits were found,

only the similarity to proteins of the PRK08581 superfamily. Further experimental work is needed to determine the true function of gene 15 in 5.1 and 6.6.

In the case of hypothetical protein 2947, three specific domain hits were produced for the gene in 5.1, 6.6 and *A. calcoaceticus* RUH 2202. These included a biofilm-associated protein (BAP) BapA prefix-like domain of the superfamily biofilm_BapA_N, a bacterial Ig domain of the Big_6 superfamily and a VC_A0849 subclass type I secretion C-terminal target domain of the superfamily Peptidase_M10_C. According to Conserved Domin Database (CDD), the BapA-prefix is conserved within several large bacterial proteins which are involved in adhesion and biofilm formation, Ig domains are found in bacterial extracellular proteins of many different varieties and in members of the Peptidase_M10_C family proteins with which the type I secretion C-terminal target domain is associated, this domain forms a corkscrew which allows the proteins to be secreted through the bacterial cell wall. This information, along with the BLASTP analysis suggests that 2947 is likely to possess similar functions related to biofilm formation in all 3 different strains and is dependent on type I secretion. There is no direct evidence, therefore, of a metaldehyde-degrading function in 2947.

PSORTb 3.0.3 was used to predict the cellular localisations of the predicted hypothetical proteins 15 and 2947 (table 4-9). Subcellular localisation sites which score confidence values of 7.5 or above are returned in the final prediction by the tool. For hypothetical protein 15 in all 3 strains, only a ModHMM hit was produced, detecting an internal helix. The final subcellular localisation predictions were unknown, however, reflected by the low confidence values demonstrated. The only hit produced for 2947 in all strains was an ECSVM extracellular localisation hit with a confidence value of . The final subcellular localisation predictions were, therefore, extracellular, aligning with the CDD searches which suggested that this protein may be secreted extracellularly by a type I secretion system in all strains.

Gene 15 in 5.1 and 6.6	
Localisation site	**Confidence value**
CytoplasmicMembrane	2.0
Cytoplasmic	2.0
OuterMembrane	2.0
Periplasmic	2.0
Extracellular	2.0
Final prediction: unknown	NA

Gene 15 in RUH 2202	
Localisation site	**Confidence value**
CytoplasmicMembrane	2.0
Cytoplasmic	2.0
OuterMembrane	2.0
Periplasmic	2.0
Extracellular	2.0
Final prediction: unknown	NA

Gene 2947 in 5.1 and 6.6	
Localisation site	**Confidence value**
CytoplasmicMembrane	0.01
Cytoplasmic	0.01
OuterMembrane	0.11
Periplasmic	0.23
Extracellular	9.64
Final prediction: extracellular	9.64

Gene 2947 in RUH 2202	
Localisation site	**Confidence value**
CytoplasmicMembrane	0.01
Cytoplasmic	0.01
OuterMembrane	0.11
Periplasmic	0.23
Extracellular	9.64
Final prediction: extracellular	9.64

Table 4-9 Subcellular localisation score results from PSORTb for genes 15 and 2947 in *A. calcoaceticus* strains 5.1, 6.6 and RUH 2202.

The genetic context of the divergent proteins was visualised using Artemis, demonstrating that genes 15 and 2947 are flanked by the same genes in 5.1, 6.6 and *A. calcoaceticus* RUH 2202, aligning with the fact that each of the genes within each strain's genome was found to be positionally homologous to one another (figure 4-7 and figure 4-8). The gene 15 variants in all three strains are immediately flanked on their left by an uncharacterized ATP-binding cassette transporter protein named YheS and on their right by a Ribosomal RNA small subunit methyltransferase named rsmB. As for gene 2947, this gene is flanked in each strain on its left by a hypothetical protein. This protein is most closely related to a hypothetical protein from *Acinetobacter calcoaceticus*, identified by BLASTP analysis, sharing 100% identity with 100% query cover (accession no. KJH65323.1) and on its right by a Type I secretion system membrane fusion protein named PrsE_2. No MGEs are flanking either gene 15 or 2947 in either 5.1 or 6.6, and because none of the other genetically different predicted proteins which were identified earlier in the section was annotated as MGEs, and none of them located near 15 and 2947, there is no direct evidence that genes 15 and 2947 were acquired through horizontal gene transfer mediated by MGEs.

Figure 4-7 Genetic context of gene 15 in strain 5.5, visualised in Artemis. The six tracks with horizontal black lines (representing stop codons) and blue coloured boxes (representing annotated genomic features) show the six frame DNA translations, the first three representing the positive strand and the bottom three representing the negative strand. The graph above the tracks indicates GC content (%) for the sliding window, with the centre line representing the chromosomal average of 38.73% with values above and below this line representing GC% above and below average, respectively.
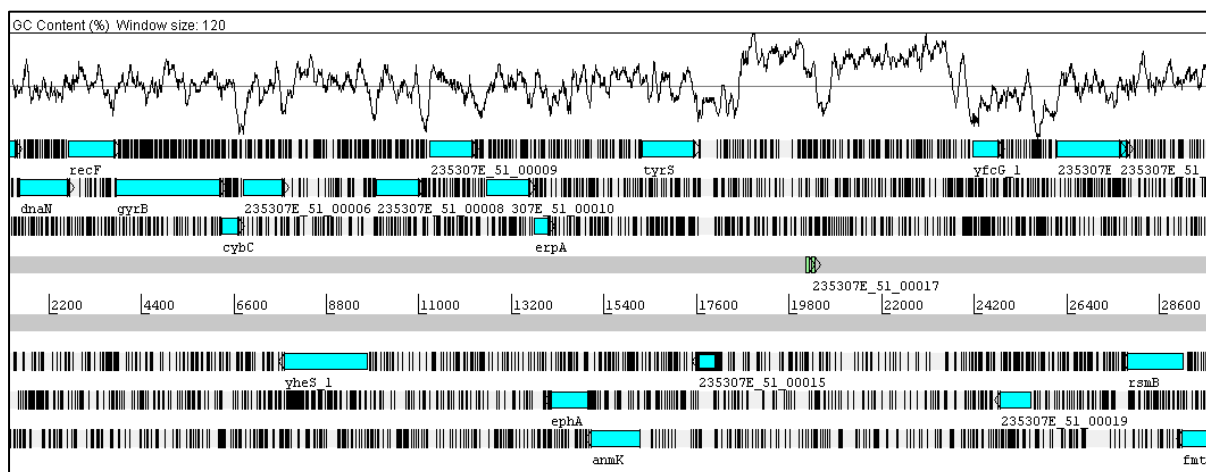
Figure 4-8 Genetic context of gene 2947 in strain 5.5, visualised in Artemis. The six tracks with horizontal black lines (representing stop codons) and blue coloured boxes (representing annotated genomic features) show the six frame DNA translations, the first three representing the positive strand and the bottom three representing the negative strand. The graph above the tracks indicates GC content (%) for the sliding window, with the centre line representing the chromosomal average of 38.73% with values above and below this line representing GC% above and below average, respectively.
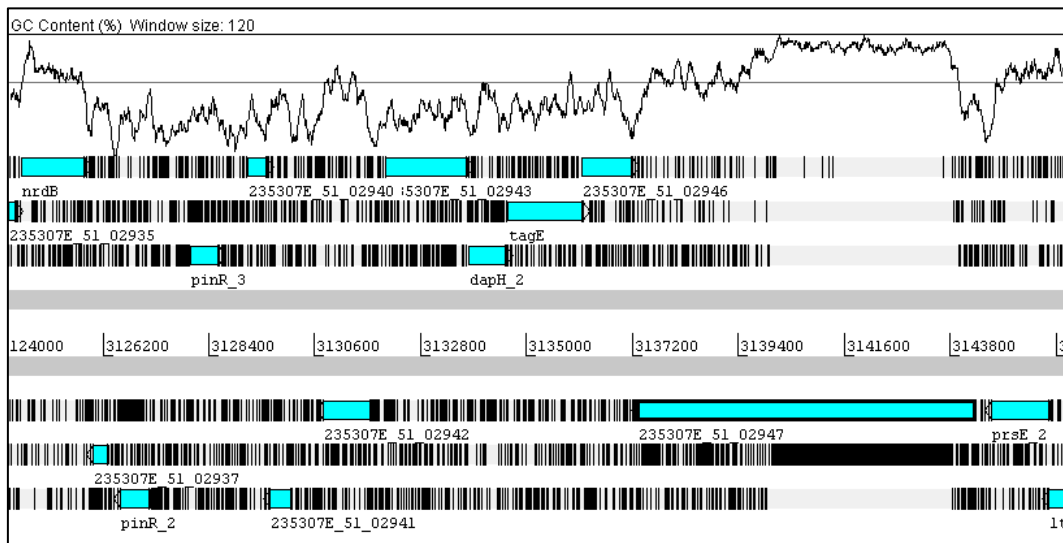
GC contents for hypothetical proteins 15 and 2947 in 5.1, 6.6 and *A. calcoaceticus* RUH 2202 were examined in Artemis as a further method for inferring their possible horizontal gene transfer (Ravenhall *et al.*, 2015). The GC contents for hypothetical protein 15 are 31.63%, 31.63% and 32.07% in strain 5.1, 6.6 and *A. calcoaceticus* RUH 2202, respectively, showing slightly lower GC contents compared to their shared chromosomal average of 38.73%. The hypothetical protein 2947 GC contents are 47.32%, 47.32% and 47.13% in 5.1, 6.6 and *A. calcoaceticus* RUH 2202, which are higher than the chromosomal average. Lack of largely different GC contents for genes 15 and 2947 rules out the possibility that these genes were horizontally acquired from distantly related organisms, because more distantly related organisms tend to display increasingly different base compositions (Lawrence and Ochman, 1997).

4.2.2 Illumina whole genome sequencing for the metazachlor-degrading strains 4.2.2, 4.5.2 and 6.4.2 and identification of potential candidate metazachlor-degrading genes

4.2.2.1 Illumina whole genome sequencing for strains 4.2.2, 4.5.2 and 6.4.2

Illumina short-read sequencing was carried out to obtain genome sequences for strains 4.2.2, 4.5.2 and 6.4.2 for comparative genomics purposes. Quality statistics for the Illumina short-read sequencing run and the assemblies are shown in table 4-10. The sequenced genomes of 4.2.2, 4.5.2 and 6.4.2 are assembled at the contig level into 41, 85 and 43 contigs of 5997823bp, 5985553bp and 5880494bp, respectively. It is of note that there are several indications that the genome sequencing assembly quality for strain 4.5.2 is lower compared to 4.2.2 and 6.4.2, including a much lower N50 value and a much higher number of contigs observed for 4.5.2 (Gurevich, 2013). Financial constraints of the project prevented long-read sequencing to be carried out, therefore, hybrid assemblies for the genomes of 4.2.2, 4.5.2 and 6.4.2 were also unable to be provided.

Strain	Median insert size (bp)	Mean coverage (fold)	No. reads	Number of contigs	Largest contig (bp)	Total length (bp)	GC (%)	N50 (bp)	#N's
4.2.2	695	77.1	984751	41	960456	5997823	62.77	362129	0
4.5.2	642	35.1	639239	85	444113	5985553	62.78	152447	0
6.4.2	630	85.7	1214063	43	580519	5880494	62.94	305004	0

Table 4-10 Quality statistics for illumina short-read whole genome sequencing and assemblies for strains 4.2.2, 4.5.2 and 6.4.2.

Automated annotation by Prokka identified 5308 coding sequences, 70 tRNA genes, and 1 tmRNA gene in strain 4.2.2, 5321 coding sequences, 68 tRNA genes and 1 tmRNA gene in strain 4.5.2 and 5180 coding sequences, 70 tRNA genes and 1 tmRNA gene in 6.4.2. These numbers indicate the genetic variation between the strains, particularly in strain 6.4.2, which contains the lowest number of protein-coding genes compared to 4.2.2 and 4.5.2.

The most frequent species to which genome sequencing reads were mapped, calculated in Kraken, was *Pseudomonas putida* for each of the three strains (appendix 6.10). A significant portion of the species that reads for each species was mapped to, however, were unclassified. Pairwise ANI calculations were performed between the genomes of 4.2.2, 4.5.2, 6.4.2 and reference *Pseudomonas* strains of the species types *P. putida, P. orziyhabitans, P. aeruginosa, P. plecoglossicida and P. entomophila* to try to determine more accurately which species each strain might belong to (table 4-11).

These analyses shed no further light on the *Pseudomonas* species types of 4.2.2, 4.5.2 or 6.4.2 because ANI values observed for each comparison were below the chosen threshold of 95% for species level assignment (Rodriguez-R and Konstantinidis, 2014). All three strains appear to be most closely related to *P. putida*, however, because the highest ANI calculation values were observed when the metazachlor degrader genomes were compared to this strain type. These results contradict the assignment of strains 4.2.2 and 6.4.2 to the *P. oryzihabitans* species and suggest the inability of 16S rRNA sequencing to provide taxonomic classifications of these strains at the species level. Further analysis is required to rectify this. ANI calculations between the strains themselves were also performed which suggested that 4.2.2 and 5.4.2 are more closely related than each other is to strain 6.4.2, with ANI of 100% for 4.2.2 vs 4.5.2, ANI of 99.65% for 4.2.2 vs 6.4.2 and 99.64% For 4.5.2 vs 6.4.2.

Comparison	ANI (%)	Mean ANI (%)	Median ANI (%)
4.2.2 vs 4.5.2	100	100	100
4.2.2 vs 6.4.2	99.65	99.65	99.87
4.5.2 vs 6.4.2	99.64	99.64	99.87
4.2.2 vs *P. putida*	86.95	86.95	87.03
4.5.2 vs *P. putida*	86.93	86.93	87.04
6.4.2 vs *P. putida*	86.88	86.88	86.99
4.2.2 vs *P. oryzihabitans*	78.40	78.4	78.5
4.5.2 vs *P. oryzihabitans*	78.39	78.39	78.53
6.4.2 vs *P. oryzihabitans*	78.43	78.43	78.52
4.2.2 vs *P. aeruginosa*	79.41	79.41	79.54
4.5.2 vs *P. aeruginosa*	79.42	79.42	79.57
6.4.2 vs *P. aeruginosa*	79.56	79.56	79.71
4.2.2 vs *P. plecoglossicida*	86.65	86.65	86.82
4.5.2 vs *P. plecoglossicida*	86.62	86.62	86.81
6.4.2 vs *P. plecoglossicida*	86.69	86.69	86.88
4.2.2 vs *P. entomophila*	85.51	85.51	85.82
4.5.2 vs *P. entomophila*	85.44	85.44	85.73
6.4.2 vs *P. entomophila*	85.5	85.5	85.78

Table 4-11. Pairwise average nucleotide identity calculations performed between strains 4.2.2, 4.5.2, 6.4.2, *P. putida*, *P. oryzihabitans, P. aeruginosa, P. plecoglossicida* and *P. entomophila* using the average nucleotide identity calculator from http://enve-omics.ce.gatech.edu/ani/.

4.2.2.2 Identification of candidate metazachlor degradation genes in strains 4.2.2, 4.5.2 and 6.4.2

Previous work has suggested that the first step of metazachlor degradation in soils is glutathione conjugation, mediated by glutathione transferase enzymes (Laue, Field and Cook, 1996). These enzymes, including glutathione-S-transferases, have also been found to be involved in the microbial degradation of other members of the chloroacetamide herbicide class, to which metazachlor belongs (Zablotowicz *et al.*, 1995; Irzyk and Fuerst, 1993). To identify possible genes conferring metazachlor degradation in 4.2.2, 4.5.2 and 6.4.2, the annotated predicted gene files of these isolates were searched manually for the presence of glutathione transferase enzymes. Four glutathione transferase enzymes were identified in each strain, as shown in table 4-12 and table 4-13. These include the predicted genes annotated by Prokka as *gstB_1*, *gstB_2*, *yibF* and three genes with no specific gene named annotation provided which were subsequently named in this study (according to the numbers in their locus tags) 4164, 3838 and 4909 in strains 4.2.2, 4.5.2 and 6.4.2, respectively. Further biological and bioinformatic analyses of strains 4.2.2, 4.5.2 and 6.4.2 could determine whether these predicted enzymes (or as yet unidentified alternatives) could be conferring metazachlor degradation capabilities in these strains.

Strain	Gene locus tag	Gene type	Gene name	Length (bp)
4.2.2	236537_422_00538	Glutathione-S-transferase	*gstB_1*	624
4.2.2	236537_422_02351	Glutathione-S-transferase	*gstB_2*	624
4.2.2	236537_422_04164	Glutathione-S-transferase	4164*	663
4.2.2	236537_422_04873	Uncharacterised glutathione-S-transferase like protein	*yibF*	624

Table 4-12 Candidate metazachlor-degrading glutathione-S-transferase enzymes identified in strain 4.2.2. Locus tags and gene names were produced by automated annotation of whole genome sequences.

* Genes named in this study rather than by Prokka annotation, based on locus tag numbers.

Strain	Gene locus tag	Gene type	Gene name	Length (bp)
4.5.2	236538_452_00359	Glutathione-S-transferase	*gstB_1*	624
4.5.2	236538_452_01093	Glutathione-S-transferase	*gstB_2*	624
4.5.2	236538_452_03838	Glutathione-S-transferase	3838*	663
4.5.2	236538_452_04894	Uncharacterised glutathione-S-transferase like protein	*yibF*	624
6.4.2	236539_642_02227	Glutathione S-transferase	*gstB_1*	624
6.4.2	236539_642_02609	Glutathione-S-transferase	*gstB_2*	624
6.4.2	236539_642_04542	Uncharacterised glutathione-S-transferase like protein	*yibF*	624
6.4.2	236539_642_04909	Glutathione-S-transferase	4909*	663

Table 4-13 Candidate metazachlor-degrading glutathione-S-transferase enzymes identified in strains 4.5.2. and 6.4.2. Locus tags and gene names were produced by automated annotation of whole genome sequences.

* Genes named in this study rather than by Prokka annotation, based on locus tag numbers.

4.3 Discussion

4.3.1 Analysis of strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202

The combined Oxford Nanopore and Illumina whole genome sequencing and assemblies were able to provide complete and fully assembled genomes for *A. calcoaceticus* strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202. The topology of all three strains was confirmed as circular, meaning that their entire genomes are each organised into one chromosome. This confirmed that neither 5.1 nor 6.6 had acquired a metaldehyde-degrading plasmid, therefore, no evidence for the horizontal transmissibility of the previously-identified metaldehyde-

degrading plasmids pAME76 or pSM1 could be provided. Furthermore, it demonstrated that the inferred metaldehyde-degrading trait in these strains was not conferred by a novel plasmid. Hybrid long-read and short-read whole genome sequencing and assembly approaches are considered the gold standard for sequencing accuracy as the long reads can help to reduce gaps in fragmented short-read sequencing assemblies by scaffolding contigs together, leading to the production of high-quality and complete genome sequences (Wick *et al.*, 2017; Ashton *et al.*, 2015). In the past, the same combined sequencing and assembly method facilitated the identification of both the metaldehyde-degrading plasmids pAME76 in *A. calcoaceticus* E1 and pSM1 in *Sphingobium* CMET-H and it is a useful approach to continue with in further studies investigating xenobiotic degradation and horizontal gene transfer (Castro-Gutiérrez *et al.*, 2022a).

The most frequent species that the whole genome sequencing reads were mapped to for strains 5.1, 6.6 and *A. calcoaceticus* RUH 2202 was *A. calcoaceticus*, confirming the species assignment results obtained from the 16S rRNA gene amplicon sequencing described in chapter 3. Average nucleotide identity (ANI) is considered a robust method for determining the relatedness between bacterial strains as it can distinguish between closely-related strains whereas 16S rRNA-based analysis can not (Jain *et al.*, 2018). ANI calculations comparing 5.1 and 6.6 to the genomes of *A. calcoaceticus* RUH 2202 and the metaldehyde-degrading strain *A. calcoaceticus* E1 confirmed that 5.1 and 6.6 more closely resemble *A. calcoaceticus* RUH 2202 than *A. calcoaceticus* E1 both in terms of nucleotide sequence similarity (100% ANI) along with the phenotypic assays described in chapter 3. These results further suggest that 5.1 and 6.6 could belong to the *A. calcoaceticus* RUH 2202 strain. It is possible, however, that 5.1 and 6.6 could belong to a different *A. calcoaceticus* strain as there are others found within soils which could have been present in and isolated from the microcosm environments which might also demonstrate high ANI values if their genomes were compared to 5.1 and 6.6 (Nemec *et al.*, 2011). Further genomic comparisons with other reference genomes of the *Acinetobacter calcoaceticus* strain types should be performed in future to determine the strain types of 5.1 and 6.6 or to verify that they are the *A. calcoaceticus* RUH 2202 type. Another possible alternative approach is multilocus sequence analysis (MLSA) which involves the comparison of selected housekeeping genes between different bacterial strains to help characterize them and has been used to characterize *Acinetobacter* strains previously (Gaiarsa *et al.*, 2019; Maiden, 2006; Diancourt *et al.*, 2010).

The progressiveMauve method can visualise respective gene gains, gene losses and structural rearrangements between genomes and allow the examination of the genetic context of genes (Darling, Mau, B. and Perna, 2010). Pairwise sequence alignments for 5.1,

6.6 and *A. calcoaceticus* RUH 2202 performed in progressiveMauve illustrated the high levels of sequence conservation between the three strains that were also observed from the ANI calculations that were performed. It allowed the identification and visualisation of the precise genetic differences between the inferred degrading and non-degrading *A. calcoaceticus* RUH 2202 variants to the level of single nucleotide polymorphisms within specific genes, making it a sufficiently precise tool for comparative genomic analysis. It also allowed the identification of potential novel metaldehyde-degrading genes at the nucleotide sequence level.

A large advantage of progressiveMauve is that it requires no prior programming knowledge or skills to perform analyses at the basic level, which was sufficient enough to provide the results gained in this study. The previous method used for the identification of metaldehyde-degrading genes involved a python-based BLAST score ratio (BSR) approach in which homologous genes present in metaldehyde-degrading strains but absent from non-degrading strains were selected as degrading gene candidates (Castro-Gutiérrez *et al.*, 2020). This method is, however, inappropriate if the candidate degrading strains being compared do not share sufficiently similar degrading genes due to the method's reliance on gene homology between strains. This was one of the reasons why not all of the metaldehyde-degrading strains' degradation genes and pathways were discovered previously as not all of the strains shared genes sufficiently similar to *mahX, mahY, and aldH.* This problem did not impact this study because the method used here only required the genomic comparison of pairs of non-degrading and degrading variants of highly similar bacterial strains whose genomes were identical for the majority, meaning that identifying candidate degrading genes was simple and the number of candidate degrading genes was small despite there only being two strains compared at one time. This method could be successful in future for comparative genomics analyses between xenobiotic-degrading and non-degrading variants of similar bacterial strains for the identification of novel xenobiotic-degrading genes when coupled with the facilitated horizontal gene transfer approach described in chapter 3.

Genes 15 and 2947 were identified as candidate metaldehyde-degrading genes based on their amino acid sequence differences compared to equivalent proteins in *A. calcoaceticus* RUH 2202 and were identical in both 5.1 and 6.6. No predicted function could be identified for gene 15, other than a possible amidase-related function which was not confidently determined. Bacterial amidase enzymes have previously been found to be involved in the bacterial degradation of xenobiotics through amide bond cleavage (Albers *et al.,* 2015; Nawaz *et al.*, 1994). Should gene 15 be found to demonstrate an amidase function,

however, this gene is unlikely to initiate the first step of metaldehyde degradation because metaldehyde's chemical structure contains no amide bonds which would be the target of its mechanism (fig. 1).

A probable biofilm-associated function was inferred for gene 2947 due to the presence of domains commonly found in biofilm-associated proteins (BAPs) including a BapA prefix-like domain, a bacterial Ig domain and a type I secretion C-terminal target domain. BAPs in different *Acinetobacter* species are secreted by the type 1 secretion system and contain type 1 secretion C-terminal target domains and Ig-like domains similar to those found in 2947 (Harding *et al.*, 2017). Additionally, BAPs which have previously been found in *Acinetobacter calcoaceticus* strains also demonstrate the presence of large SD repeat regions, much like the repeat region found in 2947 (De Gregorio *et al.*, 2015). The BAPs found within *Acinetobacter baumanii* have functions related to biofilm formation, including intercellular adhesion to human cells (Loehfelm, Luke and Campagnari, 2008).

Neither gene 15 (whose function was ambiguous) nor gene 2947 (whose function was deemed to be biofilm-related) demonstrated any resemblance to the previously-identified metaldehyde-degrading genes which include α-ketoglutarate -Fe (II) oxygenase, 2-oxoglutarate (2OG) and Fe(II)-dependent oxygenase superfamily proteins, vicinal oxygen chelate family proteins and the NAD(P)+-dependent aldehyde dehydrogenase enzymes (Castro-Gutierrez *et al.*, 2022a; Castro-Gutierrez *et al.*, 2020b). For this reason, no metaldehyde-degrading function could be inferred for either gene by the methods used in this work. This meant that no robust evidence could be provided for the success of the facilitated horizontal gene transfer method for the isolation of novel metaldehyde-degrading genes either. Further work could determine the true functions of genes 15 and 2947 and determine whether or not they are related to metaldehyde degradation. This could be done by using the methods which were used in the previously-mentioned studies on metaldehyde degradation which involved chemical mutagenesis in candidate metaldehyde-degrading strains with ethyl methanesulfonate (EMS) along with heterologous expression of the degrading genes in a non-degrading host such as *Escherichia coli*.

Through analysis of the genetic context of the candidate metaldehyde-degrading genes 15 and 2947, no direct evidence sufficient enough to infer horizontal gene transfer was identified. This means, therefore, that no direct evidence for the success of the facilitated horizontal gene transfer approach in demonstrating horizontal gene transfer of metaldehyde-degrading genes could be provided by this study. It is possible, however, that genes 15 and 2947 could have been acquired by natural transformation, as members of the *Acinetobacter*

genus including *Acinetobacter calcoaceticus* have demonstrated competence in previous studies (Johnsborg, Eldholm and Håvarstein, 2007).

During transformation, homologous sequences of DNA from a donor organism can be recombined into a host genome and replace a homologous gene, which occurs more frequently with increasing sequence homology (de Vries and Wackernagel, 2002). This form of horizontal gene transfer is difficult to detect because it leaves no specific genetic markers and because it can lead to the deletion of DNA which may be mistaken for mutations (Winter *et al.*, 2021). This could explain how both genes 15 and 2947 are homologous in part compared to their equivalents in RUH2202 but display considerable genetic differences without any visible evidence of horizontal gene transfer-mediated acquisition. The deletion of amino acids found within gene 15 and the insertions of 76 pairs of amino acids in 2947 could, therefore, be due to the replacement of the RUH2202 equivalents with an alternative gene from a similar bacterial strain. The approaches used in this study, however, could not confirm that transformation had occurred. Previous methods which have demonstrated transformation in soil environments are relevant to the work described in chapter 3 and involve facilitated transformation within soil microcosms, which has been demonstrated with *Acinetobacter* strains previously and could be utilised in future for studying the transformation of metaldehyde-degrading genes and plasmids (Nielsen, Bones and Van Elsas, 1997; Nielsen, Smalla, K. and van Elsas, 2000).

4.3.2 Analysis of 4.2.2, 4.5.2 and 6.4.2

The Illumina whole genome sequencing approach was able to provide draft genomes for the candidate metazachlor-degrading strains 4.2.2, 4.5.2 and 6.4.2 assembled at the contig level. The combined long-read and short-read sequencing approach described in the previous section performed for strains 5.1 and 6.6 could not be performed for the candidate metazachlor-degrading strains due to the financial constraints of the project. Short-read sequencing technologies which include the Illumina platforms generate read lengths between 25 and 1000 bp (depending on whether single-end or paired-end sequencing is used) and are low-cost and highly accurate up to over 99% (Goodwin, 2016). The assembly qualities for short-read genome sequencing reads, however, are often limited by unresolved repetitive regions which reduce genome assembly contiguity and result in fragmentation (Goldstein *et al.*, 2019). This means that short-read sequencing assemblies are sometimes unable to accurately resolve important genomic structures including MGEs such as plasmids and, therefore, can not always distinguish between chromosomal DNA and plasmid DNA

(Arredondo-Alonso *et al.*, 2017). Large fractions of the individual genes within genome assemblies produced by short-read sequencing, however, are successfully reconstructed at read lengths as low as 25 base pairs (Kingsford, Schatz and Pop, 2010).

The assembled genome sequences produced for 4.2.2, 4.5.2 and 6.4.2 were made up of 41, 85 and 43 contigs, respectively, meaning that investigations into their genomic structures were likely to be inaccurate due to the fragmented nature of their assemblies. Analyses of this kind were not performed as a result. It was apparent, therefore, that the methods used in this study could not detect the presence of any plasmids which could indicate horizontal gene transfer-mediated acquisition of metazachlor-degrading genes in 4.2.2, 4.5.2 and 6.4.2. In a similar fashion to the hybrid sequencing approach described in the previous section, long-read sequencing could be performed for 4.2.2, 4.5.2 and 6.4.2 to provide scaffolds for the short-reads produced in this study to resolve gaps in the genome assemblies and improve their contiguity and completeness and to determine whether there are any plasmids present which might be indicative of horizontal gene transfer (Wick *et al.*, 2017; Ashton *et al.*, 2015; De Maio *et al.*, 2019).

ANI calculations performed between strains 4.2.2, 4.5.2 and 6.4.2 and reference *Pseudomonas* strains contradicted the results gained by the 16S rRNA gene amplicon sequencing analyses described in chapter 3, and no further light was shed on their taxonomic classifications other than their assignment to the *Pseudomonas* genus. It is to be expected that 16S rRNA gene amplicon analysis and ANI results differ in terms of species classifications due to the lower resolution that 16S rRNA analysis provides for the species demarcations of bacteria (Jain *et al.*, 2018). The *Pseudomonas* genus is one of the most diverse among bacteria comprising over 300 species, therefore, future studies performing ANI calculations between 4.2.2, 4.5.2 and 6.4.2 and other *Pseudomonas* strains would likely yield more accurate species identifications (Girard *et al.*, 2021). Other analytical approaches used for species demarcation, however, could also provide more accurate species identification such as MLSA using housekeeping genes including *gyrB*, *rpoB*, and *rpoD* (along with the 16S rRNA gene) which have been used to distinguish between different Pseudomonas strains previously (Gomila *et al.*, 2015).

Identifying 4 glutathione transferase enzymes in each of the genomes of 4.2.2, 4.5.2 and 6.4.2 provided potential candidate metazachlor-degrading genes. The hypothesis that these genes could facilitate metazachlor degradation is based on the hypotheses made in previous work that microbial metazachlor degradation in soils is likely to be facilitated by glutathione conjugation, the observations of this process occurring in the metabolism of metazachlor in

plants and rats, and the fact that bacterial glutathione conjugation has been found previously to facilitate the degradation of other chloroacetamide compounds (Laue, Field and Cook, 1996; EFSA, 2008; Zablotowicz *et al.,*1995; Zablotowicz *et al.,* 2001). The analyses carried out in this study for the identification of candidate metazachlor-degrading genes, therefore, were basic. No comparative genomics analysis could be performed against previously-identified metazachlor-degrading bacterial strains' genomes because no such strains exist.

Bacterial glutathione transferases have several different functions related to the degradation and detoxification of toxic compounds including the metabolism of herbicides, protection against oxidative and chemical stresses and antibiotic resistance (Zablotowicz *et al.*, 1995; Allocati *et al.*, 2003; Arca *et al.*, 1988). It is, therefore, possible that each of the glutathione transferase enzymes identified in this study has a function unrelated to metazachlor degradation. Further work is needed, therefore, to determine whether any of the candidate metazachlor-degrading glutathione transferase genes truly confer the degrading trait. This could be done by chemical mutation of the strains using EMS and heterologous expression of the degrading genes in a non-degrading host such as *Escherichia coli* (Castro-Gutierrez *et al.* 2020).

Should the candidate metazachlor-degrading genes identified in this study prove not to confer this trait, an alternative approach could be to adapt the BSR-based approach used by Castro-Gutierrez *et al.* (2020) in the study of metaldehyde-degrading strains for the identification of novel metazachlor-degrading genes. Non-degrading reference *Pseudomonas* strains would be required for this and experiments verifying a lack of degradative capabilities in reference *Pseudomonas* strains could be performed using analytical approaches such as gas chromatography or liquid chromatography (Castro-Gutierrez *et al.* 2020; Thomas *et al.*, 2017). This would enable the identification of genes that are shared by the degrading strains but absent from non-degrading reference strains as candidate metazachlor-degrading genes. The functions of these genes could then be tested using chemical mutation on each strain with EMS and heterologous expression of the candidate genes in a non-degrading host such as *Escherichia coli* (Castro-Gutierrez *et al.*, 2020).

4.3.3 Conclusions

Comparative genomics and other bioinformatics approaches facilitated the analysis of the whole genome sequences of 5.1, 6.6 and *A. calcoaceticus* RUH 2202 leading to the identification of two candidate metaldehyde-degrading genes, 15 and 2947. Genes 15 and 2947 lacked any functional resemblance to the metaldehyde-degrading genes identified in previous studies, prompting the need for further functional analyses of these genes to determine the success of the facilitated horizontal gene transfer method for the uptake of metaldehyde-degrading genes in *A. calcoaceticus* RUH 2202. 5.1 and 6.6 were confirmed to resemble the non-metaldehyde-degrading strain *A. calcoaceticus* RUH 2202, however, this could be verified more accurately by further analysis. No evidence of horizontal gene transfer was found in either 5.1 or 6.6, meaning that no evidence of the horizontal transmissibility of metaldehyde-degrading genes could be provided by this study.

Contig-level whole genome sequence assemblies were provided for the candidate metazachlor-degrading strains 4.2.2, 4.5.2 and 6.4.2, facilitated the identification of the candidate metazachlor-degrading genes *gstB_1*, *gstB_2*, *yibF*, 4164, 3838 and 4909. It was deemed necessary that further functional analyses were performed in future to verify whether any of these genes could confer a metazachlor-degrading trait and confirm if they are in fact novel metazachlor-degrading genes. It was also suggested that if the glutathione transferase enzymes identified were shown not to confer metazachlor degradation, alternative comparative genomics approaches could be used to detect genes shared between the metazachlor-degrading strains but absent from non-degrading reference strains. Strains 4.2.2, 4.5.2 and 6.4.2 were assigned to the *Pseudomonas* genus, meaning that further analysis would be required to assign each strain to a species. Due to the fragmented nature of the genome sequence assemblies produced, no structural analyses of the genomes of 4.2.2, 4.5.2 and 6.4.2 were performed and no evidence of horizontal gene transfer of metazachlor-degrading genes could be provided. Long-read sequencing could provide better sequence assemblies for each strain if performed in future to better determine the genomic structure of the metazachlor-degrading strains' genomes.

Chapter 5: Final Discussion

The main achievements of this work include: (1) the isolation of two candidate novel metaldehyde-degrading *Acinetobacter calcoaceticus* RUH 2202 strains named 5.1 and 6.6; (2) the identification of two potential metaldehyde-degrading genes named genes 15 and 2947; (3) the isolation of three candidate novel metazachlor-degrading *Pseudomonas* strains named 4.2.2, 4.5.2 and 6.4.2; (4) the identification of several potential metazachlor-degrading glutathione transferase genes named *gstB_1*, *gstB_2*, *yibF*, 4164, 3838 and 4909 and (5) a methodological concept which could be utilised for the identification of novel xenobiotic-degrading bacterial strains in future work.

Further work is needed to verify the degradative abilities of each of the potential novel metaldehyde and metazachlor-degrading strains, their precise species and strain types and their predicted degrading genes. Further comparative genomics approaches comparing the genomes of the candidate degrading organisms to other non-degrading strain types within their genera and species could more accurately determine their identities. In the case of 5.1 and 6.6, this would determine whether these strains represent members of the *A. calcoaceticus* RUH 2202 strain type with which the microcosms that 5.1 and 6.6 were isolated from were inoculated. Analytical techniques such as gas chromatography or liquid chromatography could be used for assays verifying the metaldehyde and metazachlor degradation capabilities of 5.1, 6.6, 4.2.2, 4.5.2 and 6.4.2. Following this, additional functional analysis of the candidate metaldehyde-degrading genes 15 and 2947 and the candidate metazachlor-degrading glutathione transferases is required to determine their true degradative capabilities. This could be done via mutations of the strains with EMS and heterologous expression of the candidate genes in a non-degrading host such as *Escherichia coli*. Should these strains be proven more accurately to be capable of the degradation of the relevant xenobiotic compounds, this could lead to the proposal of novel degradation pathways and investigation of the bioremediation potential of these strains, for example, in water treatment settings (Castro-Gutiérrez *et al.*, 2022b). For 5.1 and 6.6, proper verification of their metaldehyde-degradation capabilities and strain types would provide more robust evidence of the success of the facilitated horizontal gene transfer approach for inducing the production of novel metaldehyde-degrading *A. calcoaceticus* RUH 2202 strains. Knowledge of pesticide-degrading organisms and the addition of their sequenced genomes and encoded degrading genes to reference databases can also aid in future studies on pesticide biodegradation by providing the basis for the functional annotation of further novel xenobiotic-degrading genes in other xenobiotic-degrading species.

The study was unable to provide direct evidence for the horizontal transmissibility of metaldehyde or metazachlor-degrading genes. Both the soil microcosm-based facilitated horizontal gene transfer and selective enrichment aspects of the methodology used in this study need to be adapted in future experiments to rectify this. This could include experimentation with different soil microcosm conditions to better enable gene transfer, increased periods for selective enrichment liquid culture passages to increase the selection of degrading organisms and to reduce the isolation of non-degrading organisms, and experimental functional verification of candidate degrading genes. Trialling a selection mechanism for in vitro conjugation assays between *Sphingobium* strains CMET-H and DSM 7098 using both pentachlorophenol and metaldehyde degradation could aid in the demonstration of the transfer of plasmid pSM1, which was unable to be provided in this work. Additionally, the combined ethanol and mercury resistance selection mechanism along with metaldehyde degradation assays could be used in future experiments to demonstrate the uptake of the pAME76 plasmid by strain *A. calcoaceticus* RUH 2202.

The methodological concepts used in this work, including the facilitated horizontal gene transfer of xenobiotic-degrading genes within soil microcosms between selected culturable non-degrading bacterial recipients and donor bacterial strains indigenous to soil microcosms, the selective enrichment for the re-isolation of degrading organisms from soil microcosms and whole genome sequencing and bioinformatics for the taxonomic classifications, identification of candidate xenobiotic-degrading genes and the detection of the evidence of horizontal gene transfer could all be used in future studies for the isolation of novel xenobiotic-degrading organisms and their degrading genes and pathways, to identify potential tools for bioremediation efforts. For example, the metazachlor and metaldehyde degrading strains discovered in this work, and species discovered in the future, could be used for the bioaugmentation of contaminated water in a water treatment setting, which could increase pesticide degradation within the environment through their own degradative mechanisms, as well as potentially increasing the number of degrading strains in the indigenous microbial community through horizontal gene transfer of the degrading genes.
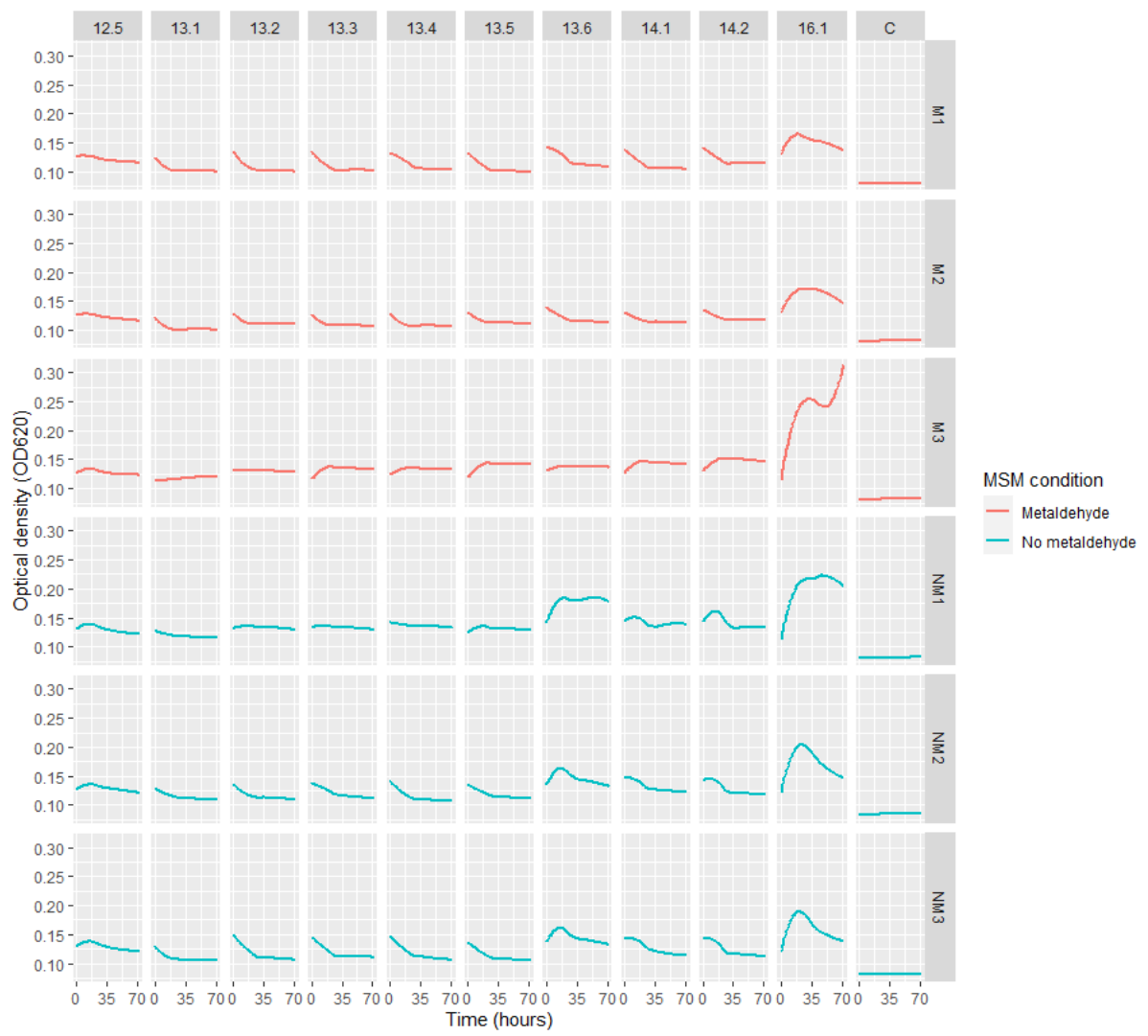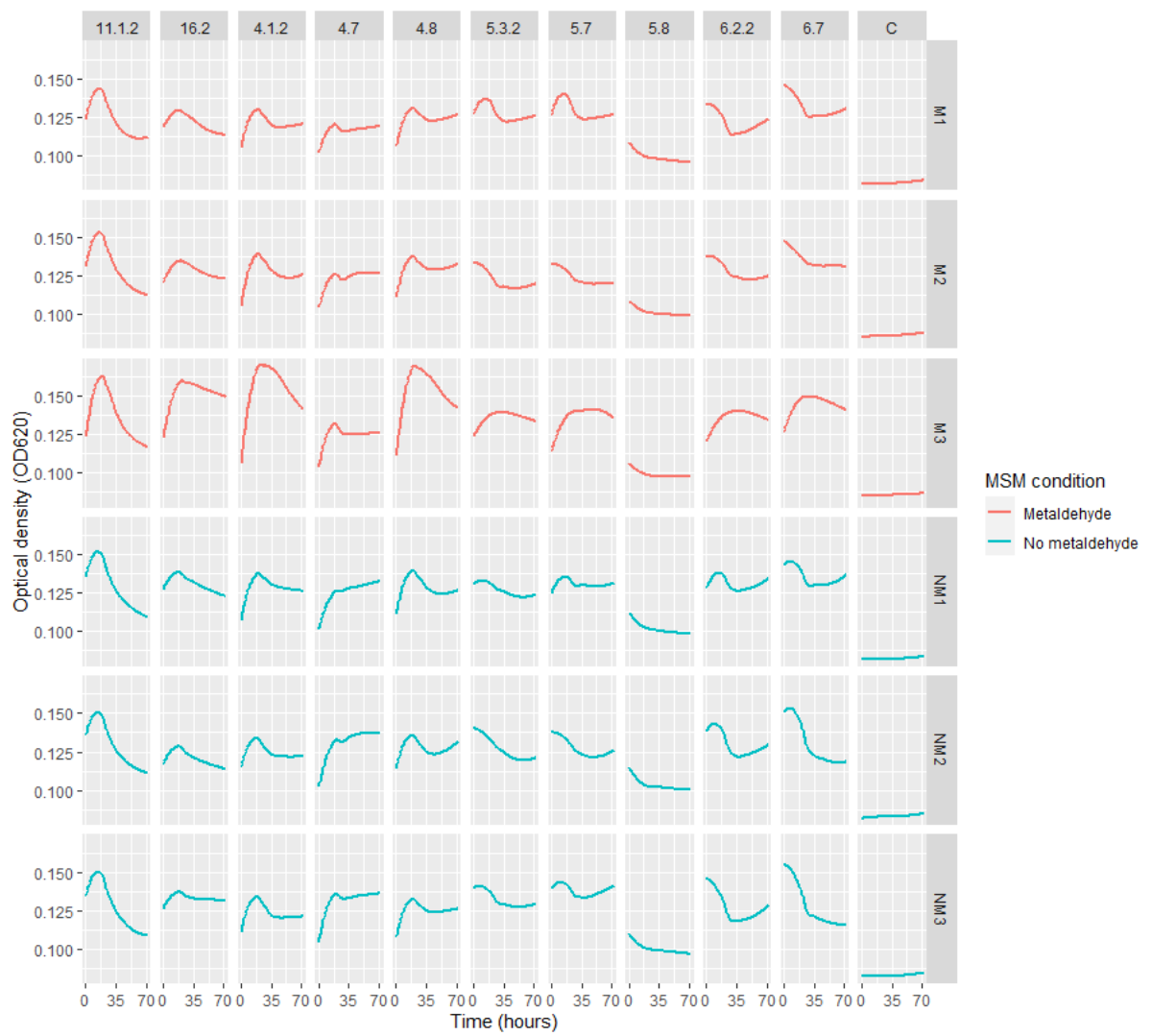
Chapter 6: Appendix

6.1 Initial triplicated growth curves for candidate metaldehyde-degrading strains grown in metaldehyde-supplemented and non-supplemented MSM
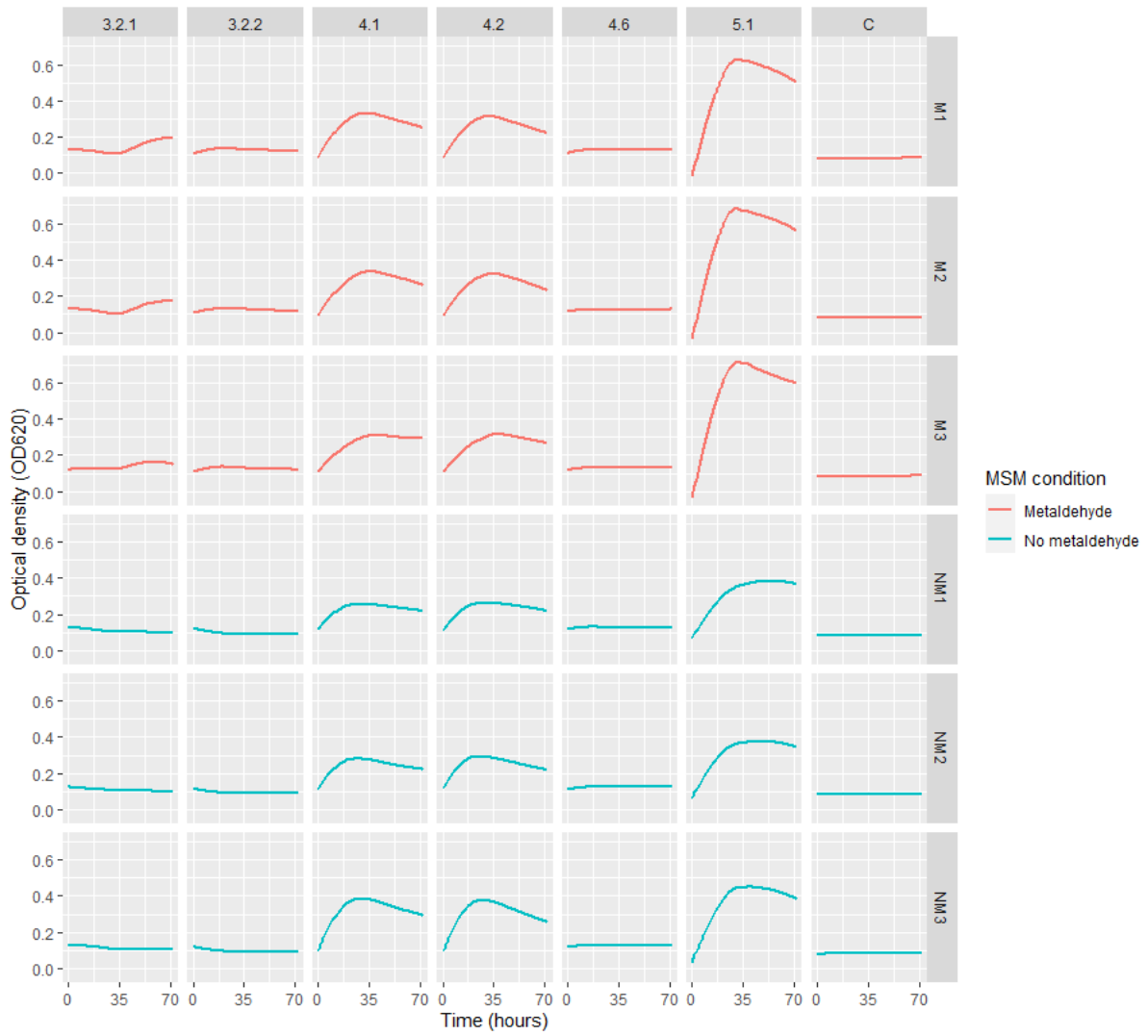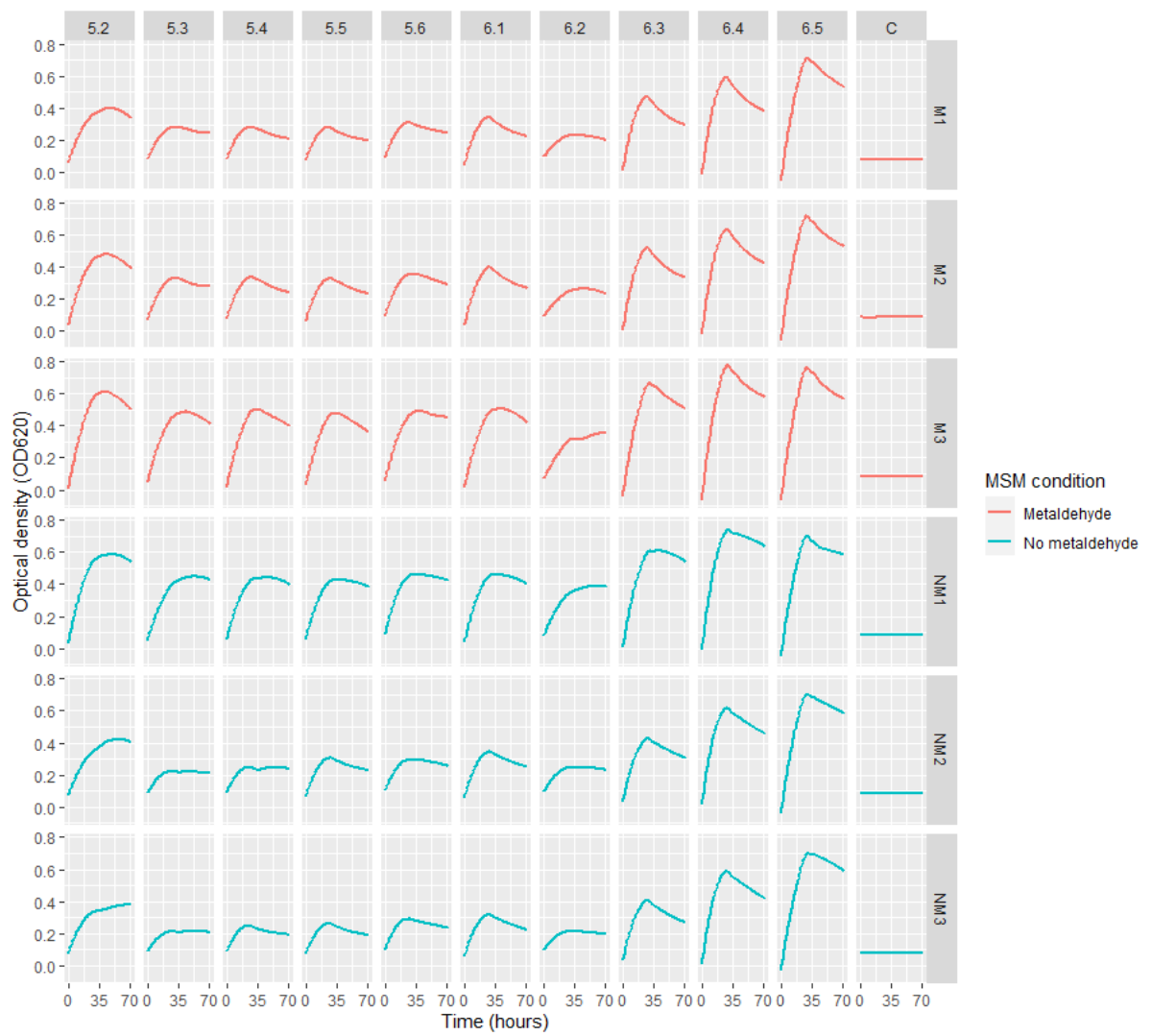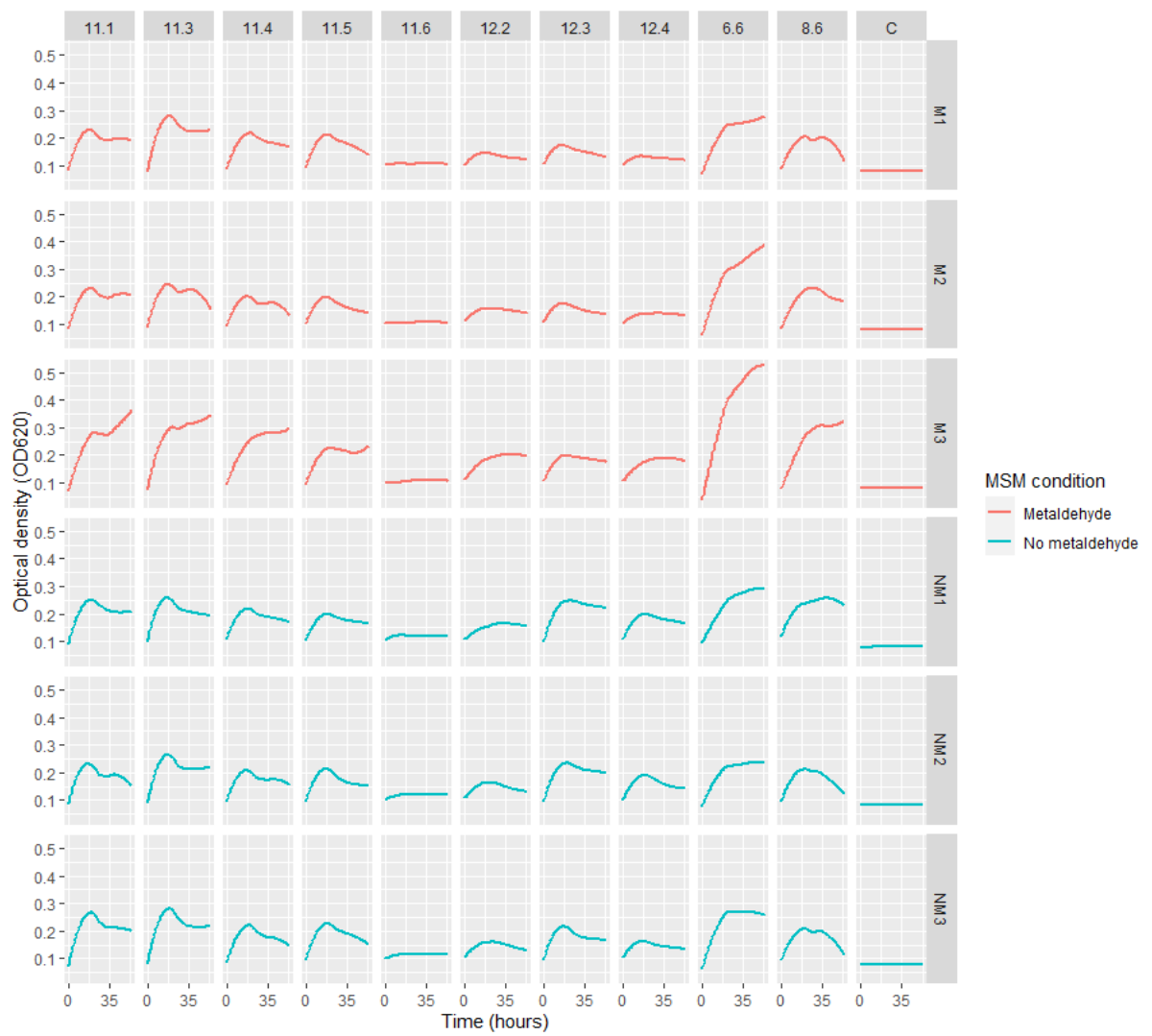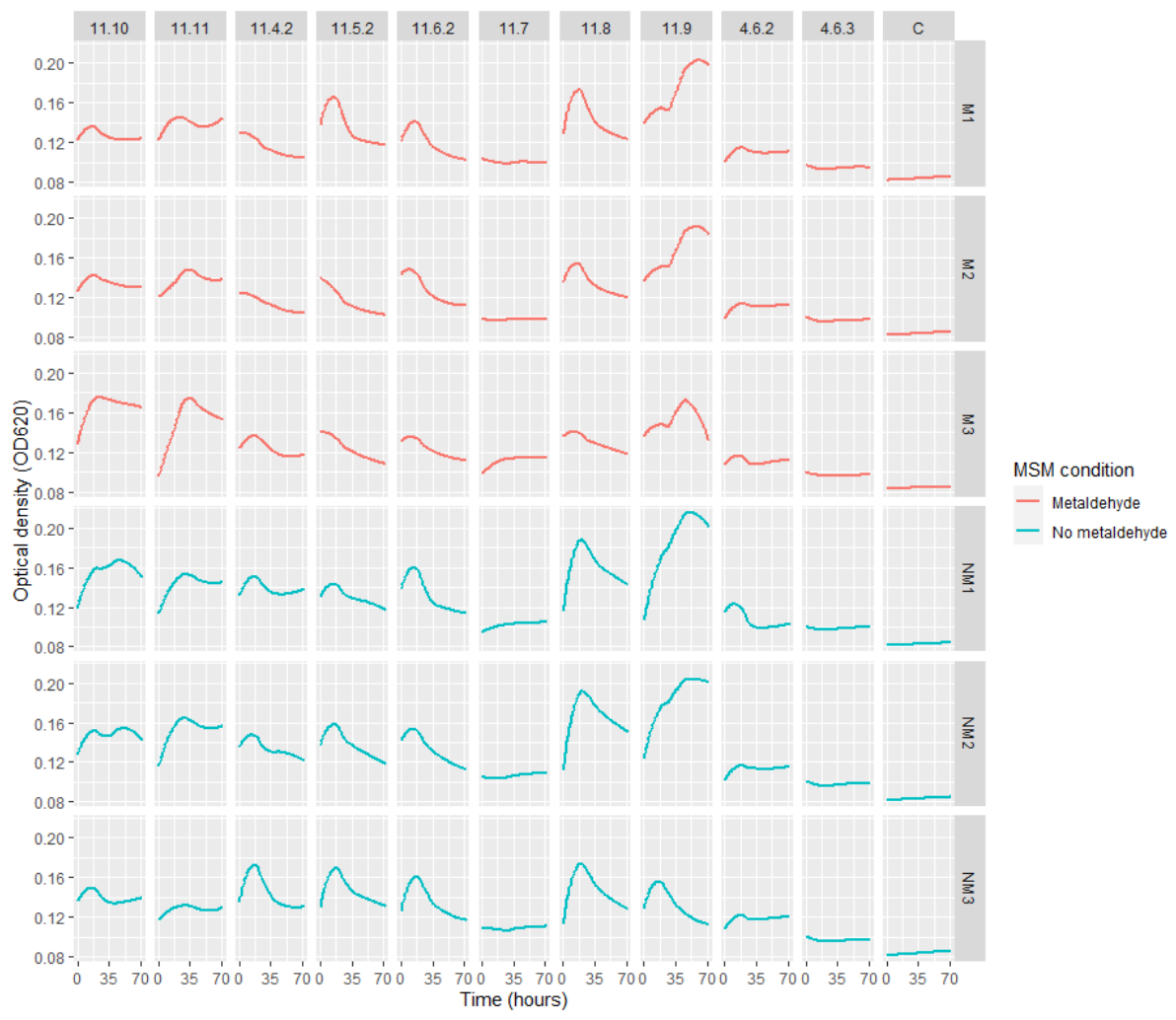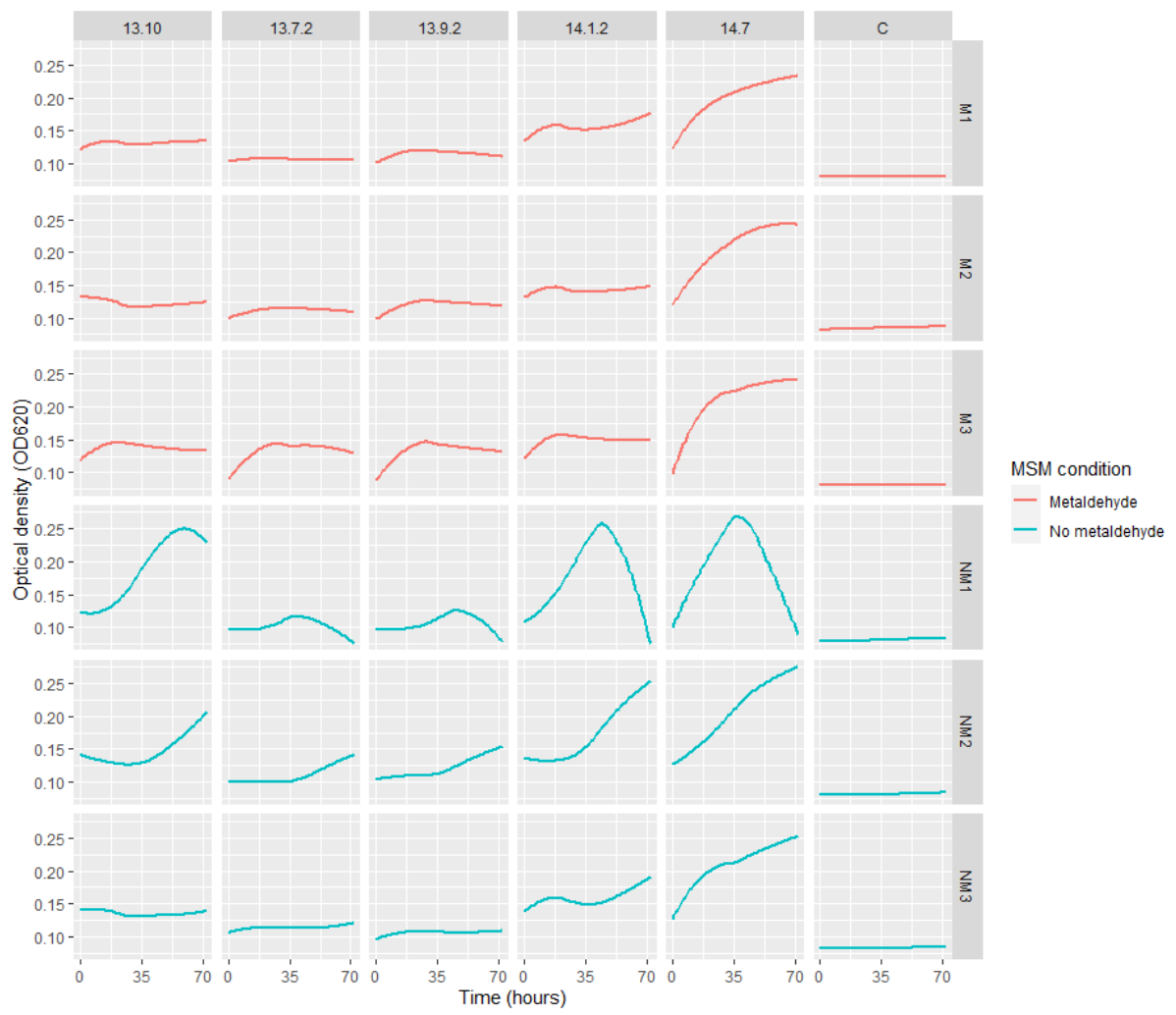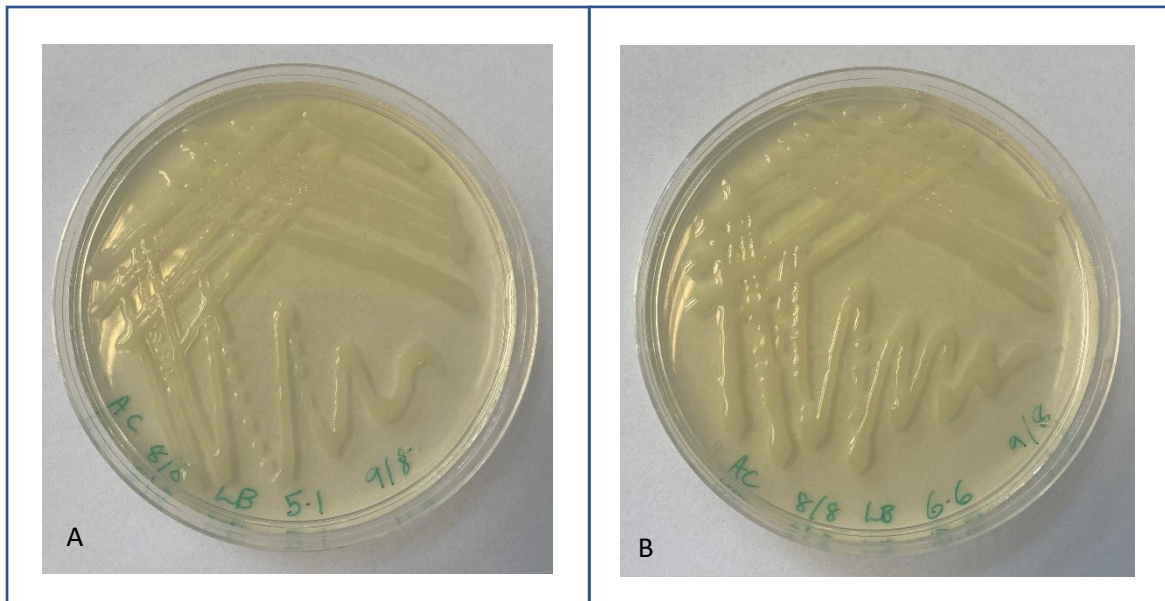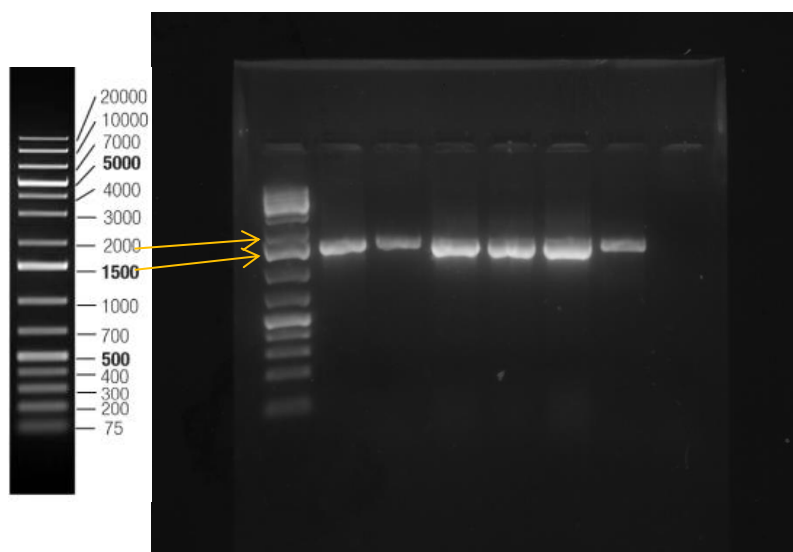
6.2 Strains 5.1 (panel A) and 6.6 (panel B) grown on solid LB agar media.

6.3 Agarose gel electrophoresis visualising 16S rRNA gene amplicons for strains 5.1 (lane 2), 6.6 (lane 3), 4.2.2 (lane 4), 4.5.2 (lane 5) and 6.4.2 (lane 6) with the GeneRuler 1kb plus ready-to-use DNA ladder in lane 1 and positive control in lane 7. Lanes run from 1 to 7 from left to right.

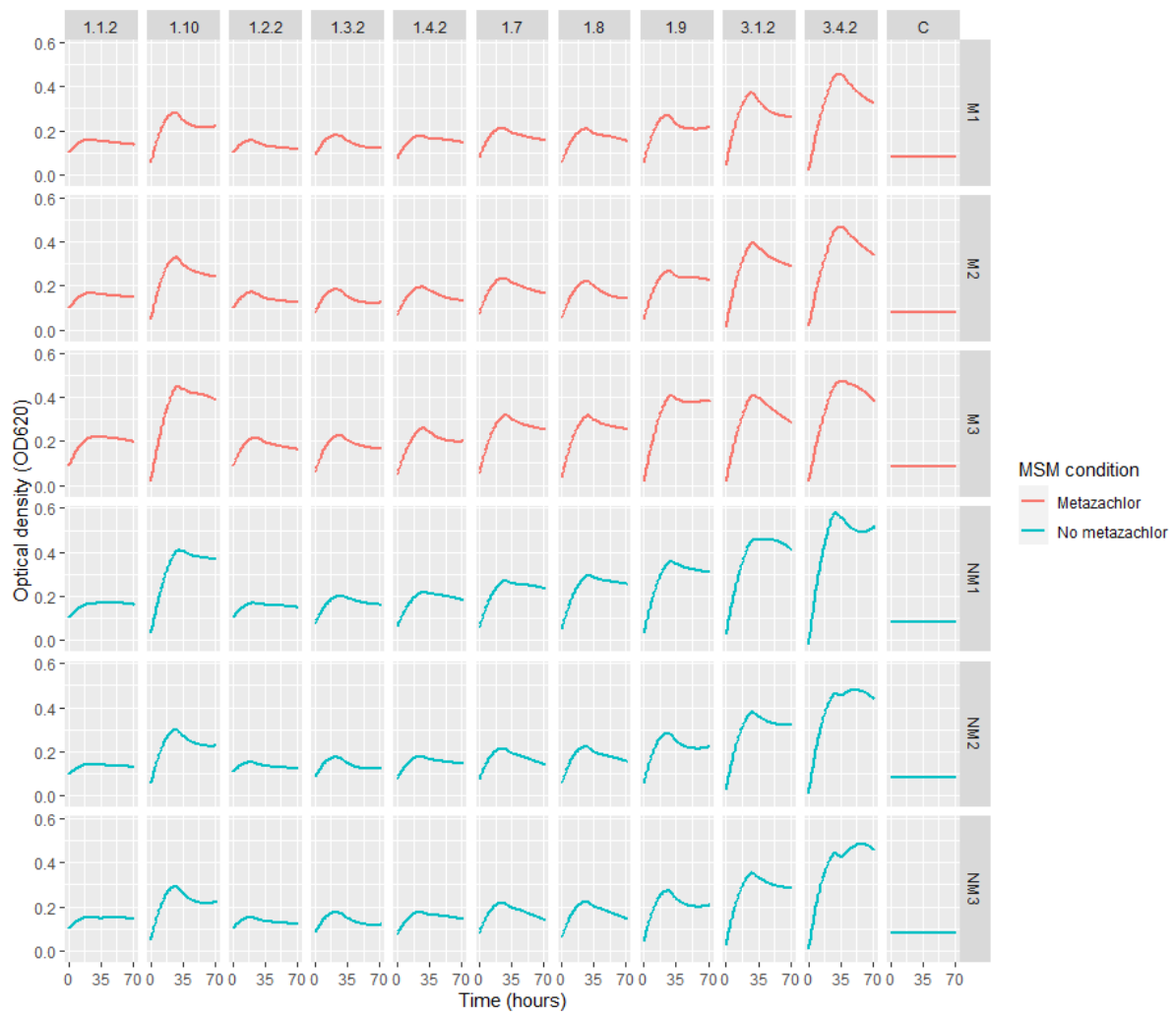**6.4 Trimmed 16S rRNA gene amplicon sequences for 5.1 and 6.6**

>5.1 16S rRNA (1190 bp)

GTCGAGCGGAGTGATGGTGCTTGCACTATCACTTAGCGGCGGACGGGTGAGTAATGCT
TAGGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGGAATGCTAATACCGCATACG
TCCTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCG
GATTAGCTAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAG
AGGATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAG
AAGGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTGAAGTTAATACCTT
CAGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCG
GTAATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGCGCGCGTAGGCG
GCTAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGG
TTAGCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGA
GATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGT
GCGAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGAT
GTCTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGA
CCGCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCA
CAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTG
ACATAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCT
GCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCA
ACCCTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACTTTAAGGATACTGCCAGTGAC
AAACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTA
CACACGTGCTACAATGGTCGGTACAAAGGTTT

> 6.6 16S rRNA (1240 bp)

CGAGCGGAGTGATGGTGCTTGCACTATCACTTAGCGGCGGACGGGTGAGTAATGCTTA
GGAATCTGCCTATTAGTGGGGGACAACATTTCGAAAGGAATGCTAATACCGCATACGTC
CTACGGGAGAAAGCAGGGGATCTTCGGACCTTGCGCTAATAGATGAGCCTAAGTCAAA
TTAGATAGTTGGTGGGGTAAAGGCCTACCAAGGCGACGATCTGTAGCGGGTCTGAGAG
GATGATCCGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAA
GGCCTTATGGTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTGAAGTTAATACCTTCA
GATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCCGCGGT
AATACAGAGGGTGCAAGCGTTAATCGGATTTACTGGGCGTAAAGCGCGCGTAGGCGGC
TAATTAAGTCAAATGTGAAATCCCCGAGCTTAACTTGGGAATTGCATTCGATACTGGTTA
GCTAGAGTGTGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGAT
CTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAACACTGACGCTGAGGTGC
GAAAGCATGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCATGCCGTAAACGATGT
CTACTAGCCGTTGGGGCCTTTGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACC
GCCTGGGGAGTACGGTCGCAAGACTAAAACTCAAATGAATTGACGGGGGCCCGCACAA
GCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACA
TAGTAAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTTACATACAGGTGCTGCA
TGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACC
CTTTTTCCTTATTTGCCAGCGAGTAATGTCGGGAACTTTAAGGATACTGCCAGTGACAA
ACTGGAGGAAGGCGGGGACGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACA
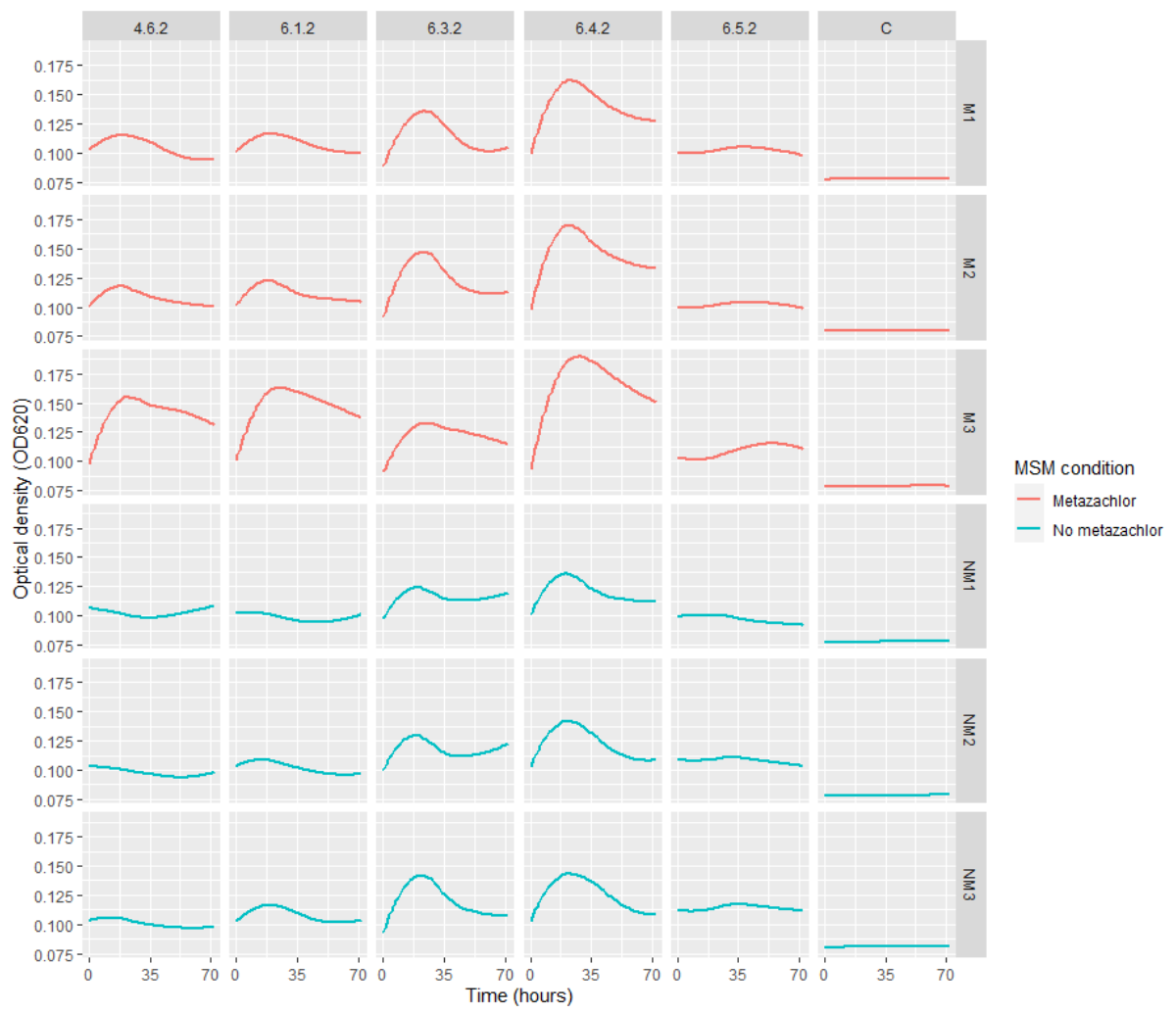CACGTGCTACAATGGTCGGTACAAAGGGTTGCTACCTAGCGATAGGATGCTAATCTCAA
AAAGCCGATCGTAGTCCGG

6.5 Initial triplicated growth curves for candidate metazachlor-degrading strains 4.2.2, 4.5.2 and 6.4.2 grown in metazachlor-supplemented and non-supplemented MSM
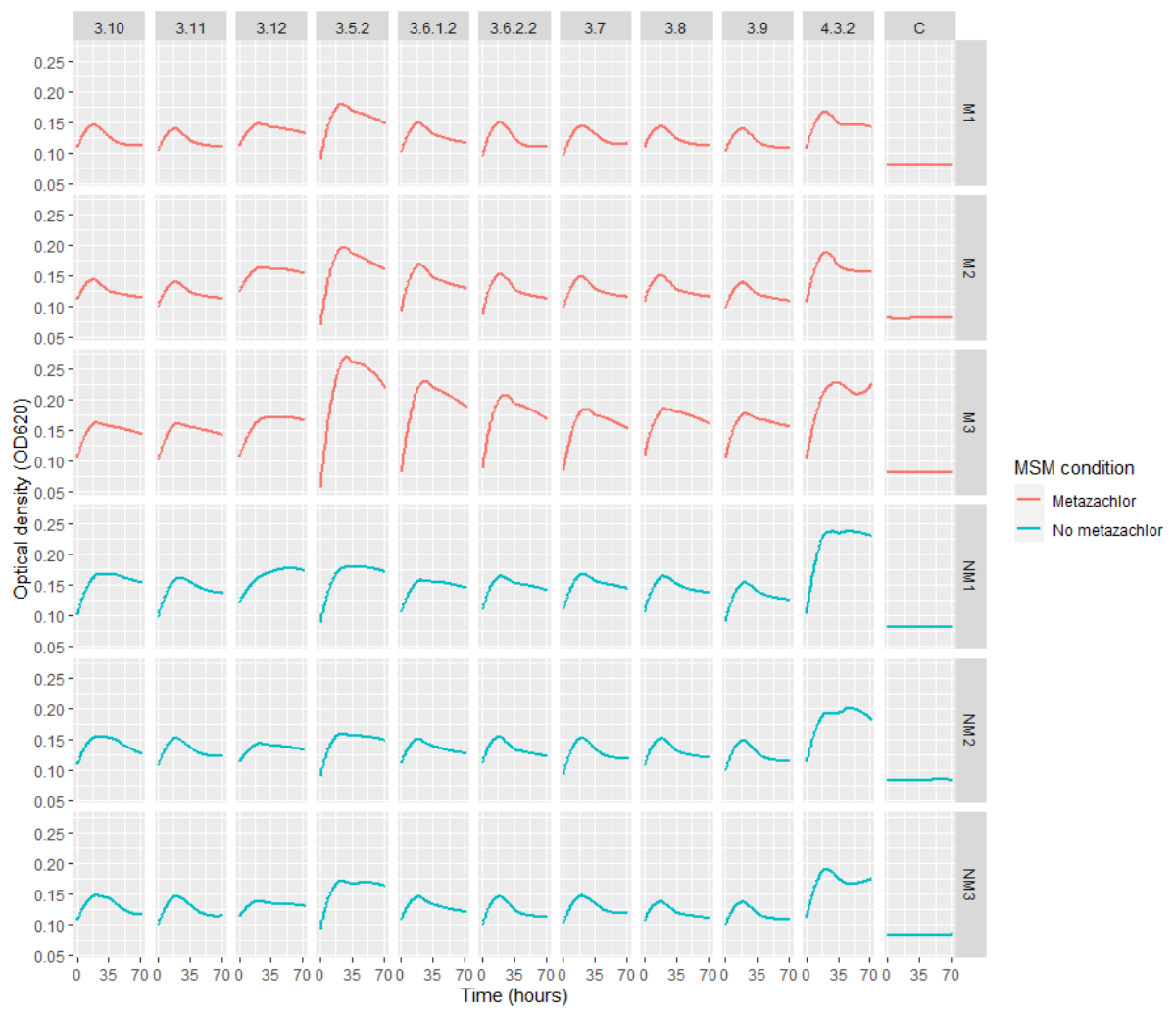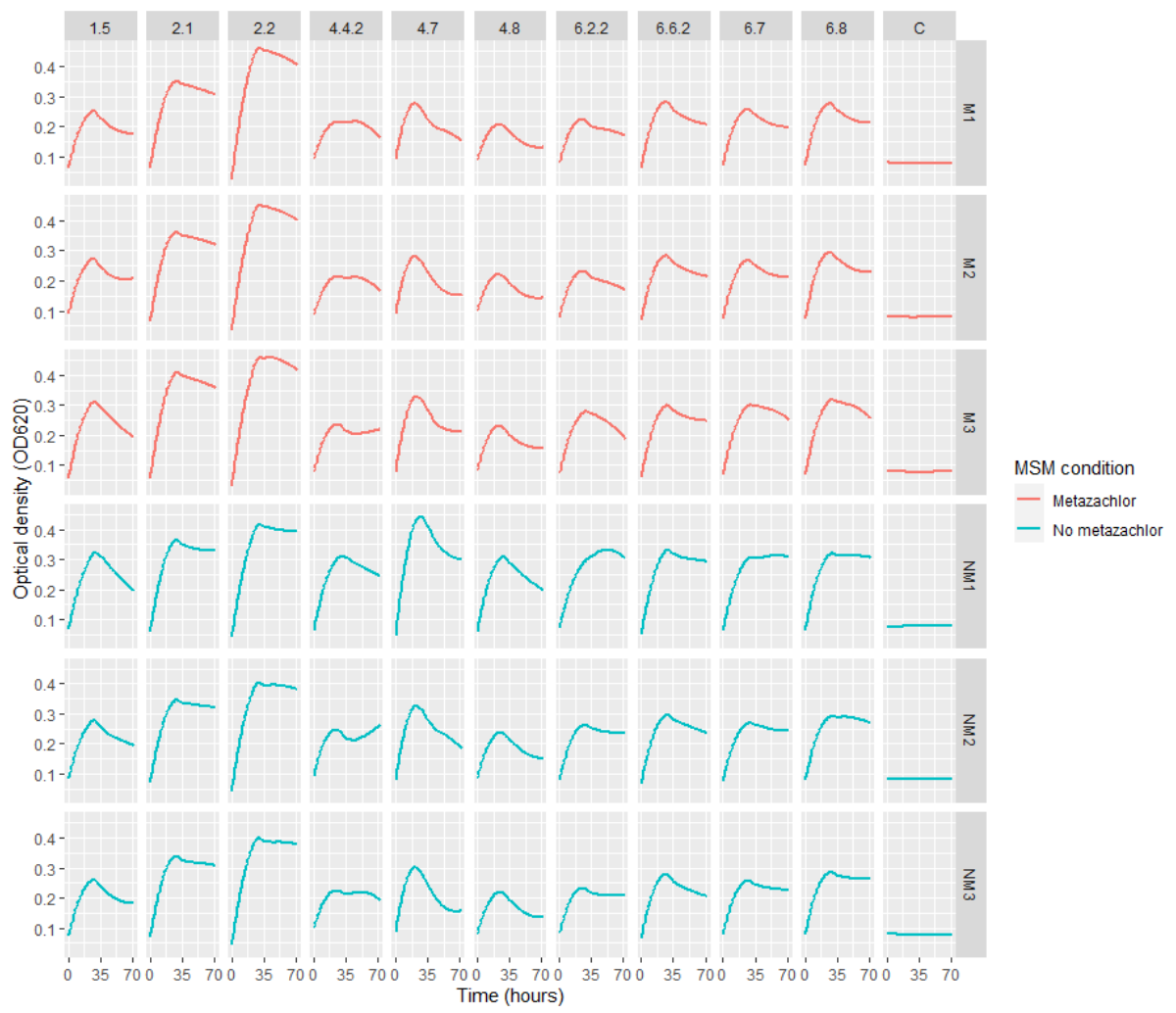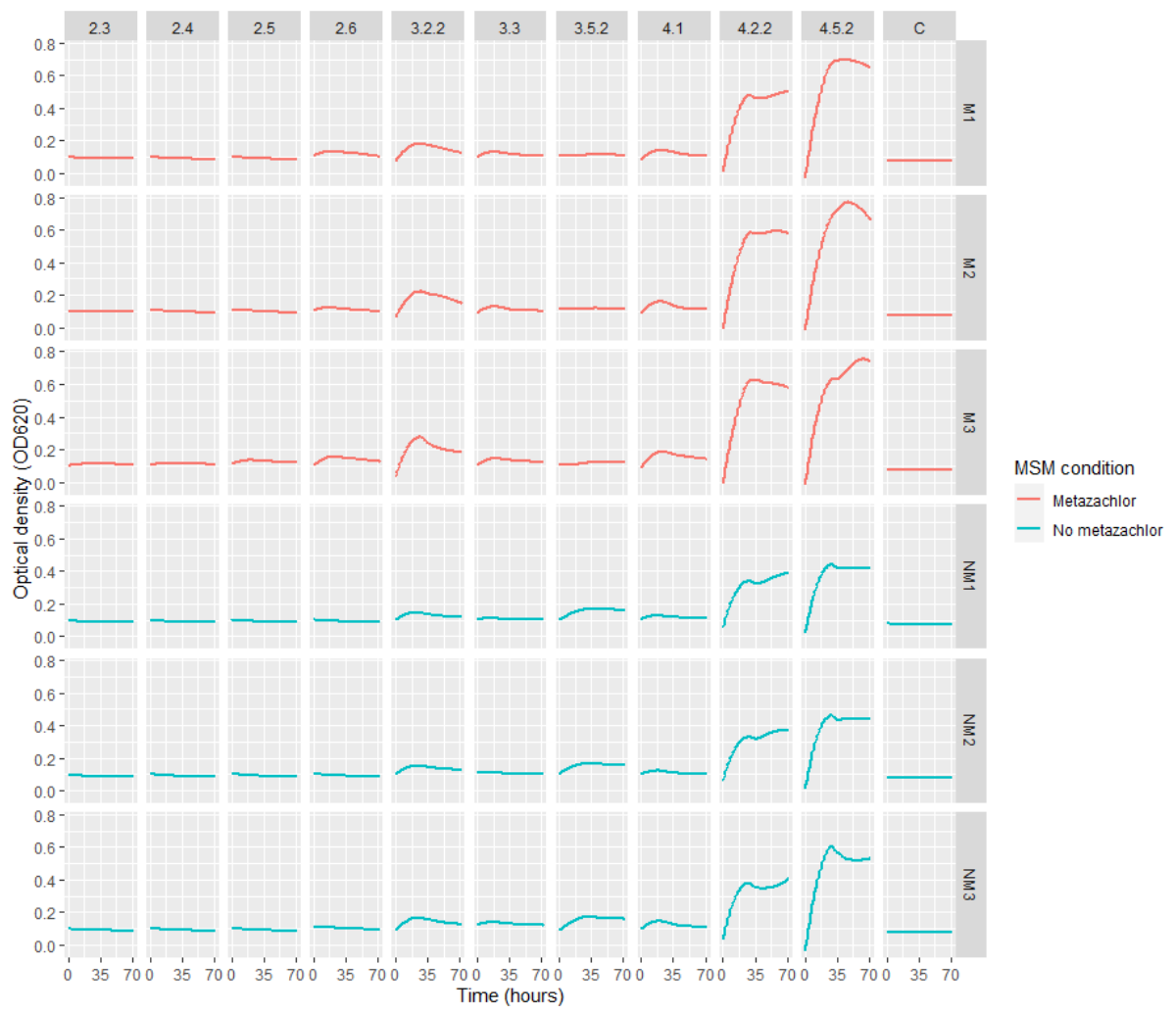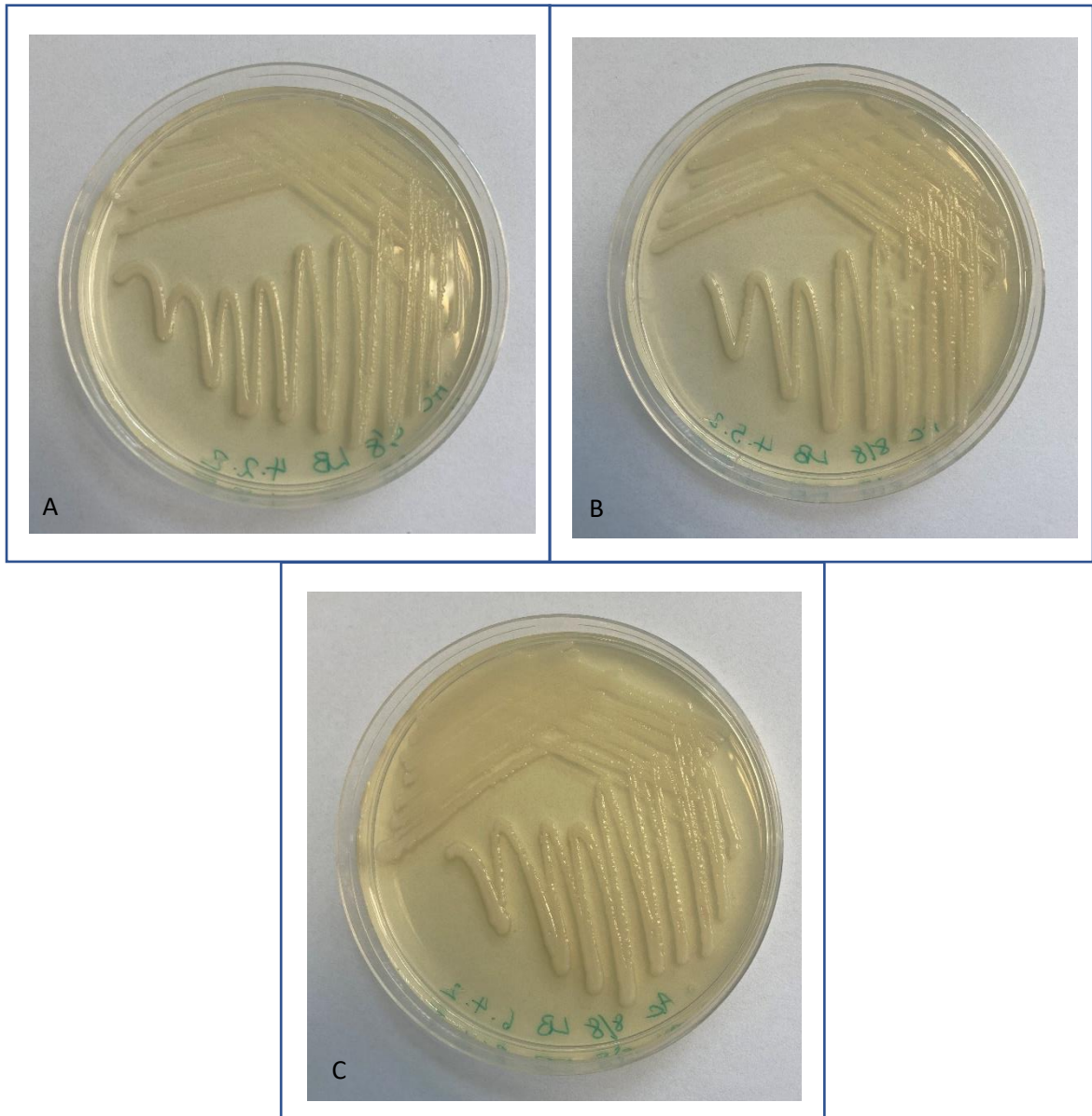
6.6 4.2.2 (panel A), 4.5.2 (panel B) and 6.4.2 (panel C) grown on solid LB agar

6.7 Trimmed 16S rRNA gene amplicons for strains 5.1, 6.6, 4.2.2 4.5.2 and 6.4.2

>4.2.2 16S rRNA (1265 bp)

GCAGTCGAGCGGATGAGAAGAGCTTGCTCTTCGATTCAGCGGCGGACGGGTGAGTAA
TGCCTAGGAATCTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGC
ATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTA
GGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT
GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCGAAT
ACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC
GCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG
GTGGTTTGTTAAGTTGAATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAA
CTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG
TAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGT
TGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCC
TTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAG
CGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCG
GTGACAAACCGGAAGAAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTG
GGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAGCCGCGAGGTGGAGC
TAATCTCACAAAACCGATCGTAGTCCGGATCGCAGTCTGCAACTCGACT

>4.5.2 16S rRNA (1205 bp)

GGATGAGAAGAGCTTGNTCTTCGATTCAGCGGCGGACGGGTGAGTAATGCCTAGGAAT
CTGCCTGGTAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGCATACGTCCTAC
GGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTAGGTCGGATTA
GCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGTCTGAGAGGAT
GATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCCGCAGTGGG
GAATATTGGACAATGGGCGAAAGCCTGATCCANCCATGCCGCGTGCGTGAAGAAGNNG
TTTCGATTGTAAAGCACTTTTTNNTTGGGNNNNNNNGGGNNNNNNNNNNNNNAATATTGC
TGGTTTGTTGTTTNNNACACAATAAAANACACGCTAACTCTGTGGNAACAGNNCGNGCT
CCCACAAAAAGGGGGAGGTTAATCGGAATTANCTGGGNNNTATAAAACNCCGGNAGGG
ATGTNNTATTATGATTGTGAAAGACCCCGGNNCCCACCCNNGGGANNNGCCTCCAAAC
NGGNNNNANNNNAGAGTACNGTAGAGGGTGGTGGAATTTCCTGTTNTAGGNGTGAANN
NGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGAC
ACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCC
GTAAACGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCAT
TAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGG
GCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCA
GGCCTTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACA
CAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAA
CGAGCGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACT
GCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGG
CCTGGGCTACACACGTGCTACAATGGTCGGTACAAAGGGTTGCCAA

>6.4.2 16S rRNA (1201 bp)

GCAGTCGAGCGGATGAGAAGAGCTTGCTCTTCGATTCAGCGGCGGACGGGTGAGTAA
TGCCTAGGAATCTGCCTGATAGTGGGGGACAACGTTTCGAAAGGAACGCTAATACCGC
ATACGTCCTACGGGAGAAAGCAGGGGACCTTCGGGCCTTGCGCTATCAGATGAGCCTA
GGTCGGATTAGCTAGTTGGTGGGGTAATGGCTCACCAAGGCGACGATCCGTAACTGGT
CTGAGAGGATGATCAGTCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGC
AGCAGTGGGGAATATTGGACAATGGGCGAAAGCCTGATCCAGCCATGCCGCGTGTGT
GAAGAAGGTCTTCGGATTGTAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGCGAAT
ACCTTGCTGTTTTGACGTTACCGACAGAATAAGCACCGGCTAACTCTGTGCCAGCAGCC
GCGGTAATACAGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCGCGTAG
GTGGTTTGTTAAGTTGAATGTGAAAGCCCCGGGCTCAACCTGGGAACTGCATCCAAAA
CTGGCAAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATGCG
TAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACTGACACTG
AGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAA
CGATGTCAACTAGCCGTTGGAATCCTTGAGATTTTAGTGGCGCAGCTAACGCATTAAGT
TGACCGCCTGGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCC
GCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGCGAAGAACCTTACCAGGCC
TTGACATGCAGAGAACTTTCCAGAGATGGATTGGTGCCTTCGGGAACTCTGACACAGG
TGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGTAACGAG
CGCAACCCTTGTCCTTAGTTACCAGCACGTTATGGTGGGCACTCTAAGGAGACTGCCG
GTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCTG
GGCTACACACGTGCTACAATGGTCGGTACAGAGGGTTGCCAAG

6.8 Genome assembly graphs for strains 5.1 (panel A), 6.6 (panel B) and *A. calcoaceticus* RUH 2202 (panel C).
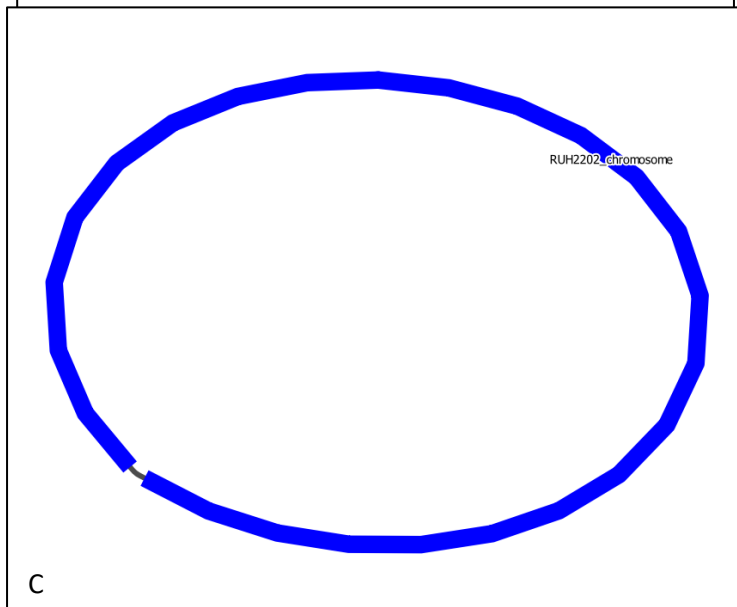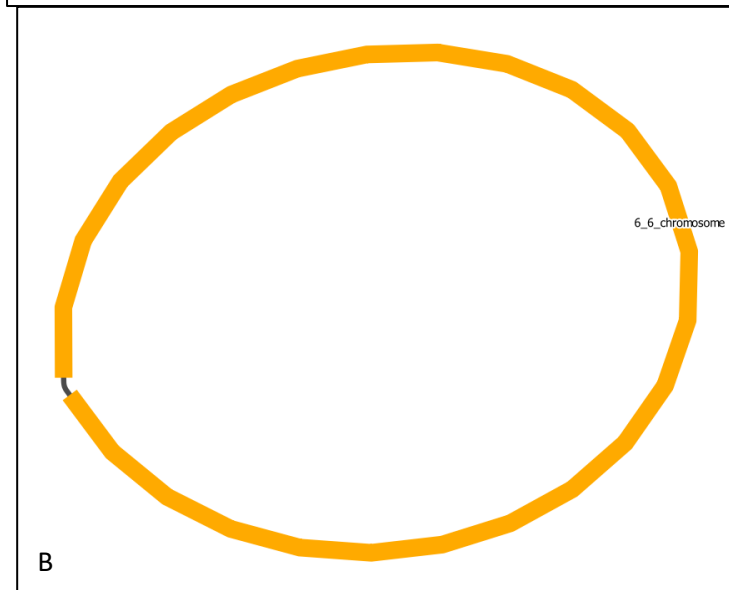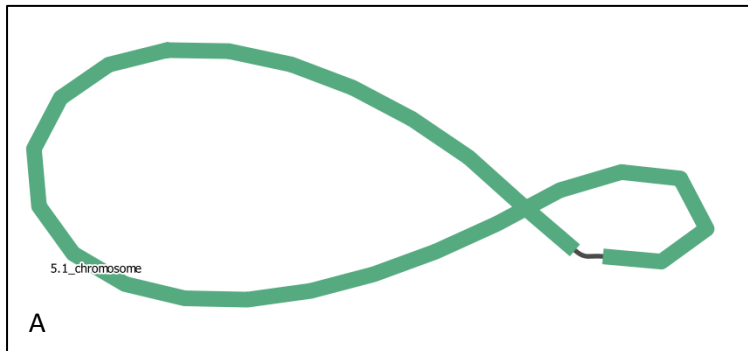
6.9 Taxonomic distributions of sequence read-mapping for 5.1, 6.6 and *A. calcoaceticus* RUH 2202

Strain	Most frequent genus	Most frequent genus (%)	2nd most frequent genus	2nd most frequent genus (%)	Most frequent species	Most frequent species (%)
5.1	*Acinetobacter*	97.82	*Klebsiella*	0.06	*Acinetobacter calcoaceticus*	85.89
6.6	*Acinetobacter*	97.59	*Klebsiella*	0.05	*Acinetobacter calcoaceticus*	85.44
RUH 2202	*Acinetobacter*	97.78	*Klebsiella*	0.06	*Acinetobacter calcoaceticus*	85.84

6.10 Taxonomic distributions of Illumina sequence read-mapping for 4.2.2, 4.5.2 and 6.4.2

Strain	Unclassified (%)	Most frequent genus	Most frequent genus (%)	2nd most frequent genus	2nd most frequent genus (%)	Most frequent species	Most frequent species (%)
4.2.2	8.84	*Pseudomonas*	90.22	*Aeromonas*	0.05	*Pseudomonas putida*	75.69%
4.5.2	15.72	*Pseudomonas*	82.90	*Streptomyces*	0.05	*Pseudomonas putida*	59.66%
6.4.2	7.47	*Pseudomonas*	91.51	*Streptomyces*	0.03	*Pseudomonas putida*	74.07%

Abbreviations

2OG	2-oxoglutarate
Acetyl-CoA	Acetyl-coenzyme A
ANI	Average nucleotide identity
ATP	Adenosine triphosphate
BAP	Biofilm-associated protein
BLAST	Basic Local Alignment Search Tool
BLASTN	Nucleotide BLAST
BLASTP Protein BLAST	Protein BLAST
CDD	Conserved Domain Database
CD-Search	Conserved Domain Search
CLP	Classification, Labelling and Packaging
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DT_{50}	Dissipation time
EMS	Ethyl methanesulfonate
EFSA	European Food Safety Authority
EUDWD	European Drinking Water Directive
GC%	Percentage guanine-cytosine
Ig	Immunoglobulin
K_{foc}	Organic-carbon normalized Freundlich distribution coefficient
K_{oc}	Organic carbon-water partition coefficient
LB	Lysogeny broth
LCB	Locally collinear block
LD50	Median lethal dose
MGE	mobile genetic element
MLSA	Multilocus sequence analysis
MPF	Mating pair formation
MSM	Minimal salts media
NAD(P)+	Nicotinamide adenine dinucleotide phosphate
NCBI	National Center for Biotechnology Information

OD	Optical density
OD_{600nm}	Optical density at 600nm
OD_{620nm}	Optical density at 620nm
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucelic acid
rRNA	Ribosomal RNA
SNP	Single nucleotide polymorphism
tmRNA	Transfer messenger RNA
tRNA	Transfer RNA
VLCFA	Very long chain fatty acid
WHO	World Health Organisation

References

Abraham, J. and Silambarasan, S., 2013. Biodegradation of chlorpyrifos and its hydrolyzing metabolite 3, 5, 6-trichloro-2-pyridinol by Sphingobacterium sp. JAS3. Process Biochemistry, 48(10), pp.1559-1564.

Ahmad, K.S., 2020. Environmental contaminant 2-chloro-N-(2, 6-diethylphenyl)-N-(methoxymethyl) acetamide remediation via Xanthomonas axonopodis and Aspergillus niger. Environmental research, 182, p.109117.

Aktar, W., Sengupta, D. and Chowdhury, A., 2009. Impact of pesticides use in agriculture: their benefits and hazards. Interdisciplinary toxicology, 2(1), pp.1-12.

Albers, C.N., Feld, L., Ellegaard-Jensen, L. and Aamand, J., 2015. Degradation of trace concentrations of the persistent groundwater pollutant 2, 6-dichlorobenzamide (BAM) in bioaugmented rapid sand filters. Water research, 83, pp.61-70.

Aldas-Vargas, A., van der Vooren, T., Rijnaarts, H.H. and Sutton, N.B., 2021. Biostimulation is a valuable tool to assess pesticide biodegradation capacity of groundwater microorganisms. Chemosphere, 280, p.130793.

Allocati, N., Favaloro, B., Masulli, M., Alexeyev, M.F. and Di Ilio, C., 2003. Proteus mirabilis glutathione S-transferase B1-1 is involved in protective mechanisms against oxidative and chemical stresses. Biochemical Journal, 373(1), pp.305-311.

Arbeli, Z. and Fuentes, C.L., 2007. Accelerated biodegradation of pesticides: An overview of the phenomenon, its basis and possible solutions; and a discussion on the tropical dimension. Crop protection, 26(12), pp.1733-1746.

Arca, P., Rico, M., Brana, A.F., Villar, C.J., Hardisson, C. and Suarez, J.E., 1988. Formation of an adduct between fosfomycin and glutathione: a new mechanism of antibiotic resistance in bacteria. Antimicrobial agents and chemotherapy, 32(10), pp.1552-1556.

Arredondo-Alonso, S., Willems, R.J., Van Schaik, W. and Schürch, A.C., 2017. On the (im) possibility of reconstructing plasmids from whole-genome short-read sequencing data. Microbial genomics, 3(10).

Asfaw, A., Maher, K. and Shucksmith, J.D., 2018. Modelling of metaldehyde concentrations in surface waters: A travel time based approach. Journal of Hydrology, 562, pp.397-410.

Ashton, P.M., Nair, S., Dallman, T., Rubino, S., Rabsch, W., Mwaigwisya, S., Wain, J. and O'grady, J., 2015. MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nature biotechnology, 33(3), pp.296-300.

Autin, O., Hart, J., Jarvis, P., MacAdam, J., Parsons, S.A. and Jefferson, B., 2013. The impact of background organic matter and alkalinity on the degradation of the pesticide metaldehyde by two advanced oxidation processes: UV/H2O2 and UV/TiO2. Water research, 47(6), pp.2041-2049.

Badische Anilin und Soda Fabrik 2020. Metazachlor and Quinmerac Water Stewardship [online]. Available at: https://www.agricentre.basf.co.uk/en/Sustainability/Water-Stewardship/Metazachlor-and-Quinmerac-Stewardship/ [accessed 14th December 2022].

Balashova, N., Hiscock, K.M., Reid, B.J. and Reynolds, R., 2021. Trends in metaldehyde concentrations and fluxes in a lowland, semi-agricultural catchment in the UK (2008–2018). Science of the Total Environment, 795, p.148858.

Beulke, S. and Malkomes, H.P., 2001. Effects of the herbicides metazachlor and dinoterb on the soil microflora and the degradation and sorption of metazachlor under different environmental conditions. Biology and Fertility of Soils, 33(6), pp.467-471.

Bieri, M., 2003. The environmental profile of metaldehyde. In BCPC Symposium Proceedings (pp. 255-262). British Crop Protection Council.

Bighiu, M.A. and Goedkoop, W., 2021. Interactions with freshwater biofilms cause rapid removal of common herbicides through degradation–evidence from microcosm studies. Environmental Science: Processes & Impacts, 23(1), pp.66-72.

Böger, P., 2003. Mode of action for chloroacetamides and functionally related compounds. Journal of Pesticide Science, 28(3), pp.324-329.

Bolger, A.M., Lohse, M. and Usadel, B., 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. Bioinformatics, 30(15), pp.2114-2120.

Bourne, N.B., Jones, G.W. and Bowen, I.D., (1988). Slug feeding behaviour in relation to control with molluscicidal baits. Journal of Molluscan Studies, 54(3), pp.327-338.

Busson, A., Boulanger, M., Meryet-Figuière, M., Perrier, S., Monnereau, A., Baldi, I., Tual, S. and Lebailly, P., 2021. O-406 Exposure to chloroacetamide herbicides in agriculture and Lymphoid Malignancies, overall and by subtypes, in the AGRIculture and CANcer (AGRICAN) cohort. Occupational and Environmental Medicine, 78(Suppl 1), pp.A37-A37.

Cabezón, E., Ripoll-Rozada, J., Peña, A., De La Cruz, F. and Arechaga, I., 2015. Towards an integrated model of bacterial conjugation. FEMS microbiology reviews, 39(1), pp.81-95.

Carver, T., Harris, S.R., Berriman, M., Parkhill, J. and McQuillan, J.A., 2012. Artemis: an integrated platform for visualization and analysis of high-throughput sequence-based experimental data. Bioinformatics, 28(4), pp.464-469.

Carver, T., Thomson, N., Bleasby, A., Berriman, M. and Parkhill, J., 2009. DNAPlotter: circular and linear interactive genome visualization. Bioinformatics, 25(1), pp.119-120.

Castro-Gutiérrez, V., 2020. Microbial Adaptation for the Degradation of Metaldehyde. University of York.

Castro-Gutiérrez, V., Fuller, E., Thomas, J.C., Sinclair, C.J., Johnson, S., Helgason, T., and Moir, J.W.B. 2020. Genomic basis for pesticide degradation revealed by selection, isolation and characterisation of a library of metaldehyde-degrading strains from soil. Soil Biology and Biochemistry 142: 107702

Castro-Gutiérrez, V., Fuller, E., Garcillán-Barcia, M.P., Helgason, T., Hassard, F. and Moir, J., 2022a. Dissemination of metaldehyde catabolic pathways is driven by mobile genetic elements in Proteobacteria. Microbial Genomics, 8(10), p.000881.

Castro-Gutiérrez, V.M., Pickering, L., Cambronero-Heinrichs, J.C., Holden, B., Haley, J., Jarvis, P., Jefferson, B., Helgason, T., Moir, J.W. and Hassard, F., 2022b. Bioaugmentation of pilot-scale slow sand filters can achieve compliant levels for the micropollutant metaldehyde in a real water matrix. Water research, 211, p.118071.

Chaudhry, G.R., Ali, A.N. and Wheeler, W.B., 1988. Isolation of a methyl parathion-degrading *Pseudomonas* sp. that possesses DNA homologous to the opd gene from a Flavobacterium sp. Applied and Environmental Microbiology, 54(2), pp.288-293.

Chen, Q., Wang, C.H., Deng, S.K., Wu, Y.D., Li, Y., Yao, L., Jiang, J.D., Yan, X., He, J. and Li, S.P., 2014. Novel three-component Rieske non-heme iron oxygenase system catalyzing the N-dealkylation of chloroacetanilide herbicides in sphingomonads DC-6 and DC-2. Applied and environmental microbiology, 80(16), pp.5078-5085.

Cheng, M., Meng, Q., Yang, Y., Chu, C., Chen, Q., Li, Y., Cheng, D., Hong, Q., Yan, X. and He, J., 2017. The two-component monooxygenase MeaXY initiates the downstream pathway of chloroacetanilide herbicide catabolism in sphingomonads. Applied and environmental microbiology, 83(7), pp.e03241-16.

Chief Inspector of Drinking Water, 2017. Drinking Water 2016 – Public Water Supplies for England and Wales, Quarter 3, July-September 2016.

Copley, S.D., 2000. Evolution of a metabolic pathway for degradation of a toxic xenobiotic: the patchwork approach. Trends in biochemical sciences, 25(6), pp.261-265.

Dalton, H. and Stirling, D.I., 1982. Co-metabolism. Philosophical Transactions of the Royal Society of London. B, Biological Sciences, 297(1088), pp.481-496.

Darling, A.E., Mau, B. and Perna, N.T., 2010. progressiveMauve: multiple genome alignment with gene gain, loss and rearrangement. PloS one, 5(6), p.e11147.

Dauner, M., Sonderegger, M., Hochuli, M., Szyperski, T., Wüthrich, K., Hohmann, H.P., Sauer, U. and Bailey, J.E., 2002. Intracellular carbon fluxes in riboflavin-producing Bacillus subtilis during growth on two-carbon substrate mixtures. Applied and Environmental Microbiology, 68(4), pp.1760-1771.

De Maio, N., Shaw, L.P., Hubbard, A., George, S., Sanderson, N.D., Swann, J., Wick, R., AbuOun, M., Stubberfield, E., Hoosdally, S.J. and Crook, D.W., 2019. Comparison of long-read sequencing technologies in the hybrid assembly of complex bacterial genomes. *Microbial genomics*, *5*(9).

Department for Environment, Food & Rural Affairs (DEFRA), 2020. Outdoor use of

metaldehyde to be banned to protect wildlife [online]. Available at:
https://www.gov.uk/government/news/outdoor-use-of-metaldehyde-to-be-banned-to-protectwildlife [accessed; 31st November 2021].

de Vries, J. and Wackernagel, W., 2002. Integration of foreign DNA during natural transformation of *Acinetobacter* sp. by homology-facilitated illegitimate recombination. Proceedings of the National Academy of Sciences, 99(4), pp.2094-2099.

Diancourt, L., Passet, V., Nemec, A., Dijkshoorn, L. and Brisse, S., 2010. The population structure of *Acinetobacter* baumannii: expanding multiresistant clones from an ancestral susceptible genetic pool. PloS one, 5(4), p.e10034.

Don, R.T. and Pemberton, J.M., 1981. Properties of six pesticide degradation plasmids isolated from Alcaligenes paradoxus and Alcaligenes eutrophus. Journal of bacteriology, 145(2), pp.681-686.

Dröge, M., Pühler, A. and Selbitschka, W., 1999. Horizontal gene transfer among bacteria in terrestrial and aquatic habitats as assessed by microcosm and field studies. Biology and Fertility of Soils, 29(3), pp.221-245.

Dunon, V., Bers, K., Lavigne, R., Top, E.M. and Springael, D., 2018. Targeted metagenomics demonstrates the ecological role of IS1071 in bacterial community adaptation to pesticide degradation. Environmental microbiology, 20(11), pp.4091-4111.

El Fantroussi, S. and Agathos, S.N., 2005. Is bioaugmentation a feasible strategy for pollutant removal and site remediation?. Current opinion in microbiology, 8(3), pp.268-275.

European Food Safety Authority (EFSA), 2008. Conclusion regarding the peer review of the pesticide risk assessment of the active substance metazachlor. EFSA Journal, 6(7), p.145r.

European Food Safety Authority (EFSA), Brancato, A., Brocca, D., Bura, L., Byers, H., Chiusolo, A., Court Marques, D., Crivellente, F., De Lentdecker, C., De Maglie, M. and Egsmose, M., 2017. Peer review of the pesticide risk assessment for the active substance metazachlor in light of confirmatory data submitted. EFSA Journal, 15(6), p.e04833.

European Food Safety Authority (EFSA), Brancato, A., Brocca, D., De Lentdecker, C., Erdos, Z., Ferreira, L., Greco, L., Jarrah, S., Kardassi, D., Leuschner, R. and Lythgo, C., 2018. Modification of the existing maximum residue level for metazachlor in Chinese cabbage. EFSA Journal, 16(1), p.e05127.

Fenner, K., Canonica, S., Wackett, L.P. and Elsner, M., 2013. Evaluating pesticide degradation in the environment: blind spots and emerging opportunities. science, 341(6147), pp.752-758.

Fera Science, 2020. Pesticide usage survey report 291: Outdoor vegetable crops in the United Kingdom 2019 [online]. Available at: https://secure.fera.defra.gov.uk/pusstats/surveys/2019surveys.cfm [accessed 5th December 2021].

Food and Agriculture Organization of the United Nations (1999). FAO specifications and evaluations for plant protection products Metazachlor 2-chloro-N-(pyrazol-1-ylmethyl)acet-2',6'-xylidide [online]. Available at: https://www.fao.org/fileadmin/templates/agphome/documents/Pests_Pesticides/Specs/metazach.pdf [accessed 13th December 2022].

French, K.E., Zhou, Z. and Terry, N., 2020. Horizontal 'gene drives' harness indigenous bacteria for bioremediation. Scientific reports, 10(1), pp.1-11.

Fuentes, M.S., Benimeli, C.S., Cuozzo, S.A. and Amoroso, M.J., 2010. Isolation of pesticide-degrading actinomycetes from a contaminated site: bacterial growth, removal and dechlorination of organochlorine pesticides. International Biodeterioration & Biodegradation, 64(6), pp.434-441.

Gaiarsa, S., Batisti Biffignandi, G., Esposito, E.P., Castelli, M., Jolley, K.A., Brisse, S., Sassera, D. and Zarrilli, R., 2019. Comparative analysis of the two *Acinetobacter* baumannii multilocus sequence typing (MLST) schemes. Frontiers in microbiology, 10, p.930.

Garbisu, C., Garaiyurrebaso, O., Epelde, L., Grohmann, E. and Alkorta, I., 2017. Plasmid-mediated bioaugmentation for the bioremediation of contaminated soils. Frontiers in microbiology, 8, p.1966.

Gautam, S.K., Sharma, R., Ahmad, A.H. and Thakur, I.S., 2003. Evaluation of pentachlorophenol-degrading potentiality of Pseudomonas sp. in a soil microcosm. World Journal of Microbiology and Biotechnology, 19, pp.73-78.

Girard, L., Lood, C., Höfte, M., Vandamme, P., Rokni-Zadeh, H., van Noort, V., Lavigne, R. and De Mot, R., 2021. The ever-expanding *Pseudomonas* genus: description of 43 new species and partition of the *Pseudomonas* putida group. Microorganisms, 9(8), p.1766.

de Gregorio, E., Del Franco, M., Martinucci, M., Roscetto, E., Zarrilli, R. and Di Nocera, P.P., 2015. Biofilm-associated proteins: news from *Acinetobacter*. BMC genomics, 16(1), pp.1-14.

Griesser, U.J., Weigand, D., Rollinger, J.M., Haddow, M. and Gstrein, E., 2004. The crystal polymorphs of metazachlor. Journal of thermal analysis and calorimetry, 77(2), pp.511-522.

Goldstein, S., Beka, L., Graf, J. and Klassen, J.L., 2019. Evaluation of strategies for the assembly of diverse bacterial genomes using MinION long-read sequencing. BMC genomics, 20(1), pp.1-17.

Gomila, M., Peña, A., Mulet, M., Lalucat, J. and García-Valdés, E., 2015. Phylogenomics and systematics in *Pseudomonas*. Frontiers in microbiology, 6, p.214.

Goodwin, S., McPherson, J.D. and McCombie, W.R., 2016. Coming of age: ten years of next-generation sequencing technologies. Nature Reviews Genetics, 17(6), pp.333-351.

Guillaume, B., 2021. Evaluation of pesticides degradation by microorganisms in the soil of a new cropping system: Isolation and characterization of pesticide-degrading strains of soil microorganisms.

Harding, C.M., Pulido, M.R., Di Venanzio, G., Kinsella, R.L., Webb, A.I., Scott, N.E., Pachón, J. and Feldman, M.F., 2017. Pathogenic *Acinetobacter* species have a functional type I secretion system and contact-dependent inhibition systems. Journal of biological chemistry, 292(22), pp.9075-9087.

Hill, K.E. and Top, E.M., 1998. Gene transfer in soil systems using microcosms. FEMS Microbiology Ecology, 25(4), pp.319-329.

Hou, Y., Dong, W., Wang, F., Li, J., Shen, W., Li, Y. and Cui, Z., 2014. Degradation of acetochlor by a bacterial consortium of R hodococcus sp. T3-1, Delftia sp. T3-6 and S phingobium sp. MEA 3-1. Letters in applied microbiology, 59(1), pp.35-42.

Huang, Y., Xiao, L., Li, F., Xiao, M., Lin, D., Long, X. and Wu, Z., 2018. Microbial degradation of pesticide residues and an emphasis on the degradation of cypermethrin and 3-phenoxy benzoic acid: a review. *Molecules*, *23*(9), p.2313.

Huber, A., Bach, M. and Frede, H.G., 2000. Pollution of surface waters with pesticides in Germany: modeling non-point source inputs. Agriculture, ecosystems & environment, 80(3), pp.191-204.

Ihlenfeldt, W.D., Bolton, E.E. and Bryant, S.H., 2009. The PubChem chemical structure sketcher. Journal of cheminformatics, 1(1), pp.1-9.

Irzyk, G.P. and Fuerst, E.P., 1993. Purification and characterization of a glutathione S-transferase from benoxacor-treated maize (Zea mays). Plant Physiology, 102(3), pp.803-810.

Ishag, A.E.S., Abdelbagi, A.O., Hammad, A.M., Elsheikh, E.A., Elsaid, O.E., Hur, J.H. and Laing, M.D., 2016. Biodegradation of chlorpyrifos, malathion, and dimethoate by three strains of bacteria isolated from pesticide-polluted soils in Sudan. Journal of agricultural and food chemistry, 64(45), pp.8491-8498.

Jain, C., Rodriguez-R, L.M., Phillippy, A.M., Konstantinidis, K.T. and Aluru, S., 2018. High throughput ANI analysis of 90K prokaryotic genomes reveals clear species boundaries. Nature communications, 9(1), pp.1-8.

Jin, Y., Gan, G., Yu, X., Wu, D., Zhang, L., Yang, N., Hu, J., Liu, Z., Zhang, L., Hong, H. and Yan, X., 2017. Isolation of viable but non-culturable bacteria from printing and dyeing wastewater bioreactor based on resuscitation promoting factor. Current microbiology, 74(7), pp.787-797.

Johnsborg, O., Eldholm, V. and Håvarstein, L.S., 2007. Natural genetic transformation: prevalence, mechanisms and function. Research in microbiology, 158(10), pp.767-778.

Joutey, N.T., Bahafid, W., Sayel, H. and El Ghachtouli, N., 2013. Biodegradation: involved microorganisms and genetically engineered microorganisms. Biodegradation-life of science, 1, pp.289-320.

Jurčíková, J., Mikula, P., Dobšíková, R., Némethová, D. and Svobodová, Z., 2007. Effects of metazachlor on vitellogenin induction in zebrafish (Danio rerio). Acta Veterinaria Brno, 76(8), pp.61-66.

Kaminski, M.A., Sobczak, A., Dziembowski, A. and Lipinski, L., 2019. Genomic analysis of γ-hexachlorocyclohexane-degrading Sphingopyxis lindanitolerans WS5A3p strain in the context of the pangenome of Sphingopyxis. Genes, 10(9), p.688.

Kanonenberg, K., Spitz, O., Erenburg, I.N., Beer, T. and Schmitt, L., 2018. Type I secretion system—it takes three and a substrate. FEMS microbiology letters, 365(11), p.fny094.

Karier, P., Kraus, G. and Kolber, I., 2017. Metazachlor traces in the main drinking water reservoir in Luxembourg: a scientific and political discussion. Environmental Sciences Europe, 29(1), pp.1-8.

Katagi, T., 2010. Bioconcentration, bioaccumulation, and metabolism of pesticides in aquatic organisms. Reviews of environmental contamination and toxicology, pp.1-132.

Kay, P. and Grayson, R., 2014. Using water industry data to assess the metaldehyde pollution problem. Water and Environment Journal, 28(3), pp.410-417.

Kerle, E.A., Jenkins, J.J. and Vogue, P.A., 1994. Understanding pesticide persistence and mobility for groundwater and surface water protection.

Kim, M., Oh, H.S., Park, S.C. and Chun, J., 2014. Towards a taxonomic coherence between average nucleotide identity and 16S rRNA gene sequence similarity for species demarcation of prokaryotes. International journal of systematic and evolutionary microbiology, 64(Pt_2), pp.346-351.

Kingsford, C., Schatz, M.C. and Pop, M., 2010. Assembly complexity of prokaryotic genomes using short reads. BMC bioinformatics, 11(1), pp.1-11.

Kong, L., Zhu, S., Zhu, L., Xie, H., Wei, K., Yan, T., Wang, J., Wang, J., Wang, F. and Sun, F., 2014. Colonization of Alcaligenes faecalis strain JBW4 in natural soils and its detoxification of endosulfan. *Applied microbiology and biotechnology*, *98*(3), pp.1407-1416.

Kreuger, J., 1998. Pesticides in stream water within an agricultural catchment in southern Sweden, 1990–1996. Science of the Total Environment, 216(3), pp.227-251.

Lapworth, D.J., Crane, E.J., Stuart, M.E., Talbot, J.C., Besien, T. and Civil, W., 2018. Micro-organic contaminants in groundwater in England: summary results from the Environment Agency LC-MS and GC-MS screening data.

Lawrence, J.G. and Ochman, H., 1997. Amelioration of bacterial genomes: rates of change and exchange. Journal of molecular evolution, 44(4), pp.383-397.

Laue, H., Field, J.A. and Cook, A.M., 1996. Bacterial desulfonation of the ethanesulfonate metabolite of the chloroacetanilide herbicide metazachlor. Environmental science & technology, 30(4), pp.1129-1132.

Lewis, K.A., Tzilivakis, J., Warner, D. and Green, A., 2016 An international database for pesticide risk assessments and management. Human and Ecological Risk Assessment: An International Journal, 22(4), 1050-1064.

Li, H., 2013. Aligning sequence reads, clone sequences and assembly contigs with BWA-MEM. arXiv preprint arXiv:1303.3997.

Liu, H.M., Cao, L., Lu, P., Ni, H., Li, Y.X., Yan, X., Hong, Q. and Li, S.P., 2012. Biodegradation of butachlor by Rhodococcus sp. strain B1 and purification of its hydrolase (ChlH) responsible for N-dealkylation of chloroacetamide herbicides. Journal of agricultural and food chemistry, 60(50), pp.12238-12244.

Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., Marth, G., Abecasis, G. and Durbin, R., 2009. The sequence alignment/map format and SAMtools. Bioinformatics, 25(16), pp.2078-2079.

Li, W., Raoult, D. and Fournier, P.E., 2009. Bacterial strain typing in the genomic era. FEMS microbiology reviews, 33(5), pp.892-916.

Loehfelm, T.W., Luke, N.R. and Campagnari, A.A., 2008. Identification and characterization of an *Acinetobacter baumannii* biofilm-associated protein. Journal of bacteriology, 190(3), pp.1036-1044.

Longstreth Jr, W.T. and Pierson, D.J., (1982). Metaldehyde poisoning from slug bait ingestion. Western Journal of Medicine, 137(2), p.134.

Maiden, M.C., 2006. Multilocus sequence typing of bacteria. Annu. Rev. Microbiol., 60, pp.561-588.

Madeira, F., Pearce, M., Tivey, A.R., Basutkar, P., Lee, J., Edbali, O., Madhusoodanan, N., Kolesnikov, A. and Lopez, R., 2022. Search and sequence analysis tools services from EMBL-EBI in 2022. *Nucleic acids research*, *50*(W1), pp.W276-W279.

Marchler-Bauer, A., Derbyshire, M.K., Gonzales, N.R., Lu, S., Chitsaz, F., Geer, L.Y., Geer, R.C., He, J., Gwadz, M., Hurwitz, D.I. and Lanczycki, C.J., 2015. CDD: NCBI's conserved domain database. Nucleic acids research, 43(D1), pp.D222-D226.

Marchler-Bauer, A. and Bryant, S.H., 2004. CD-Search: protein domain annotations on the fly. Nucleic acids research, 32(suppl_2), pp.W327-W331.

Matthes, B. and Böger, P., 2002. Chloroacetamides affect the plasma membrane. Zeitschrift für Naturforschung C, 57(9-10), pp.843-852.

Miguel, A.B.R., Jetten, M.S. and Welte, C.U., 2020. The role of mobile genetic elements in organic micropollutant degradation during biological wastewater treatment. Water Research X, 9, p.100065.

Mishra, S., Lin, Z., Pang, S., Zhang, W., Bhatt, P. and Chen, S., 2021. Recent advanced technologies for the characterization of xenobiotic-degrading microorganisms and microbial communities. Frontiers in Bioengineering and Biotechnology, 9, p.632059.

Mohr, S., Feibicke, M., Berghahn, R., Schmediche, R. and Schmidt, R., 2008. Response of plankton communities in freshwater pond and stream mesocosms to the herbicide metazachlor. Environmental Pollution, 152(3), pp.530-542.

Nawaz, M.S., Khan, A.A., Seng, J.E., Leakey, J.E., Siitonen, P.H. and Cerniglia, C.E., 1994. Purification and characterization of an amidase from an acrylamide-degrading Rhodococcus sp. Applied and Environmental Microbiology, 60(9), pp.3343-3348.

Navarro, S., Vela, N. and Navarro, G., 2007. An overview on the environmental behaviour of pesticide residues in soils. Spanish journal of agricultural research, 5(3), pp.357-375.

National Center for Biotechnology Information (NCBI). PubChem Compound Summary for CID 176, Acetic Acid [online]. Available at: https://pubchem.ncbi.nlm.nih.gov/compound/Acetic-Acid. [Accessed 15th June 2023].

Neilson, A.H., 1990. The biodegradation of halogenated organic compounds. Journal of Applied Bacteriology, 69(4), pp.445-470.

Nemec, A., Krizova, L., Maixnerova, M., van der Reijden, T.J., Deschaght, P., Passet, V., Vaneechoutte, M., Brisse, S. and Dijkshoorn, L., 2011. Genotypic and phenotypic characterization of the *Acinetobacter calcoaceticus–Acinetobacter* baumannii complex with the proposal of *Acinetobacter* pittii sp. nov.(formerly *Acinetobacter* genomic species 3) and *Acinetobacter* nosocomialis sp. nov.(formerly *Acinetobacter* genomic species 13TU). Research in microbiology, 162(4), pp.393-404.

Nielsen, K.M., Bones, A.M. and Van Elsas, J.D., 1997. Induced natural transformation of *Acinetobacter calcoaceticus* in soil microcosms. Applied and Environmental Microbiology, 63(10), pp.3972-3977.

Nielsen, K.M., Smalla, K. and van Elsas, J.D., 2000. Natural transformation of *Acinetobacter* sp. strain BD413 with cell lysates of *Acinetobacter* sp., *Pseudomonas* fluorescens, and Burkholderia cepacia in soil microcosms. Applied and environmental microbiology, 66(1), pp.206-212.

Nohynek, L. J., Suhonen, E. L., Nurmiaho-Lassila, E.-L., Hantula, J. & Salkinoja-Salonen, M. (1995). Description of four pentachlorophenol-degrading bacterial strains as Sphingomonas chlorophenolica sp. nov. Syst Appl Microbiol 18, 527–538.

Nzila, A., 2013. Update on the cometabolism of organic pollutants by bacteria. Environmental Pollution, 178, pp.474-482.

Palmen, R. and Hellingwerf, K.J., 1997. Uptake and processing of DNA by *Acinetobacter calcoaceticus*–a review. *Gene*, *192*(1), pp.179-190.

Pauling, L. and Carpenter, D.C., (1936). The crystal structure of metaldehyde. Journal of the American Chemical Society, 58(7), pp.1274-1278.

Pham, V.H. and Kim, J., 2012. Cultivation of unculturable soil bacteria. *Trends in biotechnology*, *30*(9), pp.475-484.

Quinlan, A.R. and Hall, I.M., 2010. BEDTools: a flexible suite of utilities for comparing genomic features. Bioinformatics, 26(6), pp.841-842.

Ravenhall, M., Škunca, N., Lassalle, F. and Dessimoz, C., 2015. Inferring horizontal gene transfer. PLoS computational biology, 11(5), p.e1004095

Ravintheran, S.K., Sivaprakasam, S., Loke, S., Lee, S.Y., Manickam, R., Yahya, A., Croft, L., Millard, A., Parimannan, S. and Rajandas, H., 2019. Complete genome sequence of Sphingomonas paucimobilis AIMST S2, a xenobiotic-degrading bacterium. Scientific data, 6(1), pp.1-6.

Raymond, J.W., Rogers, T.N., Shonnard, D.R. and Kline, A.A., 2001. A review of structure-based biodegradation estimation methods. Journal of hazardous materials, 84(2-3), pp.189-215.

Richaume, A.G.N.E.S., Angle, J.S. and Sadowsky, M.J., 1989. Influence of soil variables on in situ plasmid transfer from *Escherichia coli* to Rhizobium fredii. Applied and environmental microbiology, 55(7), pp.1730-1734.

Rodriguez-R, L.M. and Konstantinidis, K.T., 2014. Bypassing cultivation to identify bacterial species. Microbe, 9(3), pp.111-118.

Roesch, L.F., Fulthorpe, R.R., Riva, A., Casella, G., Hadwin, A.K., Kent, A.D., Daroub, S.H., Camargo, F.A., Farmerie, W.G. and Triplett, E.W., 2007. Pyrosequencing enumerates and contrasts soil microbial diversity. The ISME journal, 1(4), pp.283-290.

Röling, W.F. and van Verseveld, H.W., 2002. Natural attenuation: what does the subsurface have in store?. Biodegradation, 13(1), pp.53-64.

RStudio Team (2016). RStudio: Integrated Development for R. RStudio, Inc., Boston, MA. Available at: http://www.rstudio.com/

Ruiz-Perez, C.A., Conrad, R.E. and Konstantinidis, K.T., 2021. MicrobeAnnotator: a user-friendly, comprehensive functional annotation pipeline for microbial genomes. BMC bioinformatics, 22, pp.1-16.

Sarkar, S., Gil, J.D.B., Keeley, J. and Jansen, K., 2021. The use of pesticides in developing countries and their impact on health and the right to food. European Union.

Schenk, T., Müller, R. and Lingens, F., 1990. Mechanism of enzymatic dehalogenation of pentachlorophenol by Arthrobacter sp. strain ATCC 33790. Journal of bacteriology, 172(12), pp.7272-7274.

Schimel, J.P. and Schaeffer, S.M., 2012. Microbial control over carbon cycling in soil. Frontiers in microbiology, 3, p.348.

Seemann, T., 2014. Prokka: rapid prokaryotic genome annotation. Bioinformatics, 30(14), pp.2068-2069.

Sharma, I., 2020. Bioremediation techniques for polluted environment: concept, advantages, limitations, and prospects. In Trace Metals in the Environment-New Approaches and Recent Advances. IntechOpen.

Sharma, S., 2012. Bioremediation: features, strategies and applications. Asian Journal of Pharmacy and Life Science, 2231, p.4423.

Simpanen, S., Dahl, M., Gerlach, M., Mikkonen, A., Malk, V., Mikola, J. and Romantschuk, M., 2016. Biostimulation proved to be the most efficient method in the comparison of in situ soil remediation treatments after a simulated oil spill accident. Environmental Science and Pollution Research, 23(24), pp.25024-25038.

Sparling, D.W. and Fellers, G., 2007. Comparative toxicity of chlorpyrifos, diazinon, malathion and their oxon derivatives to larval Rana boylii. Environmental Pollution, 147(3), pp.535-539.

Spencer, P.S. and Barral, J.M., 2012. Genetic code redundancy and its influence on the encoded polypeptides. Computational and structural biotechnology journal, 1(1), p.e201204006.toxic

Staley, J.T. and Konopka, A., 1985. Measurement of in situ activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. Annual review of microbiology, 39(1), pp.321-346.

Takeuchi, M., Hamana, K. and Hiraishi, A., 2001. Proposal of the genus Sphingomonas sensu stricto and three new genera, *Sphingobium*, Novo*Sphingobium* and Sphingopyxis, on the basis of phylogenetic and chemotaxonomic analyses. International Journal of Systematic and Evolutionary Microbiology, 51(4), pp.1405-1417.

Tao, B. and Fletcher, A.J., 2013. Metaldehyde removal from aqueous solution by adsorption and ion exchange mechanisms onto activated carbon and polymeric sorbents. Journal of hazardous materials, 244, pp.240-250.

Thomas, J.C., 2016. The molecular basis for the biotic degradation of metaldehyde. University of York.

Thomas, J.C., Helgason, T., Sinclair, C.J., and Moir, J.W.B. 2017. Isolation and characterization of metaldehyde-degrading bacteria from domestic soils. Microbial Biotechnology 10: 1824–1829.

Top, E.M. and Springael, D., 2003. The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. Current opinion in biotechnology, 14(3), pp.262-269.

Triebskorn, R., Christensen, K. and Heim, G., (1998). Effects of orally and dermally applied metaldehyde on mucus cells of slugs (Deroceras reticulatum) depending on temperature and
duration of exposure. Journal of Molluscan Studies, 64(4), pp.467-487.

Ulrich, U., Hörmann, G., Unger, M., Pfannerstill, M., Steinmann, F. and Fohrer, N., 2018. Lentic small water bodies: Variability of pesticide transport and transformation patterns. Science of The Total Environment, 618, pp.26-38.

Ulrich, U., Pfannerstill, M., Ostendorp, G. and Fohrer, N., 2021. Omnipresent distribution of herbicides and their transformation products in all water body types of an agricultural landscape in the North German Lowland. Environmental Science and Pollution Research, 28(32), pp.44183-44199.

van der Werf, H.M., 1996. Assessing the impact of pesticides on the environment. Agriculture, Ecosystems & Environment, 60(2-3), pp.81-96.

Van Eerd, L.L., Hoagland, R.E., Zablotowicz, R.M. and Hall, J.C., 2003. Pesticide metabolism in plants and microorganisms. *Weed science, 51*(4), pp.472-495.an Eerd, L.L., Hoagland, R.E., Zablotowicz, R.M. and Hall, J.C., 2003. Pesticide metabolism in plants and microorganisms. *Weed science, 51*(4), pp.472-495.

Vartoukian, S.R., Palmer, R.M. and Wade, W.G., 2010. Strategies for culture of 'unculturable' bacteria. FEMS microbiology letters, 309(1), pp.1-7.

Velisek, J., Stara, A., Kubec, J., Zuskova, E., Buric, M. and Kouba, A., 2020. Effects of metazachlor and its major metabolite metazachlor OA on early life stages of marbled crayfish. Scientific Reports, 10(1), pp.1-9.

Verma, H., Kumar, R., Oldach, P., Sangwan, N., Khurana, J.P., Gilbert, J.A. and Lal, R., 2014. Comparative genomic analysis of nine *Sphingobium* strains: insights into their evolution and hexachlorocyclohexane (HCH) degradation pathways. BMC genomics, 15(1), pp.1-19.

Vidali, M., 2001. Bioremediation. an overview. Pure and applied chemistry, 73(7), pp.1163-1172.

Vijaya, L.P.S.D. and Venkateswarlu, K., 2008. Isolation and characterization of a chlorpyrifos-degrading bacterium from agricultural soil and its growth response. African Journal of Microbiology Research, 2(2), pp.26-31.

Vishniac, W. and Santer, M., 1957. The thiobacilli. Bacteriological reviews, 21(3), pp.195-213.

Wang, Y., Lai, A., Latino, D., Fenner, K. and Helbling, D.E., 2018. Evaluating the environmental parameters that determine aerobic biodegradation half-lives of pesticides in soil with a multivariable approach. Chemosphere, 209, pp.430-438.

Waterhouse, A.M., Procter, J.B., Martin, D.M., Clamp, M. and Barton, G.J., 2009. Jalview Version 2—a multiple sequence alignment editor and analysis workbench. Bioinformatics, 25(9), pp.1189-1191.

Wheeler, D.L., Barrett, T., Benson, D.A., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., DiCuccio, M., Edgar, R., Federhen, S. and Feolo, M., 2007. Database resources of the national center for biotechnology information. Nucleic acids research, 36(suppl_1), pp.D13-D21.

Whitman, W.B., Coleman, D.C. and Wiebe, W.J., 1998. Prokaryotes: the unseen majority. *Proceedings of the National Academy of Sciences, 95*(12), pp.6578-6583.

Wick, R.R., Judd, L.M., Gorrie, C.L. and Holt, K.E., 2017. Unicycler: resolving bacterial genome assemblies from short and long sequencing reads. PLoS computational biology, 13(6), p.e1005595.

Wick, R.R., Schultz, M.B., Zobel, J. and Holt, K.E., 2015. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics*, *31*(20), pp.3350-3352.

Wiemann, C., Goettel, M., Vardy, A., Elcombe, B.M., Elcombe, C.R., Chatham, L.R., Wang, H., Li, L., Buesen, R., Honarvar, N. and Treumann, S., 2019. Metazachlor: Mode of action analysis for rat liver tumour formation and human relevance. *Toxicology*, *426*, p.152282.

Wijewardene, L., Wu, N., Hörmann, G., Messyasz, B., Riis, T., Hölzel, C., Ulrich, U. and Fohrer, N., 2021. Effects of the herbicides metazachlor and flufenacet on phytoplankton communities–A microcosm assay. Ecotoxicology and Environmental Safety, 228, p.113036.

Winter, M., Buckling, A., Harms, K., Johnsen, P.J. and Vos, M., 2021. Antimicrobial resistance acquisition via natural transformation: context is everything. Current Opinion in Microbiology, 64, pp.133-138.

Wood, D.E. and Salzberg, S.L., 2014. Kraken: ultrafast metagenomic sequence classification using exact alignments. Genome biology, 15(3), pp.1-12.

World Health Organisation (WHO), 2019. The WHO recommended classification of pesticides by hazard and guidelines to classification 2019 [online]. Available at: https://www.who.int/publications/i/item/9789240005662 [accessed 7th December 2021].

Xu, J., Qiu, X., Dai, J., Cao, H., Yang, M., Zhang, J. and Xu, M., 2006. Isolation and characterization of a *Pseudomonas* oleovorans degrading the chloroacetamide herbicide acetochlor. Biodegradation, 17(3), pp.219-225.

Yu, N.Y., Wagner, J.R., Laird, M.R., Melli, G., Rey, S., Lo, R., Dao, P., Sahinalp, S.C., Ester, M., Foster, L.J. and Brinkman, F.S., 2010. PSORTb 3.0: improved protein subcellular localization prediction with refined localization subcategories and predictive capabilities for all prokaryotes. Bioinformatics, 26(13), pp.1608-1615.

Zablotowicz, R.M., Hoagland, R.E., Locke, M.A. and Hickey, W.J., 1995. Glutathione-S-transferase activity and metabolism of glutathione conjugates by rhizosphere bacteria. Applied and Environmental Microbiology, 61(3), pp.1054-1060.

Zablotowicz, R.M., Locke, M.A., Hoagland, R.E., Knight, S.S. and Cash, B., 2001. Fluorescent *Pseudomonas* isolates from Mississippi Delta oxbow lakes: in vitro herbicide biotransformations. Environmental Toxicology: An International Journal, 16(1), pp.9-19.

Zhang, J., Zheng, J.W., Liang, B., Wang, C.H., Cai, S., Ni, Y.Y., He, J. and Li, S.P., 2011. Biodegradation of chloroacetamide herbicides by Paracoccus sp. FLY-8 in vitro. Journal of Agricultural and Food Chemistry, 59(9), pp.4614-4621.