Environmental Fate Assessments to Understand the Legacy of Metaldehyde in Agricultural Fields and Surface Water

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Abstract

There are increasing concerns about the hazards posed to drinking water resources by persistent, mobile and toxic (PMT) substances in the environment. For example, the extensive use of metaldehyde-based molluscicide to control slug populations in agricultural fields has frequently led to pollution of surface waters and contamination of drinking water at levels exceeding the statutory limit. Regulatory environmental fate assessments and studies in the literature did not predict that metaldehyde would be persistent in the environment, contrary to observations from monitoring schemes. To understand the reasons for this disparity, this study conducted a suite of degradation experiments, covering different soil types and environmentally realistic conditions, and generated a distribution of DT_{50} values for metaldehyde to examine whether degradation rates are underestimated by current risk assessments. The results were found to vary, showing a range of DT_{50} values (3.6-4150 d), which indicated that metaldehyde had the potential to become persistent, subject to high soil moisture conditions. Additionally, leaching and dissipation assessments were conducted in lysimeters, using representative soils to understand the legacy of metaldehyde in the field. Metaldehyde concentrations were detected in leachate and soil after a period of 120 days, suggesting that its persistence in the environment could be greater than the predictions made during the regulatory environmental fate assessments. Lastly, molecular microbiology was employed to elucidate how the soil microbial population characteristics relate to the degradation potential for metaldehyde. Molecular techniques, such as qPCR and MinION sequencing revealed that the known metaldehyde degraders were very rare members of the soil communities. Hence, the trends in metaldehyde degradation may not be wholly attributed to these species and other organisms might be utilising metaldehyde that have yet to be characterised. While this research has identified environmental conditions that may lead to metaldehyde persistence in the field, further research needs to be done to understand the microbiology of metaldehyde degradation in soil. . Improved management strategies can then be developed to prevent the pollution of drinking water with metaldehyde.

Chapter 1: Introduction and Literature Review

1.1 Introduction

Pesticides are an integral part of food production to support an ever-increasing global population, but they must be used and managed properly to avoid harmful effects on human health and the environment. To ensure that possible negative effects on health and the environment are minimised, the regulatory approval process for registering agrochemicals uses stringent testing methods which have been developed over many decades. Until Brexit, pesticide regulations in relation to water quality were determined by EU directives including the Water Framework Directive (2000/60/EC); Priority Substances Directive(2008/105/EC); Amended priority substances (2013/39/EC); Groundwater Directive (2006/118/EC) (2014/80/EU); Drinking Water Directive (98/83/EC, recently supplanted by 2020/2184); Sustainable Use Directive (2009/128/EC) which serve(d) to protect human health and the environment. The ethos of the directives and the underpinning regulatory processes is centred on a cyclic risk assessment of identifying the hazards, assessing risks, taking action to eliminate or reduce any risk and monitoring the effectiveness of the entire process to ensure the regulations are/remain fit-for-purpose. This process should therefore negate compounds coming onto the market that pose an unacceptable risk to the environment.

An illustration of the continual review process is the increasing regulatory activities across Europe to address concerns about persistent, mobile and toxic (PMT) substances in the environment (Rüdel et al., 2020). For example, widespread use of molluscicide has resulted in the active ingredient metaldehyde being frequently detected in drinking water above acceptable levels. Water companies have frequently observed metaldehyde concentrations in drinking water in exceedance of the current statutory limit of 0.1 μg L^{-1} (98/83/EC; UKWIR, 2015). Current methods to remove metaldehyde from drinking water, for example by using existing granular activated carbon treatment methods, are ineffective and/or too expensive to be commercially viable (e.g., Salvestrini et al., 2017; Busquets et al. 2014). This has necessitated catchment-based interventions and modelling of metaldehyde concentrations in surface waters to predict peak concentrations in order to manage abstraction times (e.g., Asfaw et al., 2018; Kay et al., 2014; Lu et al., 2017; Castle et al., 2017). These models rely on input parameters, such as pesticide degradation rates, derived from laboratory experiments. From inspection of the currently accepted half-life of metaldehyde (< 10 days (Lewis et al., 2016)), metaldehyde would not be expected to be persistent. Standard degradation tests used for regulatory risk assessment did not predict that metaldehyde would be a pollution concern (EFSA, 2010). However, these predictions do not agree with monitoring data indicating that the current risk assessment for metaldehyde is flawed. This study aimed to investigate the cause of this disparity forming part of the cyclic risk assessment approach.

Metaldehyde enters water sources primarily as a diffuse pollutant from agricultural fields. As a small and polar molecular (Figure 1) with chemical formula $C_8H_{16}O_4$, molar mass 176.2 g/mol, an octanol-water partitioning coefficient K_{ow} of 0.12 at 20 °C, and 0.188 g L^{-1} at 20 °C water solubility (Castle et al., 2017), metaldehyde readily dissolves in water and has a relatively low binding affinity with soil (Castle, 2018). The chemical properties of metaldehyde are summarised in Table 1. In water and low oxygen environments it remains very stable, and therefore in reservoirs, metaldehyde concentrations do not decrease readily (Castle, 2018). The only significant route by which

metaldehyde is removed from the environment is through biodegradation in soil. This study aimed to examine the mineralisation of metaldehyde in soil in order to elucidate the mechanisms through which this problem compound becomes persistent in the environment, to support improved risk assessments and risk management.

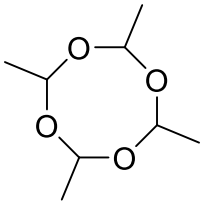

Figure 1: Chemical structure of metaldehyde.

Table 1: Physico-chemical properties of metaldehyde from Castle's review (Castle et al., 2017).

property	value
Molar mass	176.21 g mol^{-1}
CAS number	108-62-3
IUPAC name	2,4,6,8-Tetramethyl-1,3,5,7-tetraoxocane
Boiling point	112 to 115 °C
Water solubility	0.188 g L^{-1} at 20 °C
Vapour pressure	0.66 mmHg at 25 °C
Flash point	36 to 40 °C
Density	1.27 g cm^{-3}
log octanol/water partition coefficient (K_{ow})	0.12 at 20 °C
log organic carbon/water partition coefficient	0.18–0.37

1.2 Detection of metaldehyde in surface water

Metaldehyde was not included in routine monitoring until 2008 (Figure 2) as i) the regulatory risk assessment models predicted an acceptable risk, and ii) there was no reliable, cost-effective method of analysis (e.g. Colbourne, 2013; Castle et al., 2017; Kay et al., 2014). It was not until improvements in analytical methods enabled metaldehyde to be analysed routinely, was it included in screening programmes, and subsequently found to be regularly above the statutory (98/83/EC) level of 0.1 µg L^{-1} in surface waters (Chief Inspector of Drinking Water, 2009). This resulted in water companies being fined for non-compliance and thus metaldehyde rose to prominence as a pollutant of concern for drinking water supplies.

The majority of failures due to pesticides have been the result of metaldehyde pollution (Figure 2), for example, in 2013, 110 of 2269 raw water abstraction points were confirmed to have actual risk of metaldehyde contamination (Colbourne, 2013).

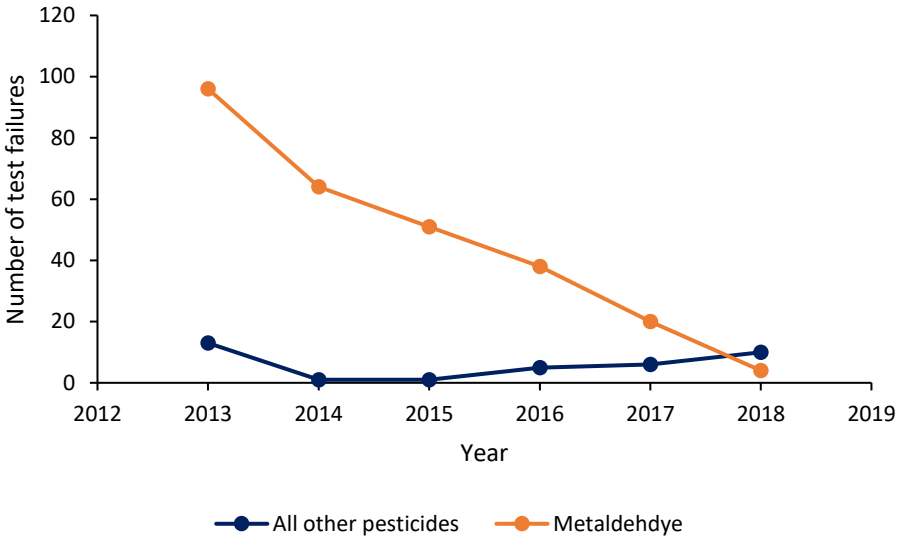

Figure 2: Total number of water quality failures at customers tap (zones) per year in the UK, due to metaldehyde or all other pesticides, compiled from the Drinking Water Inspectorate annual company reports.

The number of samples analysed for all other pesticides far exceeds the number tested for metaldehyde, yet failures due to metaldehyde in the past have represented most drinking water compliance failures. This highlights the significance of the pollution problem presented by metaldehyde. However, since the implementation of management policies, such as the metaldehyde stewardship group in 2008 (MSG, 2008), the number of failures due to metaldehyde have fallen appreciably. This trend would be expected to continue following the ban of metaldehyde use outdoors from April 2022 (DEFRA, 2020).

The prevalence of metaldehyde in surface water led to monitoring initiatives (UKWIR, 2015) and research projects to study the source of the pollution. In a study of the river Leam catchment (Asfaw et al., 2018), auto-samplers installed at a surface water abstraction site collected hourly water samples during storm runoff events, which successfully captured the short-term fluctuations of metaldehyde concentrations. Agriculture was the predominant land use in that study, hence the major influence on river quality.

Water industry monitoring data collected over a two-and-a-half-year period (Kay and Grayson, 2014) was used to quantify the presence of metaldehyde in river and finished waters. These measured concentrations were then compared with the characteristics of the catchment to identify the factors influencing pesticide losses into water. The impact of land use and hydrology of soil types was considered. It was found that eight out of the nine water treatment works found metaldehyde concentrations in excess of the 0.1 μg L^{-1}, with October-December peak values an order of magnitude higher than the regulatory limit, with a maximum value of 2.7 μg L^{-1} (Lu et al., 2017). The study did not observe any trends between catchment characteristics and metaldehyde concentration, except for the catchment comprising 93% permanent grassland having markedly lower peak concentrations of metaldehyde (0.07 μ g L^{-1}). They concluded that metaldehyde losses to water may be influenced by practices carried out on individual farms, such as the type of metaldehyde product used, application rate and timing, and washing of machinery near surface drains.

Consideration for the pollution problems arising from agricultural use of metaldehyde has been presented in review articles (Castle et al., 2017; Castle et al., 2018; Kay and Grayson, 2014, Saad et al., 2017). A review of the environmental fate of metaldehyde (Castle et al., 2017) showed that adsorption to soil organic matter (SOM) was highest four days after application, reported as 1 mg kg^{-1}, and reduced thereafter. Concentrations in soil reduced significantly after 21 days to about 0.04 mg kg^{-1}. Metaldehyde has a low organic carbon-water partition coefficient (log K_{OC} = 0.18-0.37 reported by Castle et al., 2017) which means it has a high mobility in soil (Zhang et al., 2006). Hence metaldehyde has tendency to percolate through soil and into field drains, before entering water courses, where it is persistent. According to Lazartigues et al. (2012), the transfer from field to watercourse can peak at 1-4 days after rainfall. More frequent applications are required to replace pellets that are weathered away by rain (Wilson et al., 2014), which exacerbates the problem.

Production of metaldehyde slug pellets involves two main methods. Dry processed (steamed) are the cheapest but pellets made this way are more fragile (dusty) and disintegrate more easily when wet (Hewson-Fisher, 2015) compared to wet processed pellets that are the most expensive. The manufacturing process does not appear to influence environmental fate (Bond, 2018), as the rate of loss of metaldehyde from the pellet to the environment is the same. In experiments, all the metaldehyde was released from pellets in 7 weeks. Stability of the pellet should not be confused with the release of metaldehyde from the pellet; a disintegrated pellet will continue to sequester the active ingredient, but this is no longer available to the slug, so subsequent applications would be needed, and this may have environmental consequences (Bond, 2018).

1.3 Analytical techniques for the detection of metaldehyde

The preferred analytical techniques used for detecting metaldehyde in water, as summarised in the review by Castle et al. (2017), are gas chromatography (GC) or liquid chromatography (LC) coupled with mass spectrometry (MS). Typically, GC methods use a non-polar stationary phase on which metaldehyde has a short retention time of about 8 min and high oven temperatures of 300 ^{o}C to elute all the analytes, such as dimerised metaldehyde which binds more strongly to the stationary phase. Liquid chromatography methods can analyse a wider range of compounds with low limits of detection, using LC-MS-MS systems, or time-of-flight mass spectrometry. A method using LC-MS-MS was also used by Schumacher et al., (2016), incorporating a methylamine mobile phase additive, coupled with on-line sample enrichment that enabled speedy and sensitive measurement of metaldehyde. Details of analytical techniques for detection of pesticides in water are outlined in a review by Primel et al., 2012. These instruments can be placed at surface water sites to allow rapid detection of metaldehyde and facilitate abstraction. With improved analytical techniques, concentrations of metaldehyde in water can be determined and monitored more accurately. This means that strategies to mitigate metaldehyde contamination of surface water can be assessed more accurately to determine the efficacy of catchment initiatives, which is crucial given that current methods to remove metaldehyde from finished water are too expensive to be commercially viable. Sample preparation may involve solid phase extraction prior to analysis (Environment Agency, 2009).

1.4 Metaldehyde management initiatives

Initiative schemes were put forward for the responsible use of metaldehyde. The metaldehyde stewardship group (The Metaldehyde Stewardship Group (MSG), 2013) put in place guidelines for the use of metaldehyde. It suggests using the minimum amount of active ingredient (ai), with a maximum application rate of 210 g ai ha^{-1}. Advisors may recommend rates lowered to 160 g ai ha^{-1} near water sources. The maximum total dose rate is 700 g ai ha^{-1} yr^{-1}. No pellets are to be spread

within 6 m of a water course, and growers are advised not to apply when heavy rain is forecast, or when drains are flowing.

Catchment management looks at the individual characteristics of the river catchment, such as rainfall, topography, and crop area and pesticide usage (e.g. Asfaw et al., 2018; Ibrahim et al., 2019; Kay and Grayson 2014; Lu, 2017). Metaldehyde tends to be applied in autumn when soils are wetting up and surface drain flow and runoff are high. Individual catchment strategies are needed e.g. catchment sensitive farming. Imaging methods, such as light detection and ranging (LIDAR) allow the elevation of land areas to be mapped and look at crop type and cropping history, as well as weather and soil type to assess the appropriate usage of metaldehyde. It can, therefore, be possible to have catchments where arable land usage is low, but metaldehyde exposure is high, depending on the properties of the catchment. Pesticide load was found to have reduced in monitored river catchments (CSF scheme, Natural England 2014) because of voluntary uptake of best practice by farmers e.g. only apply pellets when trapping thresholds justify it. Industry-led catchment management through the voluntary initiative (VI) is a water quality campaign focused on educating farmers on good practice to minimise pesticide pollution. Water companies also work closely with the farmers, providing them with a financial incentive to use ferric phosphate based molluscicides instead of metaldehyde, and hence reduce the risk of metaldehyde pollution and potential fines for the water company (Yorkshire Water, *pers comm*.)

1.5 Metaldehyde risk to human health and the environment

The toxicological profile of metaldehyde was determined by the European Food Safety Authority and peer review published in 2010 (EFSA 2010). A re-evaluation decision on metaldehyde was also published by Health Canada in 2008 (Health Canada, 2008), and a registration eligibility decision (RED) was published by the US EPA in 2006 (US EPA, 2006). After considerations of the environmental concerns of the products, metaldehyde was deemed eligible to use (US EPA, 2006). Concerns over wildlife poisoning led to the decision to ban outdoor use of metaldehyde in the UK (DEFRA, 2020). Metaldehyde can still be used elsewhere in Europe. A registration report was also produced by the Federal Republic of Germany (Federal Republic of Germany, 2017), which assessed the environmental risks of the product to be low-risk enough for authorisation of the products to be granted.

Most cases of poisoning are from oral administration of high doses i.e., deliberate ingestion of pellets. Levels in drinking water to not pose a risk to human health but are often more than the EC regulation level of 0.1 μg L^{-1} (98/83/EC). Furthermore, the potential level of exposure of humans to the degradation products of metaldehyde in drinking water are very low compared to the presence of acetaldehyde from other sources which can be safely metabolised at low concentrations. The current statutory limit of 0.1 μg L^{-1} (or 0.1 ppb) was set under EC directive based on a precautionary figure due to limited evidence on pesticide toxicity and the aspiration for “pesticide free” drinking water.

1.6 Metaldehyde dissipation in soil

There are few studies detailing the degradation of the pesticide metaldehyde in soil. Metaldehyde has been shown to degrade quickly in representative UK soils (Balashova et al., 2020; Bond, 2018) and fast mineralisation has also been observed in a variety of older studies (e.g., Dong et al. (2017; Zhang et al. (2011); Calumpang et al., (1995)). Pre-exposure to metaldehyde and lighter texture soils appear to favour degradation processes and metaldehyde is degradable in surface and sub-surface horizons within the laboratory setting (Balashova et al., 2020).

Zhang et al. (2011) looked at metaldehyde degradation in cabbage and soil in field studies at three locations; the disappearance half-life (DT_{50}) in soil was determined and ranged between 0.75-1.02 days when applied at two times the recommended high dose. A comparable study is that by Dong et al. (2017), who looked at the dissipation of metaldehyde in pakchoi and soil and found the half-life in soil to be 2.3-2.4 days but that was only assessed for soils at two locations. The characteristic properties of the soil in the fields at the two sites were sandy loam soil, with organic carbon 35.3 g kg^{-1}, cation exchange capacity (CEC) 29.7 C mol^{-1} kg^{-1}, pH 6.8; and the other was a sandy soil, with organic carbon 17.5 g kg^{-1}, pH 7.8, CEC 16.7 C mol^{-1} kg^{-1}. Metaldehyde degradation was found to occur quickly and was not affected by weather. The study did not find any links between degradation and soil properties. In another study, (Calumpang et al., 1995) residue trials to determine the fate of metaldehyde in a rice paddy, using 6 % metaldehyde pellets applied at 4 kg ha^{-1}, were conducted. In water, dissolution of the pellets produced residue levels that remained fairly constant between 1.58-1.47 mg L^{-1} for 1-3 days after application. Residues could only be detected up to 9 days after application, and degradation followed pseudo first-order kinetics, with a half-life of 0.27 days. In soil, metaldehyde deposition from pellets was only apparent three days after application. Residue levels ranged from 0.053-0.127 mg kg^{-1} from day 3, peaking at day 14, before undergoing rapid degradation. It was concluded that metaldehyde residues were not persistent in rice paddy ecosystems. These results are summarised in Table 2. Data in the table shows the parameters measured for various metaldehyde soil studies. These show a range of values in DT_{50} and K_{OC} for metaldehyde between studies.

Table 2: Physico-chemical properties of metaldehyde in soil collated from several literature sources.

Water solubility @ 20 ^{o}C, pH 6.5 (g/L)	**Soil adsorption constant K_{OC} (mL/g)**	**DT_{50} in soil (d)**	**Data source**
0.222	60.4	5.10	EFSA, 2010
-	34-240	3.17-223	Kay and Grayson, 2014
0.190	35	67-223	PAN pesticides database (Kegley et al., 2016)
-	240	5.10	Pesticides properties database. (Lewis et al., 2016)
-	-	2.3-2.4	Dong et al, 2017
-	-	0.75-1.02	Zhang et al, 2011
-	-	0.27	Calumpang et al., 1995
-	75-173	11.9	Safety data sheet (Chiltern Farm chemicals ltd, 2014)
0.230	240	10.0	OSU extension pesticide properties database (Vogue et al., 1994).

Several of the degradation experiments were conducted according to OECD 307, which is environmentally unrealistic. One of the main discrepancies is the use of laboratory-grade metaldehyde, when the active compound is formulated into pellets. To investigate the influence of

pellet formulation versus standard test paradigms, Bond (2018) conducted an OECD 307 degradation experiment to investigate whether degradation would be different for a pelleted metaldehyde product rather than laboratory-grade test compound. One metaldehyde pellet was dosed on the soil in the 100 ml test vessel, which equated to 135 times the recommended dose per hectare. Bond's results revealed that degradation of metaldehyde contained within pellets took slightly longer compared to laboratory grade metaldehyde, with derived DT_{50} values of 3.43 and 3.98 days for wet and dry processed pellets respectively, compared to 1.19 days for laboratory metaldehyde. Metaldehyde recovery was generally low (up to 66.9% for the loam soil and 59.7% for the clay soil). For the majority of the 30-day incubation period, recovery was <30% for all treatments. This included the lab-grade metaldehyde, so low recovery was due to extraction method, not the pellet casing retaining the compound.

There is only a small amount of data in academic literature articles on metaldehyde degradation; often only reporting a few DT_{50} values for different soil types and not providing characterisation of the soils, therefore links between degradation rate and soil properties are difficult to make. More comprehensive studies are those done for government reports, such as the Draft Assessment Report (DAR) on metaldehyde by the European Food Standards Agency (EFSA, 2010) and the Food and Environment Protection Act evaluation on metaldehyde by the Ministry of Agriculture Fisheries and Food (MAFF, 1996).

In soil laboratory incubations under aerobic conditions (Table 3), metaldehyde degrades after a lag phase of up to 19 days (EFSA, 2010). This was an important observation regarding pollution risk, as rainfall in this period could lead to high concentrations of metaldehyde being washed from the field into ditches. It exhibits low persistence, forming no major (> 10 % applied radioactivity, AR) metabolites (EFSA 2010). Mineralisation of the carbon radiolabels to CO_2 accounted for 50-78 % applied radioactivity after 22-60 days. Un-extractable residues accounted for 13-20% AR after 60 days. There was no evidence of pH dependent adsorption. The maximum recommended field application rate, 2 x 7 kg product per ha, corresponding to 2 x 0.35 kg active ingredient (ai) ha^{-1}, with at least 14 days between the two applications, was used to calculate the predicted environmental concentration (PEC) of the active ingredient in soil at various points after application for a soil layer of 5 cm depth, with a bulk soil density of 1.5 g cm^{-3}. This was followed with the metaldehyde degradation studies. The predicted environmental concentration of metaldehyde in soil decreases from 0.933 mg ai kg^{-1} to 0.052 mg ai kg^{-1} within 100 days after application (EFSA, 2010).

Metaldehyde has a very high mobility in soil; as can be interpreted from the soil adsorption constant K_{OC}, but no soil column or lysimeter studies were done for the DAR (EFSA, 2010) to assess leaching. Metaldehyde is essentially stable in anaerobic soil incubations, with only 9.7 % activity loss after 45 days for extractable residues and 4.9 % after 45 days (max 5.4 % at day 30) for non-extractable residues, compared to 50.2-77.7 % after 22-60 days (max 80.4 % at day 21) under aerobic conditions. In laboratory incubations, in dark aerobic natural sediment water systems, metaldehyde exhibited varied persistence (four systems tested). Metaldehyde had low persistence under more oxidising conditions (indicated by a negative soil redox potential) and formed the major metabolite acetaldehyde (max ca 22% AR in water, 5% in sediment) and the acetaldehyde had moderate persistence. In less oxidising systems, metaldehyde exhibited high persistence and no major metabolites were formed. The amount of AR due to mineralised CO_2 for the more oxidising system was 0.6-19 % and for the lower oxidation state system, 62-69 % at end of study. Due to the lag phase of metaldehyde degradation in all soils, degradation kinetics of test substance in soil was determined using a modified hockey stick model (i.e. the first rate constant k_1 was set to zero during the lag phase).

Table 3: Aerobic degradation under standard conditions investigated for four soil types. Degradation rates summarised, modified from EFSA (2010) draft assessment report.

Soil type	OC	pH	DT_{50} (hockey stick) (days)	Duration of lag phase (d)	DT_{50} pseudo-single first order	Chi^2 %
Silt loam	1.2	6.5	19.5	19.0	6.2	2.0
Sandy clay loam	4.2	7.0	11.6	7.5	6.3	9.6
Sandy clay loam,	3.1	6.1	15.9	13.2	6.7	9.3
Sandy loam,	1.0	7.3	6.6	5.8	2.6	7.8

*Incubation temperature of 20 ^{o}C, soil moisture maintained at pf 2.

Aerobic degradation in soil at a more environmentally realistic temperature of 10 °C can be extrapolated from data at 20 °C using the Q_{10}-method developed by the FOCUS group (FOCUS 2007). For the calculation of the DT_{50lab} (10 °C), the DT_{50lab} (20 °C) values are multiplied by the Q_{10} value of 2.58 according to the recommendations of PPR panel (2007), based on the Arrhenius equation. The extrapolated DT_{50lab} (10 °C) values range from 17.0-50.3 days. However, the activation energy used in the Arrhenius equation is based on an average value obtained from a range of plant protection products (Matthies and Beulke, 2017), and might not accurately represent the degradation processes for metaldehyde at lower temperatures.

Adsorption behaviour of metaldehyde was studied in 8 soils (EFSA, 2010) using the batch equilibrium method. K_F values of 0.432-0.977 L Kg^{-1} and calculated K_{FOC} values of 38-149 L Kg^{-1}, indicated very high to high mobility and showed no pH dependency. Freundlich coefficients, 1/n, were determined in the range 0.675-1.023. There was an observed dependency in lag phase on soil type (summarised in Table 4). Lag phase was longest in the silt loam (19 days) and shortest in the sandy loam (5.8 days), each having comparable organic carbon (OC) content of about 1%. The sandy clay had higher OC (4.2% and 3.1%) with differences in lag phase of 7.5 days and 13.2 days, respectively, which indicates that microbes are faster to adapt to metaldehyde in soils richer in OC. The DT_{50}s of the soils were comparable being 6-7 days, except for the sandy loam, which was lower at 2.6 days. Expected reasons for coarser soil to degrade metaldehyde faster could be that the coarser grains have greater spacing as they tessellate thus provide a more aerated environment which encourages aerobic transformations.

Table 4: Adsorption behaviour of metaldehyde in eight soils from studies used in the EFSA draft assessment report (EFSA 2010).

Soil type	OC %	Soil pH	K_F cm^3/g	K_{FOC} cm^3/g	1/n
Sand	0.25	7.4	0.432	173	0.9099
Sandy loam	0.4	6.5	0.644	161	0.9651
Silt loam	1.2	7.1	0.685	57	0.9918
Clay loam	1.3	7.5	0.962	75	0.9953
Humic sand	1.9	5.3	0.735	38	0.974
Sandy loam	1.56	7.7	0.633	40	1.023
Loam	1.45	7.5	0.807	56	0.961
Low humic content sand	0.76	7.8	0.675	78	0.675

In an older study for the Food and Environment Protection Act evaluation on metaldehyde (MAFF 1996), half-lives for degradation are reported for characterised soils. This allows for a certain level of interpretation of the relationships between soil properties and degradation rate. Degradation studies on three soils: sandy loam, silt loam and clay loam were conducted to determine DT_{50} values (Table 5). In a separate aerobic metabolism study on a sandy loam, the measured DT_{50} of metaldehyde was 67.2 days. Anaerobic degradation on the same soil was also studied, following the same guidelines, and found the DT_{50} to be 222 days. So metaldehyde can be classified as very persistent under these laboratory conditions, indicating the potential for persistence in anaerobic field conditions e.g. subsoil, or waterlogging. The study was not expanded to soils under field conditions. Mobility in soil was determined from adsorption-desorption and soil column studies on four soils. Metaldehyde was determined to be mobile and therefore a risk for pollution. In a separate study (MAFF, 1996), DT_{50} was lowest for sandy loam at 1.4 days and higher in clay and silt 6.6 days and 5.2 days, respectively. However, the second sandy loam tested had a longer DT_{50} of 67.2 days. Both degradation and sorption were investigated using non-standard soils, and then leaching was assessed with leaching columns, using standard German soils.

Table 5: DT_{50} and sorption coefficient values determined for the evaluation report on metaldehyde (MAFF 1996) along with characteristics for the soils used in the metaldehyde evaluation report.

Texture class	pH	% sand	%silt	%clay	%OC	Water holding capacity %	DT_{50} (d)	DT_{90} (d)	Organic carbon %	K_d cm^3/g	K_{oc} cm^3/g
Silt loam	6.2	2.2	77.3	20.5	0.73	53	5.2	57	-	-	-
Clay loam	6.7	12.2	56	31.8	1.48	37	6.6	72	-	-	-
Sandy loam	6.2	29.1	46.9	24	0.84	54	1.4	15	-	-	-
Sandy loam	6.5	54	36	10	0.8	-	67.2	-	-	-	-
Sandy loam[1]	6.5	54	36	10	0.8	-	222	-	-	-	-
Sand	7.4	92	4	4	0.5	-	-	-	0.25	0.103	41
Sandy loam	6.5	54	36	10	0.8	-	-	-	0.40	0.223	56
Silt loam	7.1	14	68	18	2.4	-	-	-	1.20	0.175	15
Clay loam	7.5	22	50	28	2.6	-	-	-	1.30	0.436	34

Samples were all field fresh soil

1(anaerobic)

The adsorption behaviour of metaldehyde was studied in eight soils (EFSA, 2010), (Figure 1.3). It is observed that metaldehyde in soils with the lowest organic carbon content has the greatest binding affinity. This is in reverse to what might be expected; more organic carbon might be expected to result in more binding of metaldehyde and a larger K_{OC} value. In the sand and sandy loam, with percentage OC of 0.25% and 0.4%, respectively, the adsorption coefficients are approximately three times larger than those of the soils with a percentage OC greater than 1 %. The effect of pH shows no discernible trend to adsorption, nor does it impact degradation rate, from the data in this study.

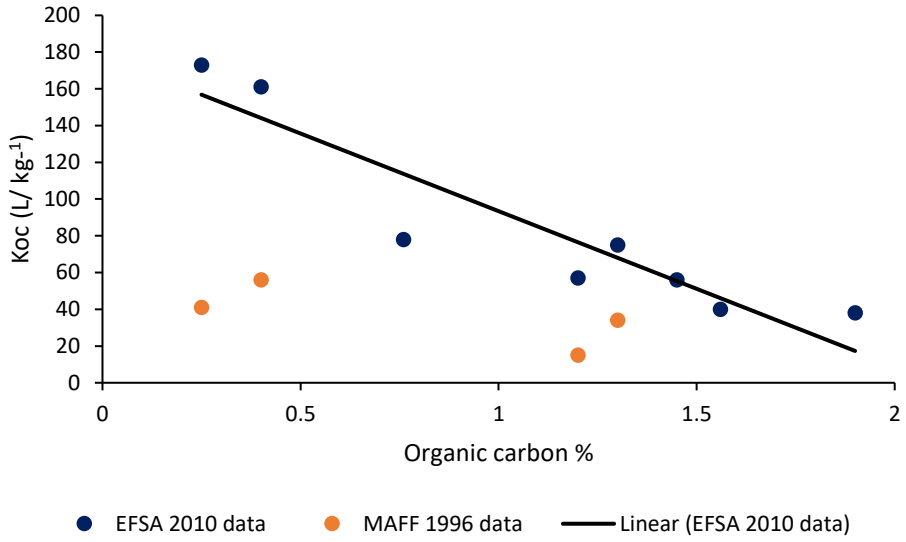

Figure 3: The relationship between organic carbon content of soil and soil adsorption partition coefficient, including data from food and environment protection act (MAFF 1996) and EFSA data (EFSA 2010).

1.7 Modelling with degradation kinetics data

Software tools such as CAKE (Computer Assisted Kinetic Evaluation) can be used to determine the kinetic parameters from environmental fate studies, such as K_{OC} and DT_{50}, which are used as input parameters for modelling and risk assessment (Gustafson and Holden, 1990). Software for chemical degradation kinetic studies were compared by Ranke et al. (2018). For the Single First Order (SFO) model, parameters obtained by kinGUII and CAKE fitted perfectly to three significant figures, and so did the goodness-of-fit criterion, chi squared. These are recommended programmes, scoring highly in the quantitative ranking developed for this report.

Pesticide degradation is typically measured at 20 °C; extrapolation of the half-lives for a more environmentally realistic temperature, such as 12 °C as with the assessment criteria under REACH (2016), will have greater uncertainty than the measured value (Matthies and Beulke, 2017). Annual average temperatures across the European climate zones range from 8 °C in northern countries to 18 °C in southern countries (European commission, 2014; FOCUS, 2015), so the variability is taken into account in the environmental exposure assessment during FOCUS scenarios. Degradation half-lives at 12 °C are derived by using the Arrhenius equation using generic activation energy of 65.4 kJ mol^{-1}, proposed by EFSA (2007), derived from an average value of activation energies from 99 datasets, at temperatures ranging from 0-30 °C.

Microbial transformations depend on the abundance and activity and population composition of microorganisms in the soil and the operations of their enzymes, which are temperature-dependent,

and will vary between species (Matthies and Beulke, 2017). Measurement of microbial biomass is not necessarily efficacious for wide-scale modelling (Ghafoor et al, 2011). Pesticide degradation generally tends to be done by certain bacteria within diverse communities, so measurement of total biomass is not necessarily accurate for model prediction. However, there is usually a positive correlation between microbial biomass and pesticide degradation rate, so the use of soil properties as a proxy for microbial activity may be a suitable method for model design according to Ghafoor et al. (2011). Biomass of soil is greatest for moist clayey soil, rich in organic carbon. This must also be related to the extent of sorption in model prediction.

Ghafoor et al. (2011) mention that, due to the variation in pore structure of soils, soil moisture influences on degradation are difficult to predict, but states that optimum microbial activity should be between 40-70 % water holding capacity. Investigating saturated soil, moisture above this range might be a determining factor for the legacy of metaldehyde if degradation is suppressed under these conditions. Nutrient availability and oxygen supply may be diminished in subsoil. These factors are also likely to lead to divergences in microbial population composition with depth, with implications on degradation rate in subsoil, which could be an important consideration for modelling (Ghafoor, 2011; Fomsgaard, 1997).

Soils are vertically stratified with greater organic content and nutrient availability at the surface. A carbon gradient can result from cultivation practices (Fromm et al., 1993) and will impact microbiology. Scow et al. (1986) and Hill and Schaalje (1985) proposed a two-compartment model consisting of two simultaneously occurring first order processes as a useful model for describing pesticide mineralization because it describes degradation for surface soil, where rate is more rapid, and subsurface, where degradation is slower. Brunner and Focht (1984), Scow et al. (1986) and Knaebel et al. (1994) described degradation of xenobiotic compounds with linear growth of the degrading microorganisms. The exponential model by Brunner and Focht (1984) shows that almost all the curves from the subsoil show an increase in the rate of formation of $^{14}CO_2$ at the beginning of the incubation, whereupon the formation of $^{14}CO_2$ becomes stable or decreases, resulting in sigmoidal curves. Most of the ploughed layer curves show only a decrease in formation of the mineralization product $^{14}CO_2$. This might suggest that certain pesticides have the potential to remain in subsoil below a certain concentration.

The first order description does not account for the catalytic aspect of biodegradation (Soulas 1982). Michaelis-Menten kinetics accounts for saturation effects, e.g. high metaldehyde local concentration of a pellet. However, enzyme concentrations will vary with the microbial population that synthesises them. Soulas' (1982) model accounts for adsorption to soil, which will impact bioavailability to microorganisms that utilise soluble substrates and therefore influence the degradation kinetics. Parameterising the Monod model was done by Sniegowski et al. (2009) for predicting mineralisation of pesticides in bio-filter systems.

1.8 Metaldehyde leaching and persistence in the environment

The predominant exposure routes of metaldehyde include surface run-off following rainfall events and exposure through field drains after leaching of the compound in soil. Both routes of exposure to surface water must be considered in modelling pollution. The amount of pesticide transport in the environment by drainage, leaching and run-off depends on the extent of degradation and sorption to soil (Børgesen et al., 2015). The relative importance of each depends on weather conditions, farming practice, soil properties etc. (Alletto et al., 2010; Reichenberger, 2007). In addition to diffuse pollution routes, point source pollution has a large impact on pesticides reaching surface water.

Soil columns were used by Bond (2018) to investigate the leaching of metaldehyde in two soil types. It was shown that clay soil leached statistically significantly higher concentrations of metaldehyde than the loam soil. Clay cores had higher mass fluxes; up to 7.16 µg m^{-2} day^{-1} for the Clay-Dry pellet treatment, and up to 164.1 µg m^{-2} day^{-1} for the Clay-Wet pellet treatment, due to preferential flow routes. For the loam cores, this was much lower; all below 0.07 µg m^{-2} day^{-1}. During the sampling period, clay control cores had the highest drainage rate, up to 0.485 mm day^{-1} for one core. All other cores had a mean drainage rate of <0.09 mm per day. Drainage rate decreased reflecting change in rainfall intensity, which decreased from 3.07 mm day^{-1} to 0.94 mm day^{-1} to 0.65 mm day^{-1}. Measurement of the flux revealed that only a small proportion of the applied metaldehyde is leached and is dependent on rainfall.

There is limited information in the literature regarding the leaching of metaldehyde, hence analogy must be made from other pesticide studies. Fenoll et al., (2011) found that a clay loam soil amended with manure significantly reduced leaching of ten pesticides. The effect of rainfall on leaching was investigated by Aslam et al., (2014). They applied simulated rain in two regimes; light frequent rain (applied 2 times a week at 6 mm h^{-1} for 20 min) and heavy infrequent rain (every 2 weeks at 20 mm h^{-1} for 24 min), with the same total amount of water being applied each month; a total of 16 mm per month. Mulch was applied to the tops of the column to examine the effect on degradation. Degradation was greater with the light frequent rainfall regime, which kept the mulch moist, while heavy infrequent rainfall tended to increase the leaching of pesticides and metabolites to lower soil.

Leaching is among the most sensitive parameters in modelling (Farenhorst 2006). Models such as VARLEACH (Trevisan et al 2000) can be used to predict the penetration and residue levels in soil are useful, since field studies are expensive and specific to one environmental scenario. VARLEACH incorporates subroutines to account for the effects of temperature and soil moisture on pesticide degradation rates. The performance of these models depends on how the laboratory data translates to the field situation. Preferential flow in clay soils is important in modelling because the presence of macro pores can cause disparities between simulated and measured data. Hence a bimodal model would be required that accounts for preferential flow and chromatographic flow (Trevisan et al 2000).

Pesticide degradation is generally positively correlated with organic carbon content, which is increased in the topsoil under conservation tillage, where the previous year's crop residue is left behind (Alletto et al., 2010). Greater pesticide interception occurs with conservation tillage, which has sorption capacities 10-60 times greater than soil, and could help reduce pollution via surface run-off. However, conservation tillage provides abundant food for slugs, meaning that the need for metaldehyde could be greater.

1.9 Microbial aspects of metaldehyde degradation

The predominant route by which metaldehyde is removed from the environment is through biodegradation in soil. Metaldehyde degrading bacteria have been extracted from soil (Thomas et al., 2017) and two bacterial isolates were cultured. One isolate belonged to the genus *Acinetobacter* and the other isolate belonged to the genus *Variovorax*. Both grew phototrophically, using metaldehyde as the sole source of carbon and energy. *Acinetobacter* E1 was able to degrade metaldehyde to residual concentrations, whereas closely related *Acinetobacter* strains could not degrade metaldehyde at all, while *Variovorax* grew and degraded metaldehyde more slowly.

In a more recent study by Castro-Gutierrez et al., 2020, eight new metaldehyde-degrading bacterial strains comprising a range of genera were isolated and characterised. They identified a highly

conserved gene cluster responsible for metaldehyde degradation that is not present in closely related genera which lack the capacity to degrade metaldehyde. The expression of this gene produces an oxygenase enzyme that cleaves the cyclic ether ring of metaldehyde, the first, and rate-determining, step of its degradation. Other metaldehyde-degrading isolates were also identified that lack this gene, suggesting that multiple degradation pathways have evolved. Castro-Gutierrez et al. (2020) demonstrated accelerated metaldehyde degradation in soils and discuss the potential for targeted bioremediation strategies.

The physico-chemical properties of pesticides affect biodegradability (Briggs, 1990). The chemical structures may be susceptible to enzymatic degradation, such as the inclusion of polar functional groups, which also increase water solubility. The soil structure affects biodegradation; properties such as clay content and organic matter content influence the microbial community (Hemkemeyer et al., 2015; Bausenwein et al., 2008) as well as the tendency for the pesticide to sorb onto the soil. Moisture content is another important factor: water serves as the solvent for the pesticide and is crucial for microbial activity. However, diffusion of oxygen is limited in water-logged soils and this may retard or accelerate biodegradation (Bausenwein et al., 2008).

Biodegradation of pesticides in agricultural soil exhibits spatial variability on a field scale (Dechesne et al., 2014). Non-random spatial patterns can depend on factors, such as pesticide classes, soil characteristics and agricultural management (Girvan et al., 2004). High organic matter content in soil tended to have a positive influence on degradation rate, linked with greater microbial activity (Kah et al., 2007). Temporal variation in surface and subsoil microbial populations was examined by Kramer et al., (2013) in an arable soil. In winter, high microbial biomass and enzyme activities were measured in all soil layers, thought to be because of increased mobilization and translocation of organic matter into deeper soil. Activities of hydrolytic enzymes decreased with depth, whereas oxidative enzyme activities showed no decrease or even an increase with depth. Different sorption mechanisms of hydrolytic and oxidative enzymes could have caused this.

Threshold concentration is interpreted as the amount of substrate where no growth of specific bacteria is observed (Toräng 2003). Biodegradation at low substrate concentration in soils high in assimilable organic carbon behave as mixed substrate assays and this has a significant effect on degradation kinetics and microbial growth yields according to Helbling et al., (2014). Anaerobic and reducing conditions in subsoil will affect biochemical processes in the soil. Redox measurements adopt a potential that is determined by the electron demand or availability at the interface with the surrounding solution (Husson et al. 2016, Rabenhorst et al. 2009) and is a measure of the tendency for a system to reduce or oxidise chemicals. This potential is affected by pH and utilisation of chemicals by plants, microorganisms and soil aeration. Redox potential can be an important parameter for determining the diversity of microbial communities because it can influence the availability of different substrates that certain genera of microorganisms need for growth (Tokarz and Urban, 2015). This can also be affected by soil type, where clay particles can be surface-catalysed redox processes and offer a microenvironment for certain microorganisms.

1.10 Ferric phosphate as an alternative to metaldehyde

Slug pellets containing ferric phosphate as the active ingredient are manufactured by several companies including Certis, Neudorff GmbH, Bayer CropScience, and Growing Success Organics Ltd among others, chiefly as the premium wet-processed product. The low water solubility (40 µg L^{-1} at 20 ^{o}C, EFSA (2020)) means that the salt will be persistent but is not thought to significantly affect iron and phosphate concentrations in the environment compared to other sources e.g. rock erosion and fertilisers (EFSA 2015). It was concluded by the EFSA (EFSA, 2015) peer review for ferric

phosphate that it does not present a risk to drinking water. EU approval of ferric phosphate has been renewed as a low risk active substance under regulation EC 1107/2009 (EEC 2009). With regards to cost, ferric phosphate pellets are more expensive than their metaldehyde competitors but use of the wet processed metaldehyde pellets are often greater than twice the cost of the dry processed equivalents. The maximum dose of ferric phosphate is 7 kg ha^{-1} and in high-pressure cases, up to four applications can be made so long as the maximum total dose does not exceed 28 kg ha^{-1} per crop. The formulated product contains chelates to solubilise the iron component to improve bioavailability to slugs. The mode of action is slower than metaldehyde and involves iron being deposited in the digestive system, which stops slugs feeding and the slug dies in 3-6 days (Speiser and Kistler, 2002).

Field tests on iron phosphate were conducted by Speiser and Kistler (2002) to test the efficacy of the treatment against slugs on lettuce and oilseed rape. Slug damage on lettuce was assessed by leaf loss and number of marketable heads. Metaldehyde was a more effective treatment over ferric phosphate in each criterion. Anderson et al., (2013) investigated the efficacy of different slug treatments in grass and clover seed crops in Oregon. The 5% iron chelate and 4% metaldehyde significantly reduced slug populations. None of the locations where 2% iron chelate was applied showed significantly reduced slug populations and the 1% iron phosphate was not an effective control. A report from Whaley (2007) showed Ferramol® (iron chelate) and metaldehyde to both have a mortality rate of >90% of slugs by day 10 while the mortality rate for the most effective iron phosphate pellets was only around 40% by day 10. The study author concluded that the pure iron phosphate baits was much less effective to kill either slugs or snails. Ecotoxicology studies by EFSA (2015) on the salt $FePO_4$ show it to be low risk to soil dwelling organisms. However, some formulations have suspected negative impact on soil invertebrates, due to the inclusion of chelate additives (Edwards et al., 2009; Langan and Shaw, 2006). There is concern about chelates mobilising toxic heavy metals in sewage and sediment (Hauser et al., 2005). EDTA, the most widely used chelate in the world (Nowack, 2002), is very recalcitrant to microbial degradation (Bucheli-Witschel and Egli, 2001), and is quite mobile in soils and readily transported to the groundwater together with the mobilized metals. The influence of EDDS and EDTA on plant uptake of heavy metals from soil is a concern for food production. Evangelou et al. (2007) investigated uptake of Cd and Cu by tobacco plant in presence of chelates. The uptake of Cu was increased by the addition of EDTA and EDDS, while no increase was observed in the uptake of Cd. Freitag et al. (2013) observed enhanced level of cadmium in cucumber plants due to mobilisation of metals from chelate present in soil from the application of ferric phosphate molluscicide products. EDTA is already used in agriculture: Manganese EDTA is a stable water-soluble chelate used in agriculture as a micronutrient fertiliser. Photochemical degradation is the main route of elimination (Kari and Giger, 1996). Biodegradation of three metal ligands including EDTA in metal-contaminated soil was investigated by Wen et al., (2009). Only 14% of EDTA was readily degraded in all three soils.

Reviews of the environmental chemistry of aminopolycarboxylate chelating agents (Nowack, 2002; Bucheli-Witschel and Egli, 2001) found their influence on metal availability and persistence in the environment causes them to be of concern. The mobilisation of toxic heavy metals produces a risk of poisoning of ground waters. In natural waters, EDTA predominantly exists complexed to Fe(III) and speciation is an important factor influencing environmental fate. The concentration of EDTA in river water is higher than any other identified organic compound (Frimmel, 1998). EDTA is poorly biodegradable, which combined with its widespread use, means that EDTA has a high background concentration in European surface waters of 10-50 ug L^{-1} (e.g. Van Dijk-Looyaard et al., 1990; Ruhr, 1994). However, exposure to EDTA from drinking-water is probably very small in comparison to exposure from other sources (WHO, 2003).

The organic chelate EDDS is biodegradable within 5-10 days in activated sludge, but degradation of the metal complexes may be less amenable (Vandevivere et al., 2001; Schowanek et al. 1997; Tandy et al., 2006). An environmental risk assessment for EDDS (Jaworska et al., 1999) explains that only the [S, S] isomer is readily biodegradable and highly water soluble. Elimination from treated sewage is 96%, determined by the activated sludge test, mostly due to rapid biodegradation. Mineralization in soil was rapid and complete in 28 days, with a DT_{50} of 2.5 days. The extent of degradation in river water was 75% with a half-life of 6.3 days, leading to a PEC of around 1 µg L^{-1}, below concentrations that will harm aquatic and terrestrial organisms.

The effect of chelates on soil quality could be indicated by microbial biomass and enzyme activity regarding mineralisation of soil nutrients. EDTA (spiked at 3 mmol kg^{-1} soil) decreased microbial activities and the utilization of substrates (Ultra et al. 2005). Epelde et al. (2008) observed a decreased activity of dehydrogenase in the presence of EDTA. Yang et al. (2013) examines the use of EDDS as a biodegradable alternative to EDTA but suggests that it might still have the effect of exaggerating the concentrations of metals in the environment.

1.11 Usage statistics for metaldehyde and ferric phosphate molluscicides

For molluscicides and repellents (not including seed treatments), the predominantly used active ingredients in 2016 were metaldehyde and ferric phosphate (DEFRA, 2016). Metaldehyde was used across 1,223,746 ha in 2016 compared to 920,317 ha treated in 2014. This 33 % increase in usage (Garthwaite, 2016) could have been due to more slug activity during the wetter winter in 2016 compared to 2014 (Met Office annual report, Kendon et al. 2014; Kendon et al. 2016). Indeed, metaldehyde was the 14th most used pesticide in 2016 (DEFRA 2016). Metaldehyde usage in terms of tonnes of active ingredient was 135,684 t vs 112,124 t in 2014: a 21 % increase on this criterion making it the 17th most used pesticide. Crop area treated with ferric phosphate in 2016 was 186,208 ha compared to 74,426 ha in 2014, which is a 150 % increase. This could have been due to pressure to use alternative molluscicides following the suggestions on metaldehyde usage from the metaldehyde stewardship group (MSG, 2008).

Comparison of molluscicide usage on different crops and the amount used is given in Tables 6 and 7. The most treated crop in 2016 was wheat, with 673,582 ha treated. However, this only represents 26% of the total crop area grown, as high slug-risk fields are treated with multiple applications, while some fields will not be treated at all. Other crops, such as oilseed rape and potatoes are highly vulnerable to slugs, and in 2016, 61% and 39%, respectively, of the crop area of these two crops were treated, with multiple application per year commonly practiced for potato growing (DEFRA, 2016).

Table 6: Main treatment statistic of crops with molluscicides and repellents in UK, 2016 from DEFRA pesticide usage survey (2016).

Crop	Area grown (ha)	Crop area treated (ha)	Crop area treated (%)	Number of treatments	Amount of metaldehyde used (ha)	Amount of ferric phosphate used (ha)	Amount of metaldehyde used (kg ai)	Amount of ferric phosphate used (kg ai)
Wheat	1,823,336	673,582	25.52	1.4	571,346	94,057	64,953	12,075
Oilseed rape	579,111	509,768	61.07	1.5	452,806	46,337	47,660	5,838
Potatoes	125,386	146,370	38.87	2.9	125,387	34,794	14,320	4,586
All crops	4,190,628	1,429,607	22.32	-	1,223,746	186,208	135,684	23,608

Table 7: Comparison of molluscicide and repellent usage in UK from 2010 – 2016 from DEFRA pesticide usage survey.

Year	Area treated (ha)	Weight applied (t)
2010	926,140	174
2012	877,965	126
2014	1,102,152	132
2016	1,429,606	160

1.12 Metaldehyde usage in other EU countries

Information about metaldehyde use in other EU countries is limited. Data from Eurostat (2014, 2018) shows the sales of molluscicides and may be used as an indicator of the amount of usage of metaldehyde. It is evident from Figure 4 that France is a large buyer of molluscicide, so it would be reasonable to assume that overall large quantities are applied to crops each year. However, France has more arable acres that some of the countries that feature in this figure, so it is difficult to predict how usage might relate to pollution risk. The climate and field conditions, such as the amount of underground pipe-draining, will also affect pollution risk. Data was not available for the UK in 2018, so cannot be compared with the other countries for this year.

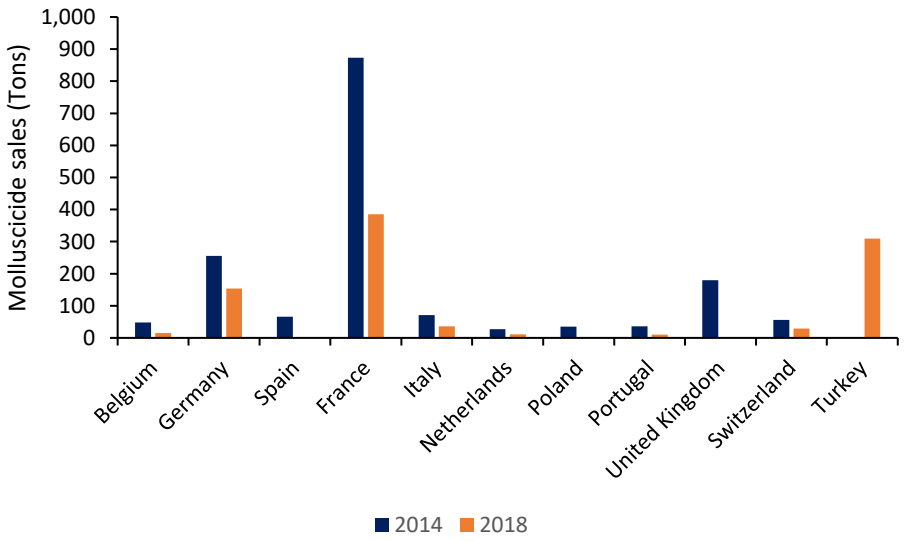

Figure 4: Molluscicide sales by quantity (tons) for the top 11 consumers in Europe over two years (Eurostat 2014, 2018).

1.13 Study proposal and research hypotheses

The hypotheses below have been created to interrogate the dynamics of metaldehyde in soil with the aim to reach an understanding of why the compound is detected in surface water at concentrations above what might be expected for a compound that, on observation of its half-life, would not be expected to be persistent in the soil environment. Current studies following OECD 307 conduct experiments at 20 ^{o}C, but this temperature is not representative of conditions when metaldehyde pellets are being applied in autumn. Degradation studies will therefore be done at lower temperatures to clarify how metaldehyde degradation is affected by temperature, which is expected to decrease with temperature. Further to these assessments, attempts will be made to establish trends between soil properties and degradation rate by directly observing microbial community compositions for variable soil properties and moisture conditions, rather than just inferring soil microbiology from chemical degradation measurements.

A correlation between soil depth and degradation rate will be established from lysimeters. Characterisation of the microorganisms' present will be undertaken for different parts of the soil profile in different soil types and related to biodegradation rates. Farming practices, such as seed bed preparation and fertiliser regime are a relevant contributing factor to determining microbial degradation and leaching of pesticides. Rapid leaching through preferential pathways like cracks to lower soil horizons may greatly increase the risk of metaldehyde loss to field drains and could be a

plausible reason for pollution in water sources being greater than predicted. If metaldehyde leaches to lower soil horizons before significant degradation occurs, greater quantities of the substance may be reaching biologically poor horizons, where degradation might not happen as readily. Proximity to field drains and reduced biodegradation could explained why current risk assessments underestimate metaldehyde pollution.

This research project will greatly benefit environmental risk assessments and pesticide pollution modelling for metaldehyde and broader application as well. Current risk assessments are based on standard guidelines, such as OECD 307 for soil degradation studies, which are not conducted under environmentally-realistic conditions and perhaps underestimates risk of pollution. Furthermore, many of the studies on metaldehyde degradation are dated and much better analytical instruments are now available.

Consideration for the microbiology of soil relating to pesticide degradation is novel to this project because no other study was found in the literature that investigates microbes in conjunction with highly detailed metaldehyde degradation experiments. Better understanding of the abundance of metaldehyde-degrading strains in different soil types and soil conditions will improve the interpretation of biodegradation test outcomes. Understanding the legacy of metaldehyde in soil will be important to water companies because it will help to inform abstraction times. Even though metaldehyde usage is due to be banned, there is evidence to suggest that it will continue to present a pollution problem into the future.

Research hypotheses:

1. The biodegradation of metaldehyde in UK scenarios is over-estimated by current risk assessment models.

    i) Degradation of metaldehyde will be slower at lower temperatures representative of conditions during application season and follows Arrhenius kinetics different to those used in standard fate assessment models.

    ii) The degradation rate of metaldehyde will show a concentration dependency, as metaldehyde present in soil at high local concentrations will result in microorganisms having a finite capacity to degrade the compound, reflected in a reduced degradation rate.

    iii) Biodegradation rates will be reduced under high soil moisture content, as less air is available for aerobic mineralisation processes.

2. Leaching to lower soil horizons will reduce the rate of metaldehyde degradation therefore metaldehyde will be measured in high recovery in leachate and deconstructed lysimeters.

    i) Residue levels of metaldehyde are expected to be detected in regions below the cultivated horizon, where soil is less well aerated, and therefore there would be a lower potential for degradation by aerobic microbes.

    ii) Significant quantities of metaldehyde will leach from the soil in lysimeters, reflecting reduced capacity for degradation under environmentally realistic conditions.

3. The degradation of metaldehyde in soil will be dependent on the presence of microorganisms with the capacity to utilise metaldehyde as an energy source. The abundance of these microbes will influence the degradation rate of metaldehyde.

    i) Analysis of soil samples taken from the degradation time points will reveal that the soils demonstrating accelerated degradation will contain a larger proportion of metaldehyde-degrading bacteria.

    ii) Microorganisms capable of degrading metaldehyde will be less abundant in lower soil horizons.

    iii) Microorganisms from agricultural soil can be isolated and characterised by inoculating an aqueous culture medium with the soil, using metaldehyde as the only source of organic carbon.

The following chapters in this thesis outline the experimental work conducted in order to interrogate these research hypotheses. Following analytical method development (Chapter 2), batch degradation assessments were conducted in the laboratory under various conditions representative of field conditions in the UK during the application season of metaldehyde pellets to assess the persistence of metaldehyde (Chapter 3). Leaching was assessed in lysimeters and concentrations of metaldehyde in leachate were compared to the predicted concentrations determined from the values determined during the batch degradation assessments (Chapter 4). Lastly, molecular microbiology was done to identify and quantify the metaldehyde degrading species in the soil samples from both the batch degradation experiments and the lysimeters to understand the mechanisms of metaldehyde degradation in agricultural soil (Chapter 5).

To date, the research has formed the basis of two publications:

Keighley, N., Ramwell C., Werner D., & Sinclair, C. "*Analytical method development and validation for the quantification of metaldehyde in soil*", *MethodsX* (2021).

Keighley, N., Ramwell, C., Sinclair, C., Werner, D. "*Highly variable dissipation of metaldehyde can explain its environmental persistence and mobility*". *Chemosphere*. 281, 131165 (2021).

Chapter 2: Analytical Method Development and Validation for the Quantification of Metaldehyde in Soil

2.1 Introduction: Analytical challenges

Quantifying metaldehyde, the active ingredient in molluscicide, in soil extracts by LCMS presented a challenge for the concentration range anticipated in biodegradation batch studies. Challenges include quantitative extraction from different soil types, and interference of matrix components from the extracts with the LC-MS analysis of the target compound metaldehyde, especially at low concentration. Several extraction procedures were trialled, and techniques were adopted to mitigate matrix effects in the LC-MS analysis. These included the use of matrix-matched analytical standards, dilution of the methanol extracts with water, and finally an optimized LCMS procedure for the improved separation of metaldehyde from interfering compounds using a BEH phenyl column (Acquity UPLC).

2.2 Materials and methods

2.2.1 Soil types, metaldehyde spiking and extraction procedures

Methods in the literature (EFSA, 2010) that detail metaldehyde extraction from soil tend to use methanol, or methanol/water solvents. This study based the extraction method on the published methods (EFSA, 2010). Initial method development to extract metaldehyde used a pasture clay loam soil, which was sampled from a livestock field in North Yorkshire and free from any known previous metaldehyde application. Soil samples (50 g dry weight basis) were weighed into 250 ml plastic screw-top containers, the moisture adjusted to 60% of maximum water holding capacity (MWHC) using deionized water and then left to settle for 30 min. The soil samples were spiked with metaldehyde (Sigma Aldrich, 99% purity) dissolved in water at three fortification levels (2.0 mg kg soil^{-1}, 0.2 mg kg soil^{-1}, and 0.02 mg kg soil^{-1}) and left to equilibrate for 30 min. Extraction of metaldehyde comprised the addition of methanol (100 ml, analytical grade from Sigma Aldrich), shaking the containers at 200 rpm for 30 minutes on a side-to-side shaker, followed by centrifuging (Beckman Coulter, Allegra) for 5 min at 3500 rpm and decanting the supernatant into a centrifuge tube. This process was repeated in a second extraction using methanol (50 ml) and the second extract was stored separately. The samples were stored in a refrigerator at 4 °C prior to analysis by liquid chromatography mass spectrometry (LC-MS). Three replicates were used as well as an untreated control.

In the second stage of method development, a range of standard soils (Lufa-Speyer), covering different texture classes and organic matter contents (refer to Table 8), were selected to validate the extraction method for a range of soil types. The soil properties were provided by Lufa-Speyer with the exception of the pasture clay loam (CL), whose properties were measured by Forest Research UK. The validation aimed to demonstrate that metaldehyde can be extracted with high recovery (70-110%) from a range of soils with different clay contents, organic matter contents and pH values. Soil samples (20 g dry weight basis) were weighed into centrifuge tubes and moisture adjusted to 60% MWHC with deionized water. Three replicates were used as well as an untreated control. The samples were spiked with metaldehyde at two fortification levels (1.5 mg kg soil^{-1} and 0.15 mg kg

soil^{-1}), thoroughly mixed with a spatula, and extracted with 25 ml methanol on a side-to-side shaker (200 rpm for 30 min), centrifuged (5 min at 3500 rpm) and the supernatant poured off and stored in a vial (Extract A). This process was repeated for a second sequential extraction (extract B). The extracts A and B were stored in separate vials in a refrigerator at 4 °C. The fortification concentrations of 1.5 mg kg soil^{-1} and 0.15 mg kg soil^{-1} were chosen as being approximately the same as the fortification concentration typically used in batch experiments, then a tenth of that value, respectively. Further validation samples were produced at a one-hundredth dose (0.015 mg kg^{-1}), to test the limits of detection.

Table 8: pH, organic carbon (OC) content and texture of the validation soils

Soil sample	pH ($CaCl_2$)	OC %	Sand %	Silt %	Clay %
Pasture CL	6.3	2.73	43.0	29.0	28.0
2.1 soil	4.7	0.67	86.1	10.2	3.7
2.3 soil	5.9	0.66	59.1	33.3	7.6
2.4 soil	7.4	1.99	32.1	41.3	26.3
6S soil	7.2	1.78	23.8	35.3	40.9

CL = clay loam

2.2.2 Method optimization

Initially, complications arose when metaldehyde extractions were performed at low spiking concentrations of 10% and 1% of the fortification level, representative of expected concentrations to occur at 90% and 99% degradation. At low concentration, recovery of metaldehyde was very poor (<10%). These results are shown in Table 2. A concentration-dependent process was apparently suppressing the metaldehyde signal during LC-MS analysis, and further experimental work was undertaken to understand the phenomenon.

Table 9: Recoveries (% quantified relative to application) of metaldehyde for the validation of extraction at different concentrations in the pasture clay loam soil, not accounting for matrix suppression.

Fortification level (mg/kg)	Extraction	Mean recovery (% application)					Mean	RSD
		Rep 1	Rep 2	Rep 3	Rep 4	Rep 5		
0.015	A	0	0	0	0	0	0	n/a
	B	0	0	0	0	0	0	n/a
0.15	A	3.79	4.41	1.22	1.38	0	2.70	1.42
	B	0	0	0	0	0	0	n/a
1.5	A	54.4	53.4	52.0	-	-	53.27	0.98
	B	22.9	21.9	-	-	-	22.43	0.41

The extraction method was trialled with other solvent systems, namely water alone and acetonitrile, concurrently to see if other solvents would perform better, but again, recoveries were equally poor (<10%). It was hypothesized that the observed poor recovery could be due to irreversible sorption to the soil, or matrix effects influencing the LC-MS analysis. Experiments were designed to test these ideas. Further extractions were done on a sandy soil, with low organic carbon content, compared to the organic-rich clay loam initially used in the study, with the expectation that sorption would be less relevant for the sandy soil. In this experiment also, recovery was poor for both soils, indicating that sorption of metaldehyde was not the main cause for poor recoveries. Direct spiking of blank extracts (50 ml) with 0.01 mg of metaldehyde was done to avoid sorption to soil. Despite the expected

concentration being double, and no soil available to cause sorption, recovery was still below 20%. It was concluded that matrix effects were influencing the LC-MS analysis.

To better understand how matrix suppression might be influencing the LC-MS results at lower concentration in the supernatant, standard addition samples were prepared and run on the LC-MS instrument. The standard addition samples were made up from metaldehyde stock solution (0.01 mg/ml in methanol) then diluted in 10 ml volumetric flasks using the supernatant from extraction of 0.01 mg fortification of metaldehyde in 50 g soil with 100 mL methanol. This was done in accordance with the aforementioned extraction method, using the supernatant from the first extraction. By diluting different quantities of the stock solution, standards of 0.01, 0.05, 0.1 and 0.25 µg ml^{-1} were prepared, not accounting for the metaldehyde present in the supernatant, along with a sample of the supernatant alone. A standard addition plot (Figure 1) was produced of the peak area (from the chromatograms, such as the one in Figure 2) against the concentrations of the standards and supernatant alone to give a linear plot from which the concentration of metaldehyde in the supernatant could be calculated without influence from matrix suppression. This technique yielded a recovery of 110.9% for the extraction of 0.01 mg metaldehyde from 50 g soil, which was vastly improved from previous attempts.

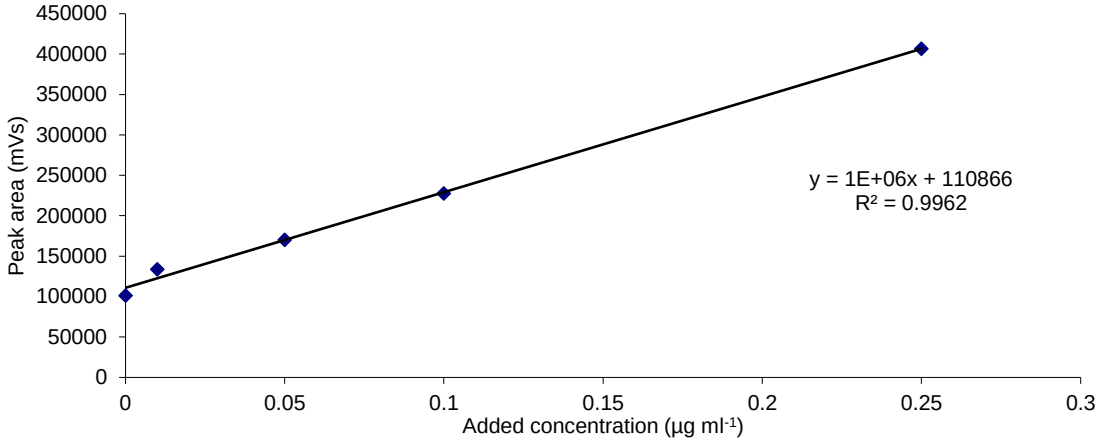

Figure 5: Standard addition plot (external standardization) for metaldehyde standards made up in the supernatant of a 0.01 mg fortification in 100 ml supernatant extraction from soil.

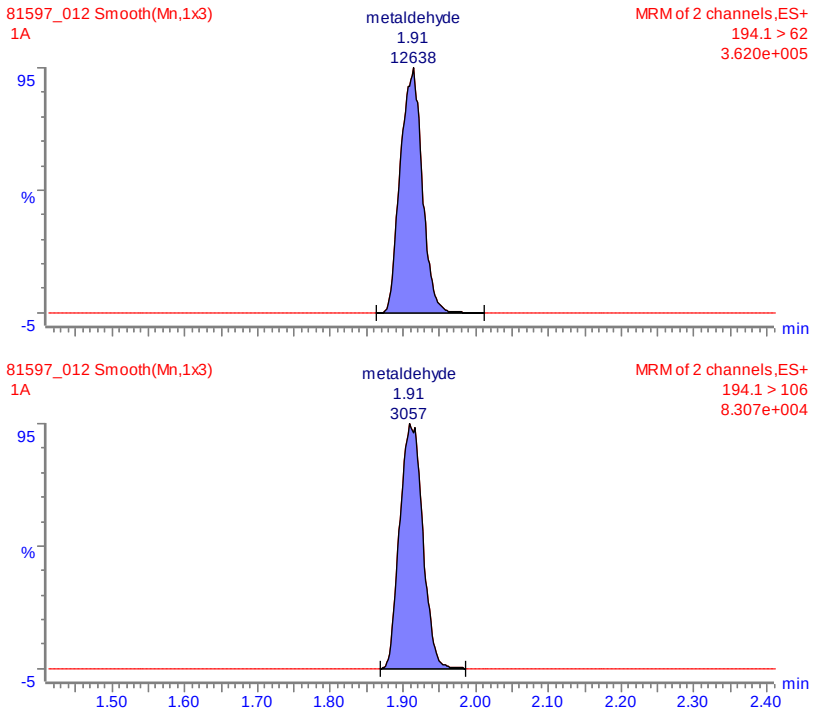

Figure 6: Chromatograms from the optimized LC-MS method showing both transitions at m/z values of 62 and 106 for a matrix-matched calibration standard of 0.25 µml^{-1} metaldehyde in methanol.

However, producing standard additions greatly increases the number of samples to be measured. Given that matrix suppression had been confirmed to be an issue in the analysis of metaldehyde, work on the LC-MS method was undertaken for optimization against matrix effects. Different LC columns were trialled (Kinetex XB-C18 50 × 2.1 mm column (Phenomenex, Macclesfield, UK and BEH phenyl (Acquity UPLC)), and alterations to the LC-MS method adapted from Thomas (2016) were made to optimize the analysis of soil extracts, rather than extracts from bacterial cultures, as was the case in the referenced study.

Samples for LC-MS analysis were prepared by diluting 1/10 with water and filtered through a (*Whatman*) 0.2 *µ*m pore nylon filter (*Thermo Fischer Scientific*). Calibration standards were matrix matched, produced from the same metaldehyde stock solution used for spiking the batch experiments, spiked into blank soil extract. The calibration range used included the following standards: 0.01 µg ml^{-1}, 0.05 µg ml^{-1}, 0.1 µg ml^{-1}, 0.25 µg ml^{-1}, 0.5 µg ml^{-1}, 0.75 µg ml^{-1}, 1.0 µg ml^{-1}, and 2.5 µg ml^{-1}. Calibration standards were produced by serial dilution of the 0.1 mg ml^{-1} metaldehyde (aq) stock solution used for the soil spiking. These were matrix-matched by amending the standards to 1/10 methanol used in a blank extraction of the test soil. A calibration curve is shown in Figure 3.

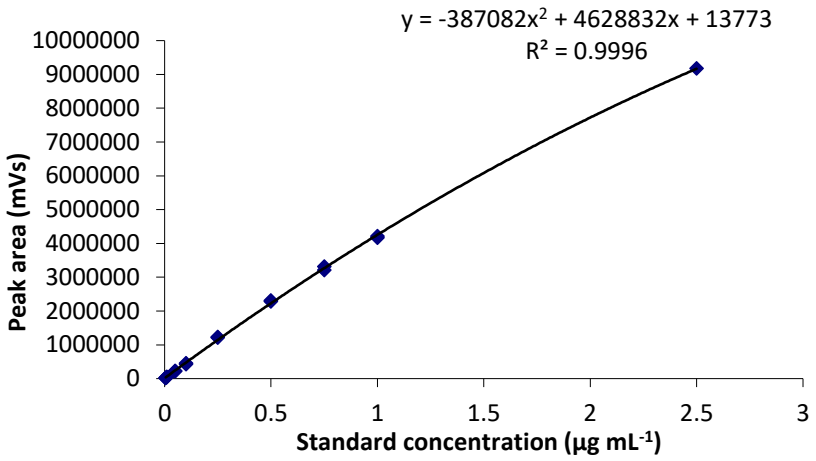

Figure 7: Calibration curve used for the method validation developed for the extraction of metaldehyde from soil.

The LC-MS instrument used initially, during the analysis of these samples, was a Shimadzu Nexera X2-SCIEX Qtrap 5500. The LC-MS method was developed from the one described by Thomas (2016) and optimized for metaldehyde in soil extracts. A Kinetex XB-C18 50 × 2.1 mm column (Phenomenex, Macclesfield, UK) was used with 2.6 µm particles, with a flow rate of 0.5 ml min^{-1}, using a mixture of 1 mM ammonium acetate, prepared with ultrapure water, and methanol as the organic component, and these two solvents comprised the mobile phase with an elution gradient flow detailed in Table 4. Samples were stored in a refrigerator at 4 ^{o}C prior to injection into the instrument. Mass spectrometry involved an optimized protocol with greater response and improved precision that used 0.5 ml min^{-1} flow rate, 5.25 kV capillary voltage and 325 °C solvation temperature. To observe product ions (transition at m/z 106, $C_4H_8O_2 + NH_4^+$, and m/z 62, $C_2H_4O + NH_4^+$), multiple reaction monitoring with a dwell time of 160 ms was used.

For the final method, a different LC-MS instrument (Waters Acquity-Quattro Premier XE) was used with an optimized methodology for detecting metaldehyde in soil extracts. The methodology was scaled down from the previous trial to save laboratory consumables. To avoid the issues with matrix suppression, the column was changed during the development of the optimized method. Changing the C18 column used on the SCIEX instruments to a BEH phenyl column on the Waters instruments helped to notably reduce matrix suppression of the metaldehyde signal, which caused a loss of response at low metaldehyde concentrations. The method used a BEH phenyl (Acquity UPLC) 1.7 µm particles 2.1 x 100 mm column, with a flow rate of 0.4 ml min^{-1}, using the same mobile phase as previously described: 1 mM ammonium acetate, prepared with ultrapure water, and methanol as the organic component, with the same gradient flow (Table 4). For mass spectrometry, the original parameters used with the SCIEX instruments were selected, with the following amendment: opened the acquisition window earlier (between 0.5-5.00 min) with a 0.6 s solvent delay. This LC-MS method proved successful in overcoming the issues presented by matrix suppression of the metaldehyde signal, and the complete analytical method was then validated for use in environmental fate studies in metaldehyde.

Table 10: Gradient flow used in the LC-MS methodology to elute metaldehyde.

Time (min)	*% Ammonium acetate*	*% Methanol*
Initial	*95.0*	*5.0*
1	*50.0*	*50.0*
3	*10.0*	*90.0*
7	*10.0*	*90.0*
7.10	*95.0*	*5.0*
12.00	*95.0*	*5.0*

2.2.3 Method validation

Method validation results are presented in Table 3. The recovery of applied versus quantified metaldehyde was 100-132% for all soil types, 109% on average, with acceptably low relative standard deviation of <20%. The proportion of metaldehyde present in the second sequential extraction (extract B) was higher for the more clayey, high organic content soil types, soil 2.4 and soil 6S, illustrating the need for the second extraction step. These results demonstrated that applied metaldehyde could be extracted and quantified with a high recovery from different soil types.

Table 11: Method recoveries for the extraction of metaldehyde from different soil types (specifications in Table 1). RSD = relative standard deviation.

Soil	**Mean recovery (n = 3) for 1.5 mg kg^{-1} fortification**			**Mean recovery (n = 3) for 0.15 mg kg^{-1} fortification**		
	Extract A %	Extract B %	RSD for combined extracts	Extract A %	Extract B %	RSD for combined extracts
2.1 soil	82.6	27.7	9.52	113.3	0	8.54
2.3 soil	76.5	23.9	12.08	106.5	0	20.6
2.4 soil	85.6	17.4	8.33	52.2	52.5	12.18
6S soil	86.3	19.1	10.19	41.4	90.8	13.34

Chapter 3*:* Highly Variable Soil Dissipation of Metaldehyde Can Explain its Environmental Persistence and Mobility

3.1 Introduction: the variable persistence of metaldehyde

In the literature, metaldehyde has been shown to demonstrate a varied persistence in soil (e.g., EFSA, 2010). The dissipation of metaldehyde was measured in three representative soils during a series of batch degradation experiments conducted during this study to identify whether varied experimental conditions, more representative of the range of field conditions in the UK and Northern Europe, would significantly affect the degradation of metaldehyde in soil. It was hypothesised that trends between the soil conditions and the observed persistence would be identified and thus facilitate an understanding of why highly variable dissipation of metaldehyde is observed. This information could potentially offer a rationale for the disparities between the regulatory approval process conducted under standardised laboratory conditions and the observed persistence of metaldehyde in the environment.

3.2 Materials and methods

3.2.1 Batch degradation experiments

Batch degradation studies were performed with three field fresh soils collected from the same district in North Yorkshire: a clay loam (CL) soil collected from a permanent pasture field on 02/07/19, an arable clay loam collected on 08/11/19, and an arable sandy loam (SL) collected on 16/01/20. The pasture clay loam was taken from a field that had historically been used for sheep farming and was therefore free of any residual metaldehyde, based on land-use history. The arable clay loam was of the same texture class (refer to Table 12), collected from the adjacent field. It was selected to investigate how land management practices might influence biological processes occurring in the soil. The field had been used for growing wheat in the previous harvest. The sandy loam was selected from a local field to represent a contrasting soil type to elucidate whether any observed trends in metaldehyde degradation would continue in other soil types. This field also had wheat as the previous crop.

Table 12: Properties of the soils collected from the field for use in the batch degradation experiments.

Soil sample	MWHC (%)	pH ($CaCl_2$)	OC %	Sand %	Silt %	Clay %
Pasture CL	34.4	6.3	2.73	43.0	29.0	28.0
Arable CL	32.6	6.02	3.3	29	34	37
Arable SL	26.4	5.46	1.76	48	32	20

CL= clay loam; SL = sandy loam

The batch degradation experiments tested the effects of 1) temperature, 2) soil moisture content and 3) metaldehyde concentration on degradation rates, compared to a reference experiment conducted in accordance with OECD 307 guidelines at an incubation temperature of 20 °C and soil moisture of 60% MWHC. The experimental methods to test each of these parameters were kept the same, other than for the use of a different soil type. Metaldehyde (0.3 ml or 1 ml of a 0.1 g L^{-1} aqueous solution) was dosed into soil (20 g or 50 g) in accordance with OECD 307 guidelines, unless

otherwise stated. The soil moisture content was adjusted to the required value using deionised water, based on the sample weight and amended weekly. Metaldehyde was extracted, in accordance with the validated method, 0, 1, 3, 7, 14, 21, 28 and 60 days after treatment and subsequently quantified using LCMS.

Using the experimental procedure described above, metaldehyde degradation was measured in soils at five temperatures. The batch soil samples were assembled, dosed, and then moved to separate incubators at temperatures of 20 °C, 16 °C, 12 °C, 8 °C and 4 °C to test the effect of decreasing temperature on the degradation rate of metaldehyde. Degradation experiments at different soil moisture contents were also assembled. A degradation experiment using the pasture clay loam was set up with the soil moisture content amended to 100% MWHC, incubated at 20 °C. Further experiments to test the effect of soil moisture content on metaldehyde degradation were set up using the arable clay loam. The field fresh soil was allowed to dry to a moisture content of 40% MWHC in trays before been weighed into containers, and, in separate experiments, adjusted to moisture contents of 40%, 60%, 80% and 100% MWHC, with a further two experiments (soil moistures of 60% and 100% MWHC) incubated at 12 °C. Experiments with soil moisture contents of 40 %, 60 % and 100 % MWHC were replicated in the sandy loam soil. Additionally, experiments were set up with varying metaldehyde application doses: 100 mg/kg dry soil (arable clay loam; arable sandy loam) and 50 mg/kg soil (arable sandy loam only). The fortification concentration of 100 mg/kg soil equates to the amount of metaldehyde contained within a single 3% slug pellet (0.375 mg active ingredient, e.g., AXCELA pellets) on a square centimetre of soil and 2.5 cm depth, i.e., representative of field conditions. The metaldehyde was applied as a solid powder mixed into silica sand rather than as an aqueous solution because the amount being applied would not readily dissolve in water. A summary of the various experimental conditions is presented in Table 2. Each degradation experiment comprised 3 replicates plus an untreated control. Additionally, a degradation experiment was conducted using soil that had been sterilised in an autoclave to serve as a control.

3.2.2 Data Manipulation

The measured degradation data from individual time points were analysed using the software, Computer Assisted Kinetic Evaluation (CAKE) (Tessella, ALTRAN GROUP) to calculate degradation rates. The measured temperature-effect data were used to investigate the accuracy of the Arrhenius model which is commonly used in environmental fate models to extrapolate from temperatures used to conduct standard laboratory fate studies (20°C and 25°C) to field conditions. The Arrhenius equation was used to construct the plots in Figure 1, from which the value of the activation energy was derived. This value was then used to predict the degradation rates at temperatures <20 °C and compared with the predictions using the standard activation energy that is utilised in regulatory fate models. The Arrhenius model was then extended to different soils and varied soil moisture contents to examine how predicted degradation rates might differ under different test conditions.

3.3 Results and discussion

3.3.1 Overview of the Batch Degradation Experiments

The measured degradation rates for metaldehyde were greater for the permanent pasture clay loam soil, while in the arable soils, metaldehyde was shown to degrade quickly, in line with findings from the literature (EFSA, 2010). The batch degradation experiments demonstrated a highly variable set of degradation rates with DT_{50}s ranging from 3.6-4150 days (Table 13). These could not always be linked to the parameter under investigation, and the greatest variability, generally, was observed between soil types as opposed to the other tested parameters. It was found that Single First Order

(SFO) kinetics was the best fitting model for calculating degradation rates in each experiment. The most conspicuous result was that of the pasture clay loam under high soil moisture (100 % MWHC) conditions, where the DT_{50} was two orders greater than the respective reference experiment at 60 % MWHC moisture content. This demonstrated that metaldehyde had the potential to become persistent in high moisture content soil.

Table 13: Summary of the batch degradation experiments; experimental conditions and calculated disappearance half-lives using Single First Order (SFO) kinetics.

Soil type	Soil moisture (% MWHC)	Fortification level (mg kg^{-1})	Incubation temperature (^oC)	DT_{50} (days)
Pasture Clay Loam	60	2	20	46.5
	60	2	16	46
	60	2	12	58.3
	60	2	8	71.7
	60	2	4	129
	100	2	20	4150
	60	100	20	116
Arable Clay Loam	60	2	20	2.97
	100	2	20	5.6
	80	2	20	3.62
	40	2	20	13.8
	100	2	12	9.26
	60	2	12	14.7
	60	100	20	7.39
	60	50	20	7.76
	60	2	20	>10,000*
Arable Sandy Loam	60	2	20	10.8
	100	2	20	7.65
	40	2	20	4.53
	60	100	20	13.5

*Sterile control

3.3.2 Temperature dependency of metaldehyde degradation

The temperature-dependency of metaldehyde degradation was assessed by constructing Arrhenius plots (Figure 8) to determine the agreement of the experimental data with the Arrhenius model and activation energies. A value for the activation energy for metaldehyde degradation was determined in the pasture clay loam soil over a range of temperatures from 4-20 °C. At temperatures <12 °C, the kinetic profile was biphasic, with an initial short period (<3 days) of faster degradation, followed by a slower secondary phase, the latter having a greater influence on the overall rate of dissipation. The Arrhenius plot (Figure 1) that was constructed with the data fitted with Single First Order (SFO) kinetics demonstrated a greater temperature dependency (E_a = 37.5 kJ mol^{-1}) than the plot fitted with the second phase of the Double First Order Parallel (DFOP) kinetics (E_a = 15.5 kJ mol^{-1}). However, the ln of the rate does not depend linearly on 1/T. There is a relatively narrow linear range in each curve, for which the E_a is less than the standard value. While the data do not support the hypothesis that the standard temperature correction results in overestimated degradation rates, the data do raise questions about the applicability of Arrhenius theory to metaldehyde degradation in soil. Both derived values suggested a lower temperature dependency for metaldehyde degradation in soil than the standard value (E_a = 65.4 kJ mol^{-1}) that is used in regulatory risk assessment. These

results therefore did not support the initial hypothesis that temperature dependency was a source of error leading to overestimation of degradation rates in soil.

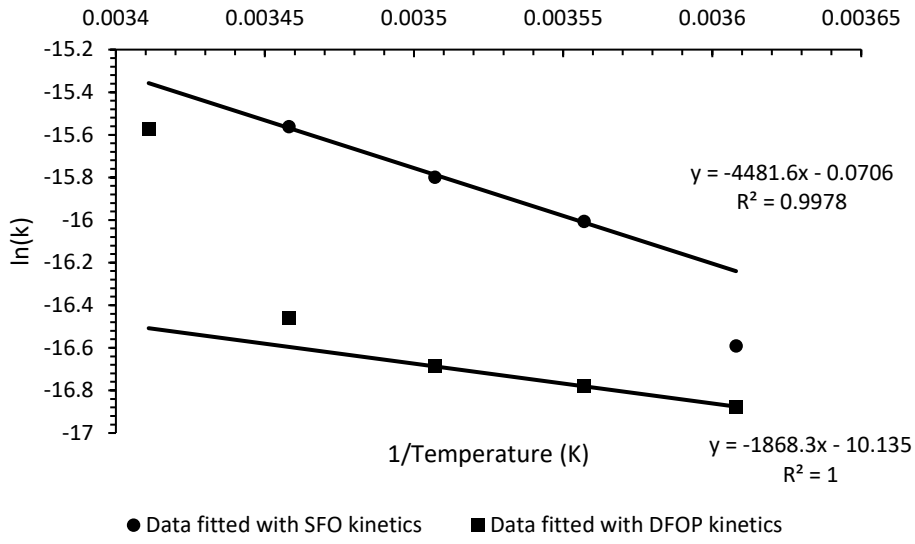

Figure 8: Arrhenius plots derived from the linear range of the experimental results from the batch incubation tests conducted over five temperatures from 4-20 ^{o}C, using Single First Order (SFO) kinetics and Double First Order Parallel (DFOP) kinetics to derive an activation energy for metaldehyde degradation in soil.

The predictions deviated from the experimentally determined values by an appreciable amount for standard activation energy used in regulatory modelling, but not the experimentally derived activation energy using SFO kinetics (Figure 9). The SFO-derived activation energy was chosen for further modelling because this kinetic model generally fitted the data best. The deviations in the data set in the Arrhenius plots likely represent a change in kinetics towards a more biphasic pattern at lower temperature, which was observed in the degradation profiles of the incubation experiments. All the predicted DT_{50} values in Figure 2 were calculated from the experimental rate constant measured at 20 °C to replicate the approach used in standard modelling work. Investigations into the suitability of the Arrhenius equation were then expanded to the arable clay loam soil at two moisture contents.

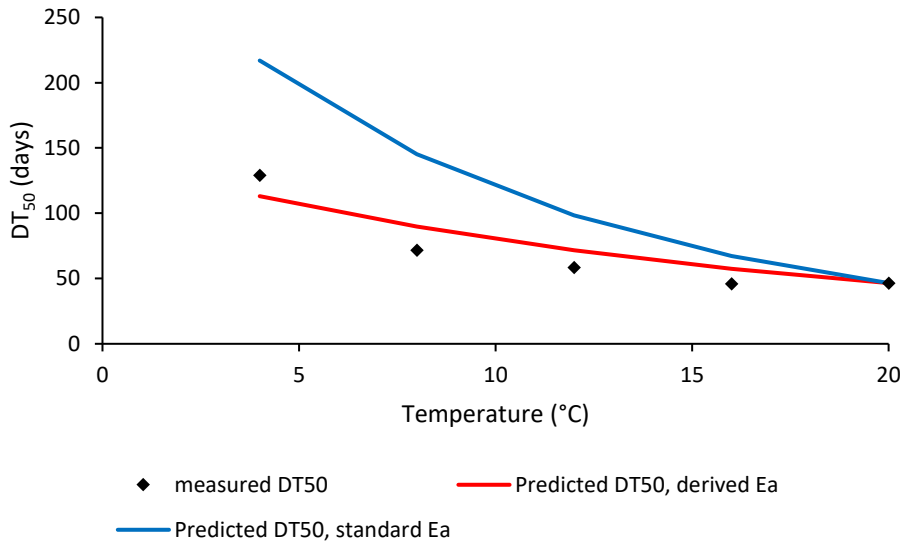

Figure 9: Comparison of measured metaldehyde degradation rates (DT_{50} vales) to the predicted metaldehyde degradation rates at different temperatures using both the standard activation energy (E_a) and the derived activation energy.

3.3.3 Effects of soil moisture content on metaldehyde degradation

Of the parameters investigated, soil moisture content had the greatest effect on metaldehyde degradation rate. The degradation profiles for metaldehyde in three soils adjusted to 100% of their maximum water holding capacity are shown in Figure 10. The most prominent result across the entire suite of batch degradation experiments was that of the pasture clay loam soil under high moisture (100% maximum water holding capacity (MWHC)) conditions where metaldehyde became persistent, with a half-life of 4150 days, far exceeding measured degradation times for any other experiment. On the arable clay loam, the difference was less marked with a DT_{50} of 5.6 d under high soil moisture conditions (100% MWHC) relative to the reference experiment (DT_{50} = 2.97 d; 60% MWHC). Additionally, rapid metaldehyde degradation was observed during experiments using the sandy loam soil, which was also collected field fresh under arable management. Metaldehyde degradation at high soil moisture content (100% MWHC) resulted in faster degradation (DT_{50} = 7.7 d) compared to the reference experiment (10.8 d).

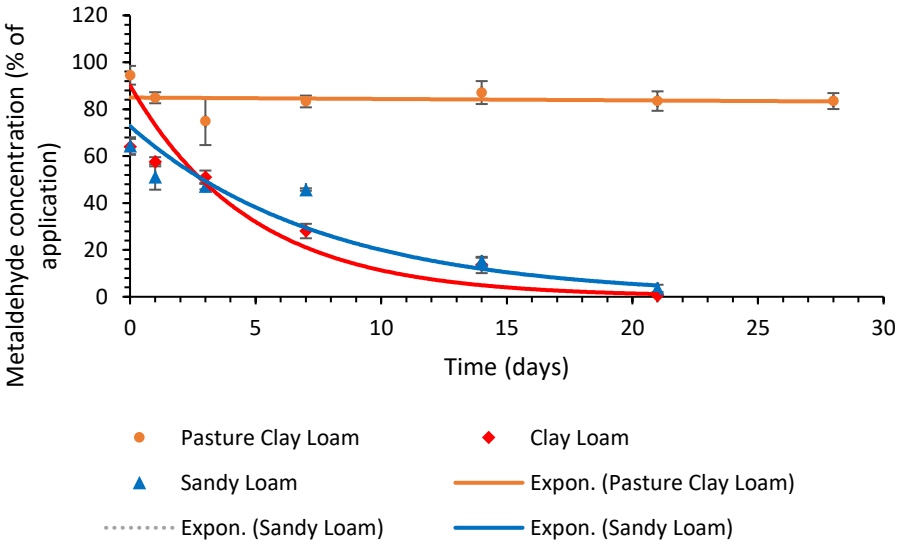

Figure 10: Comparison of metaldehyde degradation profiles in three soils at high soil moisture content of 100% MWHC showing the mean ± 1 standard error

The combined effect of high soil moisture conditions and lower temperature on degradation rate was measured. Figure 4 displays how DT_{50}s in the arable clay loam soil varied under these two parameters. The data show that DT_{50} was greater when measured at 12 ºC, as opposed to 20 ºC in soils at two moistures (60 and 100% MWHC), as expected from the temperature-dependency study. However, the higher moisture content soil did not always display slower degradation, as suggested by the hypothesis.

To examine the predictability of these results, the Arrhenius equation was used to predict rate constants at 12 °C for metaldehyde degradation occurring at two soil moisture contents: 60% MWHC and 100% MWHC in the arable clay loam soil (Figure 11). The results generally deviated from the experimental results by an appreciable amount for both the experimentally derived E_a and the standard E_a. The experimentally derived activation energy in this investigation was not substantially more accurate than the standard value, with only the high soil moisture experiment fitting the model well. It was therefore concluded that the activation energy derived for the permanent pasture soil was not applicable to the arable soil of the same texture class. Therefore, it was decided that the standard value for activation energy is a good approximation for environmental fate modelling.

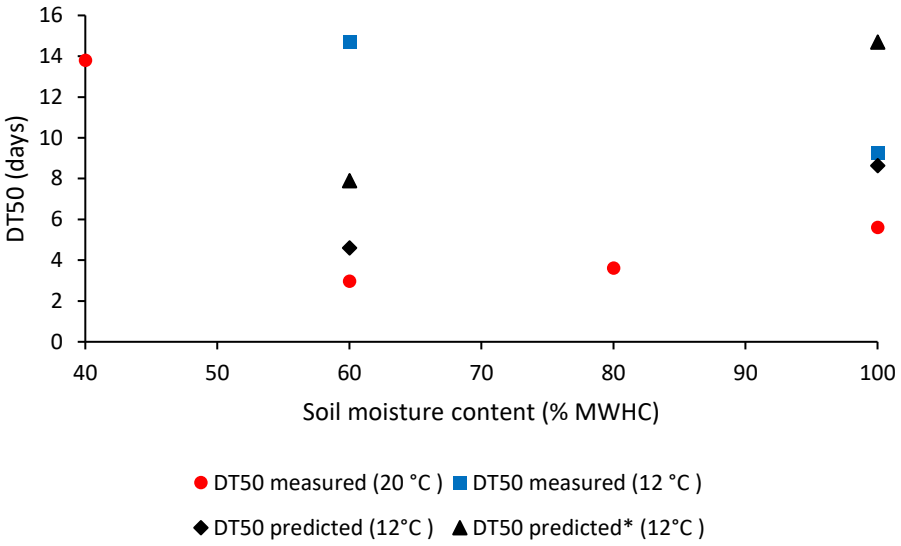

*Figure 11: Distribution of metaldehyde DT $_{50}$ values in an arable clay loam soil at different soil moisture contents along with predicted values using the experimentally derived activation energy for metaldehyde and the standard activation energy (*predicted with standard E_a).*

3.3.4 Metaldehyde degradation at high concentration

The rate of metaldehyde degradation was highly variable across some batch experiments examining varied metaldehyde soil concentration, where the soil moisture content was kept constant, set to 60% MWHC and an incubation temperature of 20 °C. With reference to Figure 12, the most outlying result was that of the pasture clay loam conducted with high metaldehyde concentration. The degradation half-life deviates the most from the rest of the data set, with at DT_{50} of 116 days, compared to the reference value of 46.5 days. Degradation rate was in every case quicker in the clay loam and sandy clay loam arable soils than in the pasture clay loam experiments. During these incubations, high metaldehyde concentration resulted in reduced degradation rate.

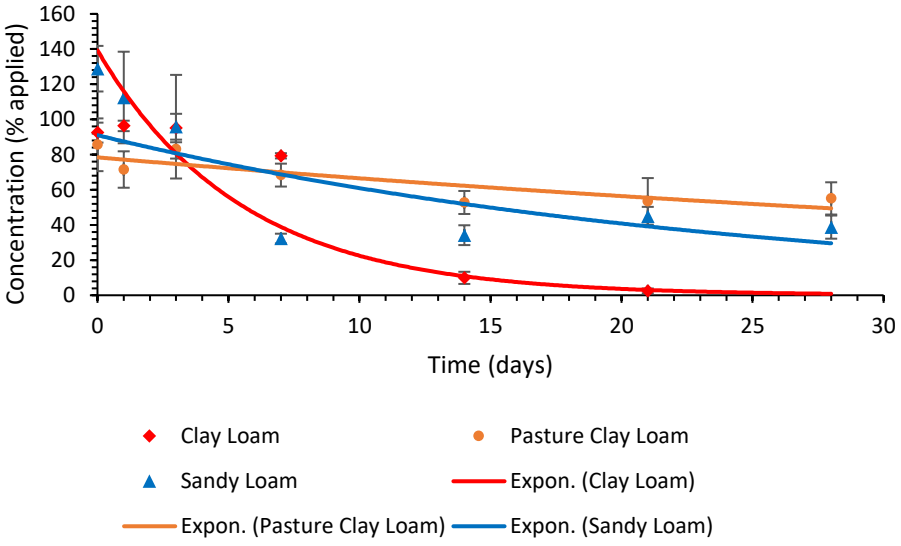

Figure 12: Comparison of metaldehyde degradation profiles in three soils at high metaldehyde soil concentration (100 mg/kg soil) showing the mean ± 1 standard error.

It was hypothesised that metaldehyde degradation rate would not be affected by high metaldehyde concentration, and the process would continue to follow SFO kinetics. While SFO kinetics was found to be a suitable model, metaldehyde showed a reduced rate of degradation compared to the reference experiment in all three soils: most notably in the pasture clay loam. As in previous experiments, degradation of metaldehyde proved faster in the arable clay loam and sandy loam (DT_{50} of 7.4 d and 13.5 d, respectively. Metaldehyde degradation rate was observed to be faster in the clay loam and sandy loam arable soils. One explanation could be that the abundance of microorganisms capable of degradation metaldehyde is the limiting factor. The microbial community in the arable soils may be conditioned to previous exposure to xenobiotics, hence will degrade synthetic chemical more readily (Yale et al., 2017).

Additionally, there are implications of soil type and management, where aeration from cultivation and treatment with fertiliser might enhance the abundance of microorganisms with the capacity to degrade metaldehyde (Hemkemeyer et al., 2015). This might also have a relevance to the observed trends in metaldehyde degradation. The formulation of metaldehyde-based molluscicide as pellets could result in heterogeneous concentrations in the field; metaldehyde might be present in localised pockets of high concentration, where growth controlling factors such as nutrient availability become relevant. In scenarios where metaldehyde concentrations are in gross excess of normal nutrient substrates, following the application of slug pellets, the capacity for the soil microbes to degrade high concentrations of metaldehyde could be limited by their abundance (Ghafoor et al., 2011). If only a fraction of the metaldehyde present in the soil is subjected to the normal SFO degradation processes, this could have implications for the quantities of the chemical leaching to lower soil horizons.

Across the suite of batch incubation experiments, the greatest variability in metaldehyde degradation was generally observed between different soil types, rather than in response to the tested parameter. In the case of the permanent pasture soil, for example, the microbial community of this soil is likely to be conditioned for more anaerobic conditions, created as a result of livestock activity compacting the soil to create a pan and this soil is not disturbed during cultivation; a process which aerates the soil and therefore may facilitate the degradation of metaldehyde, which has been shown to be an aerobic process (ESFSA 2010). The seasonal dynamics of soil microbial communities, as they become habituated to certain environmental conditions might also have an impact on pesticide degradation (Paganin et al., 2010). Analysis of the microbial dynamics associated with metaldehyde degradation represents future work in this project. However, for the purpose of improving environmental fate assessments, degradation rates should be measured empirically for appropriate soil types/moisture/temperature conditions in a country, rather than theoretically inferred from data measured under unrepresentative standard conditions.

3.3.5 Wider implications of the research

Protection of agricultural crops is of crucial importance for society, as populations continue to grow. It is also important to ensure sustainable farming, with a consciousness for the environmental impact of farming practices. Slug damage to crops can cause significant losses in agricultural production. While slug damage can be mitigated by agricultural practices such as soil cultivation by ploughing and deeper seed drilling, these are often insufficient and chemical control is required (Glen et al., 2006). Oilseed rape and wheat are among the most susceptible crops to slugs, with 59% and 22% of the total crop area affected, respectively, and if all molluscicides were withdrawn, the calculated annual tonnage lost equates to around a value of £42m for these crops alone in the UK (Nicholls, 2014). It has been estimated (Dewar, 2016) that the cost to UK crop production if chemical control methods were unavailable could be £100 million per year (Dewar, 2016). While molluscicides

are crucial for protection of agricultural crops and are needed for efficient food production, sustainability also demands that pollution should be within acceptable levels. The conclusions from this study aim to contribute to the knowledge basis that can be used to improve risk assessment and help to inform the responsible use of molluscicide.

Concerns over wildlife poisoning recently led to the decision to ban outdoor use of metaldehyde in UK (DEFRA, 2020) with no applications permitted beyond March 2022. Metaldehyde can still be used elsewhere in Europe. Despite the ban on the outdoor use of metaldehyde, there is still a risk of exceedance of regulatory drinking water limits as metaldehyde is persistent in water and has been observed in water sources from catchments that have not had recent applications (UKWIR, 2015).

With the withdrawal of metaldehyde, growers will probably have to switch to using ferric phosphate. Ferric phosphate has low solubility in water and the environmental fate of the product is not thought to significantly raise the natural concentration of iron and phosphate ions in water, compared to artificial fertiliser usage. However, it is unclear how the product, formulated with chelate molecules, interacts with the environment and there are concerns over toxicity to soil invertebrates, such as earthworms (Edwards, 2009). Furthermore, the discrepancy between low water pollution risks predicted by the testing regime used for regulatory approval of metaldehyde and the real-world occurrence of metaldehyde in drinking water poses fundamental questions about the robustness of current testing procedures for chemicals, and the need to improve them. Depending on the efficacy of ferric phosphate products and the costs to agriculture associated with the ban on metaldehyde, the future for metaldehyde-based molluscicide is unclear. Metaldehyde exemplifies growing concerns about mobile, persistent, and toxic compounds in the environment and the findings support calls for more robust methods to determine biodegradation rates.

3.4 Conclusions

The impact of temperature, moisture content and initial concentration on the degradation rate of metaldehyde in soil varied with soil type. However, a high soil moisture content and high metaldehyde concentration resulted in longer DT_{50} values for metaldehyde than those derived during regulatory assessment for some soils. Based on the results of this study, lack of prior metaldehyde exposure, high moisture content, low temperature and high metaldehyde concentration were identified as high-risk conditions for low pesticide biodegradation in UK soils. It can be easily envisioned how such conditions might locally co-exist in the UK during the metaldehyde application season, and then lead to metaldehyde peaks in surface water affecting drinking water supplies. The findings will have implications for environmental fate modelling and risk assessment, indicating that metaldehyde has the potential to be more persistent in the environment than regulatory assessments might suggest.

Chapter 4: Field Dissipation Assessments for Metaldehyde in Lysimeters

4.1 Introduction: the leachability of metaldehyde

Metaldehyde leaching was assessed in lysimeters composed of intact soil cores. It was hypothesised that the physical condition of the soil would be more influential with regard to leaching of metaldehyde than soil type. Mechanisms such as preferential flow routes were expected to have the greatest impact on leaching, where porous columns would release metaldehyde the soonest. These mechanisms were considered to have a significant effect on the predictability of metaldehyde contamination of surface waters during modelling scenarios. By comparing the results from the leaching experiments with the model predictions using the laboratory-derived degradation rates, an explanation of how circumstances could arise where metaldehyde becomes persistent in surface waters could be determined.

It should be recognised that leaching is a means of chemical dissipation from soil. The dissipation of metaldehyde referred to in this study refers to the non-recoverable metaldehyde, which is essentially lost from the system i.e., is not present in either soil or leachate from the measurements taken using LCMS. This might include metaldehyde present as non-extractable residues, lost via volatilisation, and degraded through the biotic processes of microbes. Therefore, dissipation is referring to the system holistically i.e., soil and leachate in order to derive a mass balance from the total amount of metaldehyde that is recoverable.

4.2 Materials and Methods

4.2.1 Field sampling and soil selection for the study

Field sites comprised three contrasting soil types: a sandy loam (SL), a clay loam (CL) and a silty clay loam (SCL). These soils were used to construct lysimeters. Collection of 15 intact soil columns from the three field sites in North Yorkshire was done on 8/2/2020 and 12/2/2020. To examine the effects of cultivation on leaching, parallel samples were prepared in triplicate between recently cultivated soil and established soil conditions for the clay loam and sandy loam field sites. The silty clay loam field site had been ploughed in autumn to over-winter as fallow. All three field sites were used for growing cereals in the previous crop rotation. The soil-filled columns were stored in open bags inside an outdoors container unit prior to installation. The columns were made of PVC tubes, formally purposed for underground pipe, with an inner diameter of 160 mm and cut to lengths of 350 mm. The columns were packed with intact soil by compressing them into the ground with a sledgehammer, with the columns being bevelled at one end to facilitate forcing them into the ground, then the columns were dug out manually. The ends of the cores were covered to protect the columns while in transit to the laboratory.

Soil selection for the lysimeters included a clay loam soil and a sandy loam soil, sampled from the same location as the soil used for the degradation experiments in chapter 3, a heavy silty clay loam was also selected. Characterisation of soil properties was done externally by Forest Research Ltd. The lysimeter study aimed to compare ploughing vs min till soil management regarding effects on leaching, which is perhaps dependent on the depth of cultivated layer. The simulated plough cultivation conditions were prepared by digging with a spade to a plough depth of ca. 20 cm, then breaking up the soil to a till with a rake to represent fresh cultivation. Paired samples were produced

in triplicate, with three columns having replicated cultivation and the other three taken as the intact soil from the previous year's crop. The leachability was then compared between the lysimeters that had simulated fresh cultivation and those collected from soil with an established till from the previous year, which had a stubble cover. Three replicates of each were collected, with three contrasting soil types being tested: a total of 15 columns. The columns were pre equilibrated with water in accordance with the simulated rainfall application stated in the installation method before dosing and initial leachate was analysed for metaldehyde as a control.

The lysimeter experiment aimed to observe differences in metaldehyde leaching between contrasting soil management practices. The sampled fields were under wheat stubble from the previous year's crop, with re-growth present. First, the stubble was removed from the soil surface, then the simulated cultivation was done. The appearance of the disturbed soil was cloddy, which is typical for clayey soils, where it is more difficult to achieve a fine till and hence slugs can thrive in the crevices. For the non-cultivated soil columns, the stubble and re-growth on the surface was preserved, but with excess material removed by hand from the column surface. This was in order to be representative of slug pellets applied to an established crop or stubble scenario in the field versus applying slug pellets immediately after seed drilling. The depth of these columns was such that the columns penetrated the subsoil level, which appeared lighter in colour and was more compacted. This provided a holistic representation of leaching in the field from the cultivated horizon to subsoil. The clay loam and sandy loam fields had not been treated with metaldehyde for over two years.

The silty clay loam soil columns were the heaviest soil type used in this study, and were collected as three replicates from the field, which had already been cultivated at the back end of the year, before the weather turned inclement, which makes the field conditions such that travel with machinery is not possible. The conditions at the time of sampling were indeed very wet, with water stood in places. Sampling was undertaken at a risen area of the field to avoid digging in pools of water. The cultivated soil had large clods from ploughing (not worked to a fine till with a harrow) and these clods had settled to form a dense, coarse topsoil. The field had been treated with metaldehyde two years ago, and high levels (80 mg L^{-1}) had been detected in the field drains in a previous sampling excursion.

4.2.2 Installation and operation of the lysimeters

The columns were assembled at the laboratory on 23/3/2020. The bottoms were back picked level with the bottom of the pipe to remove smears that might block pores and affect leaching. The bottom of the columns was then covered with an endcap that had an 8 mm hole drilled into it centrally in which a connector to a length of PEX flexible pipe was fixed, which fed to a 250 ml bottle for collecting leachate. The endcap contained a space between the socket and coupler, which produced a void of 80 mm depth, which was filled with silica sand, which was wetted with 300 ml DI water to remove air pockets and ensure good contact with soil and to promote free draining. A layer of muslin cloth was placed at the bottom of the endcap beforehand to filter large particles from the leachate and stop the pipes from blocking. The columns were placed into two wooden crates, made from 60 cm wide x 120 cm long x 40 cm high boxes raised on legs, filled with building sand to modulate variations in temperature. The catchment vessels were placed underneath the boxes. Air was withdrawn from the 250 ml collection bottle with a 50 ml syringe (13 ml air withdrawn) via an extra pipe containing a valve to ensure a small continuous vacuum of approximately 50 mbar was applied at the bottom of the column to prevent waterlogging. Eight columns were contained per box (note that one was a dummy to fill the space). Columns were produced in triplicate to assess repeatability of results. The set-up of the columns is shown in Figure 13. The soil from each column was weighed accurately and moisture content determined following destructive sampling after the

experiment was stopped at 120 days. The completed lysimeters were stored outside under a shelter for the duration of the experiment. The set-up is summarised in Table 14.

Figure 13: Lysimeters set-up showing: Sandy loam columns (top left, with established soil on above row and cultivated soil on the bottom row; the bottom right column is a silty clay loam); silty clay loam columns (top right picture, the two on the far left); clay loam columns (top right, with cultivated soil on the above row and established soil on the bottom row); lysimeter crates (bottom left) and the coupler endcaps (bottom right).

Once installed, the columns were equilibrated with rainfall application on 27/3/2020. Originally, an "artificial rain" solution (140 ml of 0.01 M $CaCl_2$ in DI water) applied every 2-3 days over 14 days was to be used. However, restrictions to laboratory access due to the COVID-19 outbreak meant that the laboratory set up of the lysimeters was done at home and deionised water had to be used following the same application protocol. The application was done in accordance with the UK monthly average (MA) rainfall amount for March, which is when metaldehyde pellets would be applied to fields to protect spring crops. The artificial rainfall (140 ml water) was applied, and then the blank columns were allowed to drain before the next rainfall application. This initial leachate was collected and stored in a freezer at -4 °C to be analysed for residual metaldehyde within the field-collected soil and thus serve as a control. A blank water sample for each rainfall application was also saved to use as a control. It was noticed that rainfall soaked into the SL and CL columns relatively quickly, but the water pooled on the surface of the SCL columns. The applied suction, however, was not adjusted for these columns and was kept constant for all columns to enable cross-comparison.

Metaldehyde was then applied as an aqueous solution (this is instead of using solid powder mixed with silica sand (0.42 mg mixed in 5 g sand, with three applications) which was the original plan, again due to restrictions in laboratory access for weighing out chemical). The aqueous solution was applied centrally to the column by decanting 4.2 ml of a 0.1 mg ml^{-1} stock solution from a measuring cylinder on 6/4/2020. The dose is equivalent to the maximum allowed application at any one time of 210 g metaldehyde per hectare. Two further dosing events were applied at fortnightly intervals on 19/4/2020 and 2/5/2020. This overall fortification is approximately equivalent to the maximum recommended application rate suggested by the metaldehyde stewardship group (MSG) of 700 g

ai/ha/year, calculated from the area of the column surface, in order to represent the maximum allowed applications in a season. Due to the use of an aqueous solution of metaldehyde, rather than a solid formulation, initial leaching was expected to be faster and representative of a worst-case scenario of 100% chemical leaching in the first rainfall event.

Artificial rain was applied (140 ml per rainfall event, with 14 rainfall events in the month for March, spaced evenly every 2-3 days: amount, frequency and duration determined from UK monthly averages from Met Office data). The application method of the rain was done using a showering head to be even across the column surface and to avoid disturbance of the soil surface by the simulated rain. The leachate was collected in the vessels (250 ml bottles), placed under the column, at regular intervals. The volume removed was measured using a measuring cylinder. Samples were stored in a freezer at -4 or -18 °C prior to metaldehyde analysis by LC-MS.

Table 14: summary of the columns used in the experimental design of the lysimeters.

Column number	Details
C1	Clay loam, uncultivated
C2	Clay loam, uncultivated
C3	Clay loam, uncultivated
C4	Clay loam, cultivated
C5	Clay loam, cultivated
C6	Clay loam, cultivated
C7	Sandy loam, uncultivated
C8	Sandy loam, uncultivated
C9	Sandy loam, uncultivated
C10	Sandy loam, cultivated
C11	Sandy loam, cultivated
C12	Sandy loam, uncultivated
C13	Silty clay loam, cultivated
C14	Silty clay loam, cultivated
C15	Silty clay loam, cultivated

4.2.3 Destructive sampling of soil columns

At the end of the 120-day experiment, the soil columns were removed from the lysimeters, and the soil was manually removed by digging with a trowel. The soil was separated into five fractions according to depth: 0-15 cm, 15-25 cm, 25-30 cm, 30-35 cm, and the sand fraction at the column base. These specific sections were chosen with decreasing thickness to provide greater resolution with column depth to account for the variability in subsoil properties versus the homogeneity of the cultivated layer at the top. Thus, trends between soil properties with depth and metaldehyde accumulation could be later inferred. The soil from each fraction was weighed separately, then mixed thoroughly in a bucket using the trowel to homogenise as well as possible. A subsample of the soil (approx. 80 g collected in a sample tube) was then taken back to the laboratory for analysis. Analysis was performed the next day and involved extraction of the soil samples (20 g subsamples in triplicate) in accordance with the validated methodology from chapter 2. The extracts were then analysed by LC-MS, using the established protocol from chapter 2. The soil moisture content was also measured for the surface layer (0-15 cm) and the subsoil layer (30-35 cm) for each soil column. Additionally, a sub sample from each of the soil samples collected from the lysimeter experiment were saved for molecular microbiology analysis. Any soil saved for further analysis, including the samples for molecular analysis were stored in a freezer at -18 °C.

4.2.4 Model comparison to lysimeters using measured degradation data

This study aimed to determine improved predicted environmental concentrations (PECs) in surface water. Environmental fate modelling was undertaken using input parameters, summarised in Table 15, and included biotransformation rates derived during the batch degradation assessments from chapter 2. It was hypothesised that measured degradation data obtained for a range of environmentally realistic temperatures and soil moisture contents would help to produce more reliable environmental fate assessments, which reflect the metaldehyde soil concentrations measured from the lysimeters.

To create simulations, three crop scenarios were selected: winter and spring cereals and oil seed rape, winter sown. These two crop types were chosen based on the reasoning that they are at the most risk from slug damage and therefore where most metaldehyde is used. Surface water pollution was simulated for drain flow water. This information could then be compared to actual pollution monitoring data from the field and lysimeter experiments. For the modelling, relevant environmental scenarios were selected, as detailed in Table 16 of the results section. The metaldehyde application method was amended to be soil incorporated, with one application of the maximum allowed dose of 0.7 kg per hectare. Soil incorporation was adjusted to be soil incorporated uniformly to a depth of 20 cm (uniform wash-in to surface cultivated soil after rainfall degrading the pellets). The application window was left as the default: 15 days prior to drilling. With these conditions set, the simulations were run.

The simulations were exported to (1) PRZM (surface water model), and (2) MACRO (drain flow model). The pesticide root zone (PRZM) simulation was run with the environmental fate parameters outlined in Table 14. In MACRO, to calculate drain flow, the individual crop scenarios were selected, namely cereals, spring and winter, and oilseed rape (winter sow) as well as the predefined soil and rainfall scenarios, D1-D6 outlined in Table 16. The defined application was selected for each scenario, then the simulations were executed as a batch. The results from the calculation to determine the predicted environmental concentrations of metaldehyde in drain flow were added to the final report. The simulations were then run again using modified values for disappearance half-life obtained from the batch laboratory experiments outlined in chapter 3. For crop processes, wash-off from canopy was set to the minimum value (1E-6 m^{-1}), the half-life on crop canopy was set to the minimum value of 1 day, and the coefficient for uptake by plants was set to zero. These are worst-case scenario values.

Table 15: Physico-chemical properties of metaldehyde input parameters for SWASH_5.3 modelling and for sorption properties of metaldehyde under soil equilibrium conditions and transformation processes in the environment.

Property	Value
Molar mass	176.21 g mol^{-1}
Saturated vapour pressure	87.99 Pa (measured at 25 °C)*
Molar enthalpy of vaporisation	41.3 kJ mol^{-1}†
Solubility in water	188 mg L^{-1} (measured at 20° C)*
Molar enthalpy of dissolution	27 kJ mol^{-1}‡
Reference diffusion coefficient in water	4.3E-5 m^2 day^{-1}‡
Reference diffusion coefficient in water	0.43 m^2 day^{-1}‡
K_{OC} (pH independent)	50 cm^3 kg^{-1}
Freundlich sorption exponent (1/n)	0.9918‡
Surface water (equilibrium sorption in suspended solids)	50 L kg^{-1}
Soil aerobic transformation half-life (not biphasic)	46 days (measured at 20 °C)
Soil moisture content at which transformation was measured (relative to field capacity)	60%
Exponent for the effect of liquid	0.7‡
Q10 factor for the effect of temperature on transformation	2.58
Temperature effect K^{-1}	0.0948‡
Half-life in water body	1000 days‡
Half-life in sediment	1000 days‡

***** Data taken from pesticide properties database.

† Data from Chemspider, predicted values using ACD/Labs calculator

‡ Default parameters from SWAH_5.3 model

Metaldehyde is described in the literature as being a relatively mobile compound, with moderate sorption to soil organic matter. The selected organic carbon sorption coefficient of 50 L^3 kg^{-1} (lower range from the EFSA (2010) data set) was to represent metaldehyde as a mobile compound and serve as a worst-case for leaching. The permanent pasture soil used for batch experiments (A)1.0-4.1 had a soil organic carbon content of 2.79 %, which, based on Figure 1.3 would be expected to have a small organic carbon coefficient, therefore the lower values were taken to be most realistic for the scenario-based investigation using half-lives derived from the laboratory. A range of DT_{50} values were chosen that covered the broadest ranges of the results from the batch degradation experiments (chapter 2) and used as input parameters for the model. Namely, the worst-case pollution risk with the highest measured DT_{50} of 4150 days from experiment (A)3.1, and the appropriate reference experiment (A)1.0, with a DT_{50} of 46 days for comparison.

PECs for soil and drain water were also derived using excel models (available at https://www.hse.gov.uk/pesticides/pesticides-registration/data-requirements-handbook/fate/environmental-fate-models.htm). The output of these models was to be compared with the results from the MACRO drainage water simulations, so included the same degradation rate input parameters to enable direct comparison. Namely, dissipation half-lives of 46 days and 4150 days, which were derived for batch experiments (A)1.0 and (A)3.1 respectively.

To calculate the PEC for soil, the DT_{50} value was entered into the spreadsheet (either 46 days or 4150 days) along with the application rate and number of applications. For these calculations, an application rate of 240 g ha^{-1} was used with three application events at 14-day intervals. This was in fitting with the method implemented for the lysimeter experiment and in line with the guidelines for metaldehyde application in the field (MSG, 2018). The model assumed a soil bulk density of 1.5 g cm^{-3} and an equal distribution of the pesticide in the 0-5 cm layer. Because metaldehyde is applied as pellets, the FOCUS crop interception values used in the model may not apply. Therefore, these were adjusted in the spreadsheet to zero in order to generate a worst-case scenario. The results from these calculations were used to generate a graphical representation of how metaldehyde concentrations in the soil might change over the course of four months, depending on the dissipation half-life. Furthermore, the calculations were used to predict an accumulation concentration of metaldehyde over the course of twenty years, with the aforementioned annual application regime.

To calculate the PEC in drain water, parameter settings were adjusted to reflect the experimental conditions of the lysimeter experiment. The application rate was set at 240 g ha^{-1}, with three applications at 14 day intervals, as per the method implemented for the lysimeters. The first day of drain flow assumed by the model was set to 01-March, to reflect the timing and nature of the lysimeter experiment, and to represent spring-time application of metaldehyde, before drain flow decreases with the onset of dryer summer conditions. The application timings were then set to the appropriate dates, according to the lysimeter experiment. An organic carbon sorption coefficient (50 L^3 kg^{-1}) was selected based on the lower end of values determined in the literature (refer to Figure 1.3 in chapter one) to reflect metaldehyde as a mobile compound, giving a worst-case for leaching risk.

This modelling exercise, to calculated PECs in drain water, aimed to assess the influence of degradation rate on metaldehyde concentrations that might be expected in drain water. Therefore, a range of DT_{50} values determined during the batch experiments were selected to generate a range of predicted concentrations in drain water to compare with the findings from the lysimeters. The model assumes degradation between the application intervals, but no degradation after the final application. This is when drain flow begins, which is assumed to be a 100,000 L drain flow event.

4.3 Results and Discussion

4.3.1 Hydraulic observations

The CL uncultivated columns (C1-C3) drained very quickly following rainfall application, which immediately soaked into the soil. Leachate drained into the catchment flask immediately following application initially, then the drainage rate subsided to a dropwise flow after ca. five minutes. The column surfaces had small pores visible due to the activity of soil macro fauna, which is likely to have facilitated the drainage via preferential flow routes.

The CL cultivated columns (C4-C6) also drained quickly; water immediately soaked into the soil, but drainage into the capture flask was slower than that of the uncultivated CL columns. All three of the CL cultivated columns were apparently free draining, but the drainage pattern was slightly more fluxional than the uncultivated columns, which was attributed to disruption of the pre-established preferential flow routes. The cultivation process may have disturbed the eggs/larvae of the soil macro fauna, therefore there was less visible activity from these organisms. Additionally, the cultivated till was cloddy in nature and these clods could have amalgamated to an extent due to the sticky nature of the soil and therefore the columns would be less porous and preferential flow, which is key to the drainage of heavy soils, is reduced.

Of the columns selected for the lysimeter study, the clay loam columns (C1-6) were the freest draining. These columns were free draining from the start of the 120-day experiment, as demonstrated from the bars in Figures 14 and 15, which show that most of the columns drained at a rate >0.02 L day^{-1} and so leachate could be collect from the start of the experiment. There was no obvious disparity in drainage rates between cultivated and uncultivated columns for the clay loam soil.

The SL uncultivated columns (C7-C9) demonstrated a varied drainage pattern. Column C7 was successful in yielding a leachate early in the experiment, but the other columns C8 and C9 did not. Eventually, however, these columns started to drain freely (see Figure 16). These columns were not as free draining as the CL columns (C1-C6); rainwater applied to the surface of the column stood for a while before soaking in and some evaporation is likely to have taken place as well. The surface of the columns was hard and smooth, with no visible pores, therefore there was unlikely to be significant preferential flow processes occurring, when compared to the CL columns, illustrating the importance of this water transport mechanism with regards to leaching potential. Because the columns did drain well in general, it is likely that the standing water must first pass soil that has been subjected to surface compaction, then can drain more freely in deeper soil.

Some of the SL columns were reluctant to drain initially. The uncultivated columns (C7-9, Figure 16) generally did not begin to provide leachate until collection on day 49 of the experiment. Column 7 did start to drain earlier, but this may have been a result of some inconsistency, such as preferential flow down the sides of the column. These intact soils were well-compacted; thus, transport of water was with lower velocity. For the cultivated parallel samples (C10-12), except for column 12, which may have suffered with blockages, drainage was freer, and leachate could be collected earlier in the experiment. The SL cultivated columns (C10-C12) where the compacted surface soil was freshly disturbed did not have standing water after rainfall application and drained freely. The broken-up soil probably had a greater propensity for preferential flow pathways than the compacted undisturbed columns. After a short lag time, all these columns drained freely, as shown in Figure 17.

Lastly, The SCL soils (C12-C15) were the least permeable of all. Water pooled on the column surfaces and barely drained. Consequently, every other rainfall application day had to be omitted during the course of the experiment. This gave the pooled water an opportunity to disappear by slow drainage and evaporation from the surface. Once the columns had dried, desiccation cracks on the surface provided a route for water transport and preferential flow then meant that the columns could start to drain freely, as shown in Figure 18. The extent of the cracking determined the flow velocity. Column 15 had the largest cracks and consequently had a greater flow rate than the other replicates. Column 13 did not crack and did not drain, and therefore this replicate was abandoned.

Edge effects as a means of preferential flow might be a factor contributing to the observed fast drainage of some of these columns. The interface between the soil and the edge of the plastic pipe is likely, albeit to different extents between the columns, to provide a route for water movement with less resistance compared to movement through soil pores alone. This can be particularly true for the clay loam soils, which exhibit greater contraction in periods of dehydration than sandy soils, thus increasing the gapping at the edge of the columns. This is one reason why metaldehyde was applied centrally to the column, to maximise the amount of the compound traveling through the soil as opposed to being flushed down the edges. The SL columns presumably exhibit lower preferential flow; this judgement being based on the observation that water stands on the column surface for a period, so tight contact between the soil and the column edge can be assumed. This was a consideration that had to be kept in mind for analysis of the results. However, analysis revealed that

the drainage and resulting concentration of metaldehyde in leachate were in line with model predictions, so edge effects may be negligible in this instance.

Table 16: Overview of results and experimental design summarising the hydraulic observations and concentration of metaldehyde in leachate from the different soil columns used during the lysimeter experiment.

Soil type	Column number	Peak drain flow (L/day)	Mean drain flow (L/day)	RSD	Peak conc (µg/L)	Mean conc (µg/L)	RSD
Clay loam (intact)	C1	0.057	0.031	0.012	14.760	3.408	4.611
	C2	0.056	0.031	0.014	8.291	0.568	1.854
	C3	0.063	0.034	0.014	3.821	0.468	1.032
Clay loam (cultivated)	C4	0.048	0.027	0.013	4.374	0.354	0.966
	C5	0.063	0.018	0.021	2.104	0.157	0.486
	C6	0.050	0.025	0.015	1.974	0.362	0.543
Sandy loam (intact)	C7	0.037	0.025	0.010	17.118	1.746	4.120
	C8	0.031	0.008	0.012	146.905	12.373	34.373
	C9	0.041	0.013	0.015	239.000	29.185	61.596
Sandy loam (cultivated)	C10	0.041	0.025	0.013	1.803	0.220	0.476
	C11	0.042	0.025	0.015	1.148	0.060	0.256
	C12	0.056	0.005	0.014	2.533	0.207	0.631
Silty clay loam	C13	0	0	0	0	0	0
	C14	0.013	0.003	0.004	164.414	19.673	50.224
	C15	0.031	0.008	0.012	3.602	0.575	1.075

4.3.2 Metaldehyde breakthrough in leachate

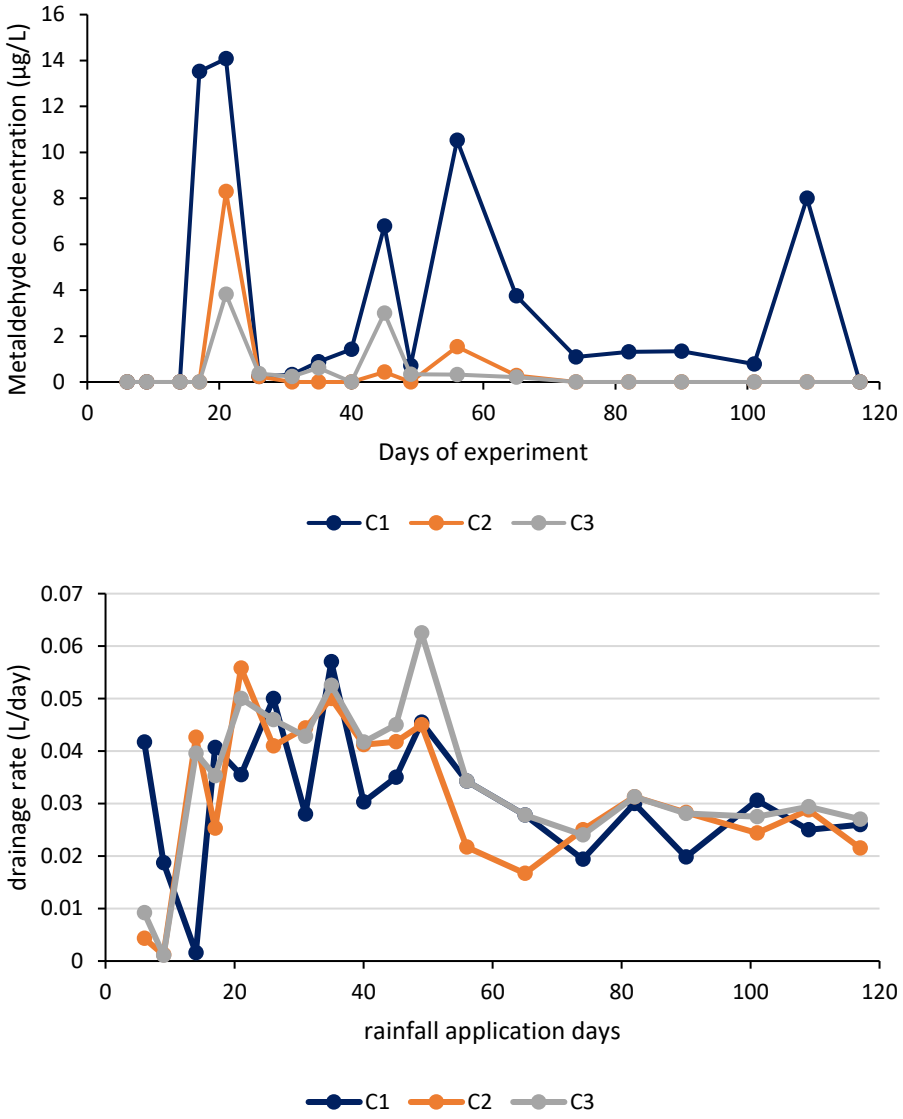

Figure 14: Breakthrough curve for metaldehyde in leachate from the clay loam uncultivated soil columns, with average drainage rate between the collection points shown by the chart below.

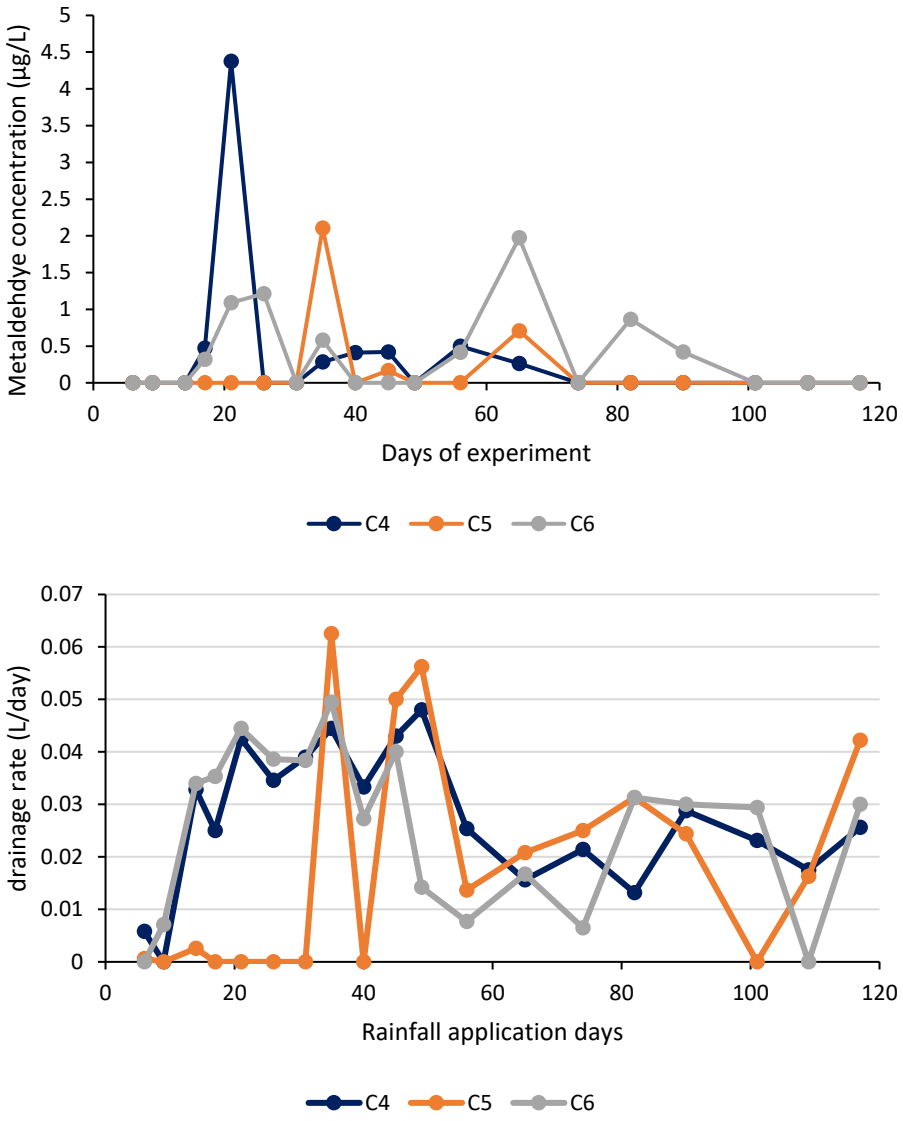

Figure 15: Breakthrough curve for metaldehyde in leachate from the clay loam cultivated soil columns, with average drainage rate between the collection points shown by the chart below.

Metaldehyde breakthrough in the leachate occurred as three peaks, mirroring the application regime. Metaldehyde was applied on day 10, day 13 and day 36 of the experiment. The peak concentration for metaldehyde in the leachate from the clay loam columns occurred on day 21 of the experiment, suggesting a lag phase of 11 days between application of aqueous metaldehyde and peak concentration in drain flow. However, it must be noted that the leaching process would be retarded by the disaggregation of slug pellets applied to the field before they release metaldehyde, and the lysimeters represent a "worst case" for metaldehyde leaching in drain flow.

The peak concentrations on day 21 of the experiment varied between the cultivated and uncultivated samples. The highest concentration detected in leachate for the clay loam soils was 14.1 µg L^{-1} from column 1, compared to a peak concentration of 4.4 µg L^{-1} for the cultivated columns, despite these replicates having comparable drainage rates. The latter two peaks occurred on days 45 and 56 for the uncultivated columns, and on days 35 and 65 for the cultivated columns and generally comprised a lower concentration of metaldehyde than was measured on the initial break though. However, there was a degree of variability between the three replicates.

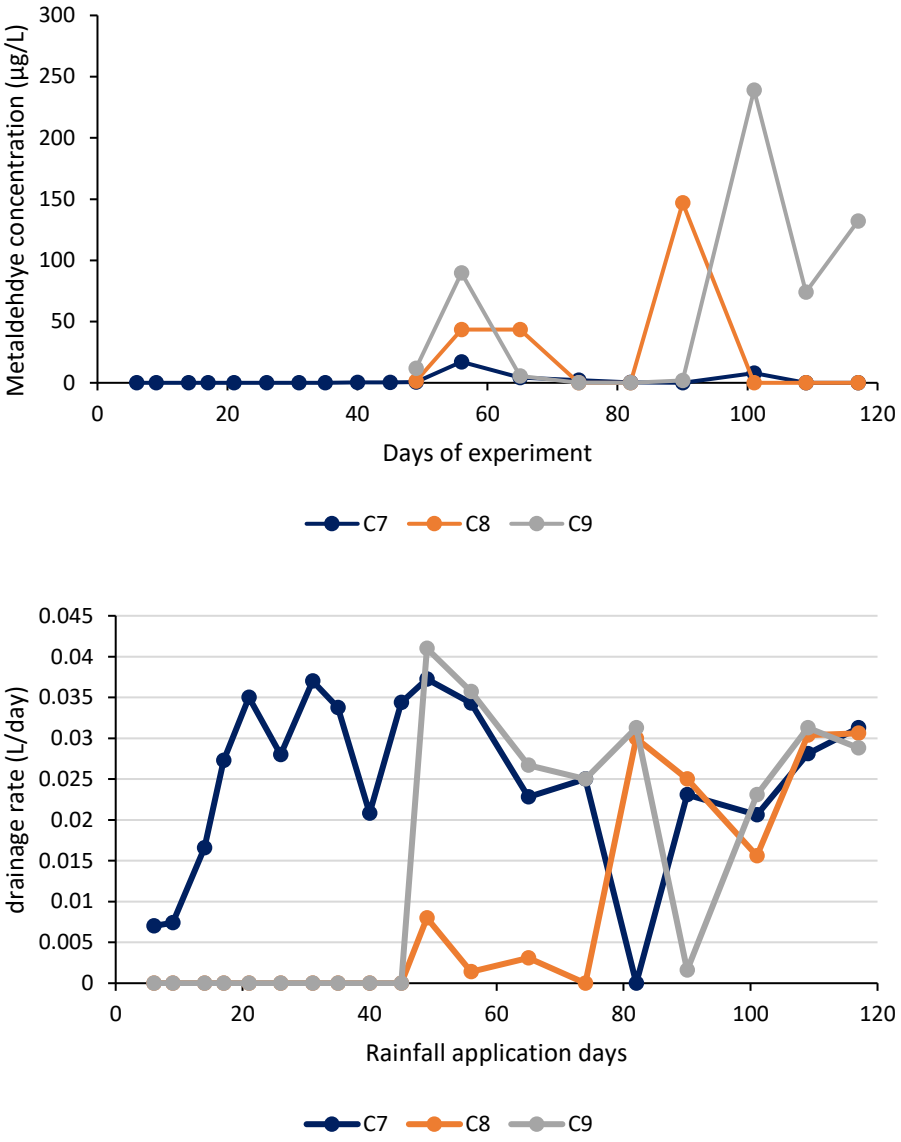

Figure 16: Breakthrough curve for metaldehyde in leachate from the sandy loam uncultivated soil columns, with average drainage rate between the collection points shown by the chart below.

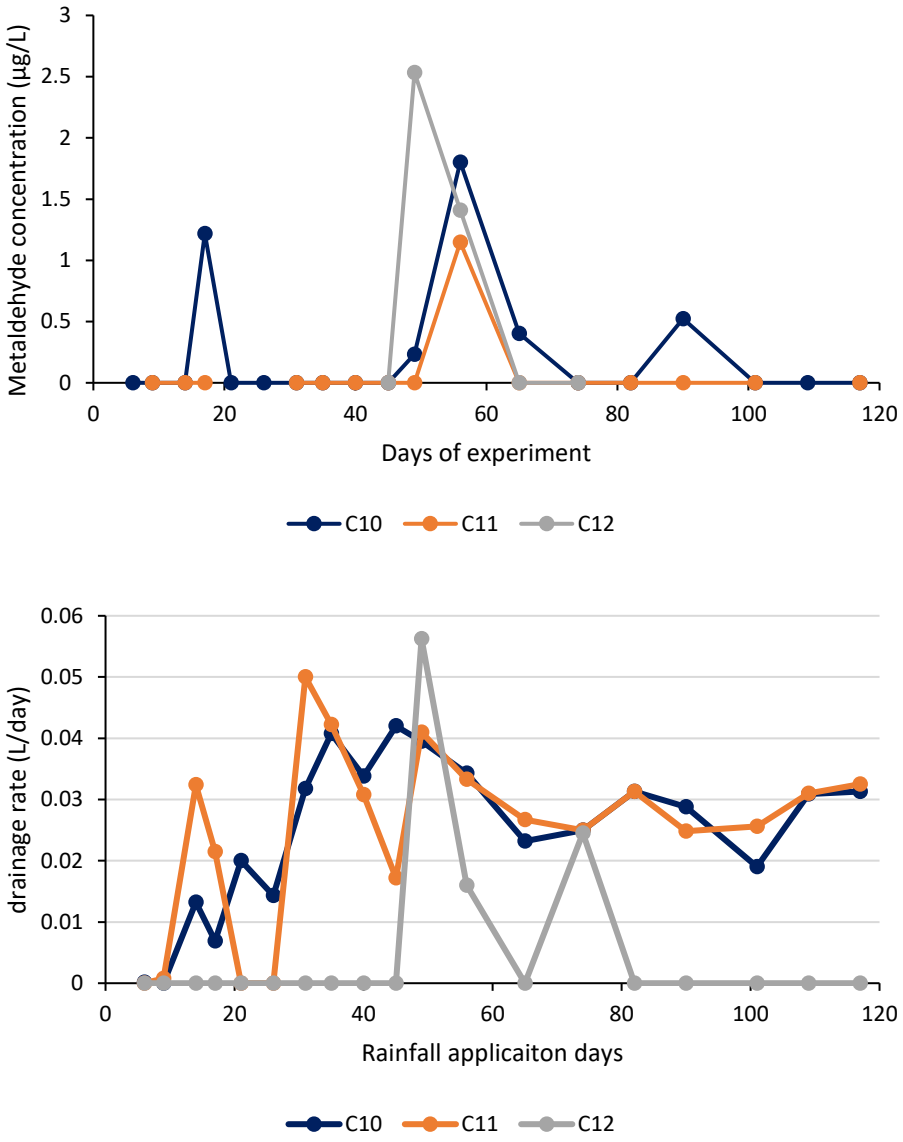

Figure 17: Breakthrough curve for metaldehyde in leachate from the sandy loam cultivated soil columns, with average drainage rate between the collection points shown by the chart below.

There was a greater variability in the drainage output of the sandy loam columns compared to the clay loam columns. Only one of the uncultivated columns drained throughout the experiments; the other two replicates commenced drain flow from day 49 and eventually drained with a comparable velocity to the first replicate. Consequently, metaldehyde breakthrough did not occur until after this day. Intriguingly, metaldehyde was not detected at comparable concentration in the free draining column 7; being much lower. Peak metaldehyde concentrations of 146.9 µg L^{-1} and 89.8 µg L^{-1} in the leachate occurred on days 90 and 101 for columns 8 and 9, respectively. These measured concentrations were approximately ten times greater than those measured for the free draining clay loam columns, which leached metaldehyde over the duration of the experiments, rather than during a surge towards the end.

For the cultivated sandy loam columns, drainage was freer; apparently disturbing the soil in the simulated cultivation process broke up the compaction observed for the undisturbed soil surface, which was attributed to retarding the drainage processes in the uncultivated columns. Collection of leachates from columns 10 and 11 started from day 14 of the experiment. Column 12 drained less

well and leachate was only collected on three sampling occasions. Metaldehyde concentration measured in column 10 leachate exhibited the characteristic three peaks associated with the metaldehyde dosing regime, with peak breakthrough concentrations being measured on day 17, day 56 and day 90 of the experiment. The highest measured concentration for this replicate was 1.8 µg L^{-1}. Columns 11 and 12 demonstrated a single breakthrough peak on day 49 and 56, respectively, with measured concentrations of 1.1 µg L^{-1} and 2.5 µg L^{-1}.

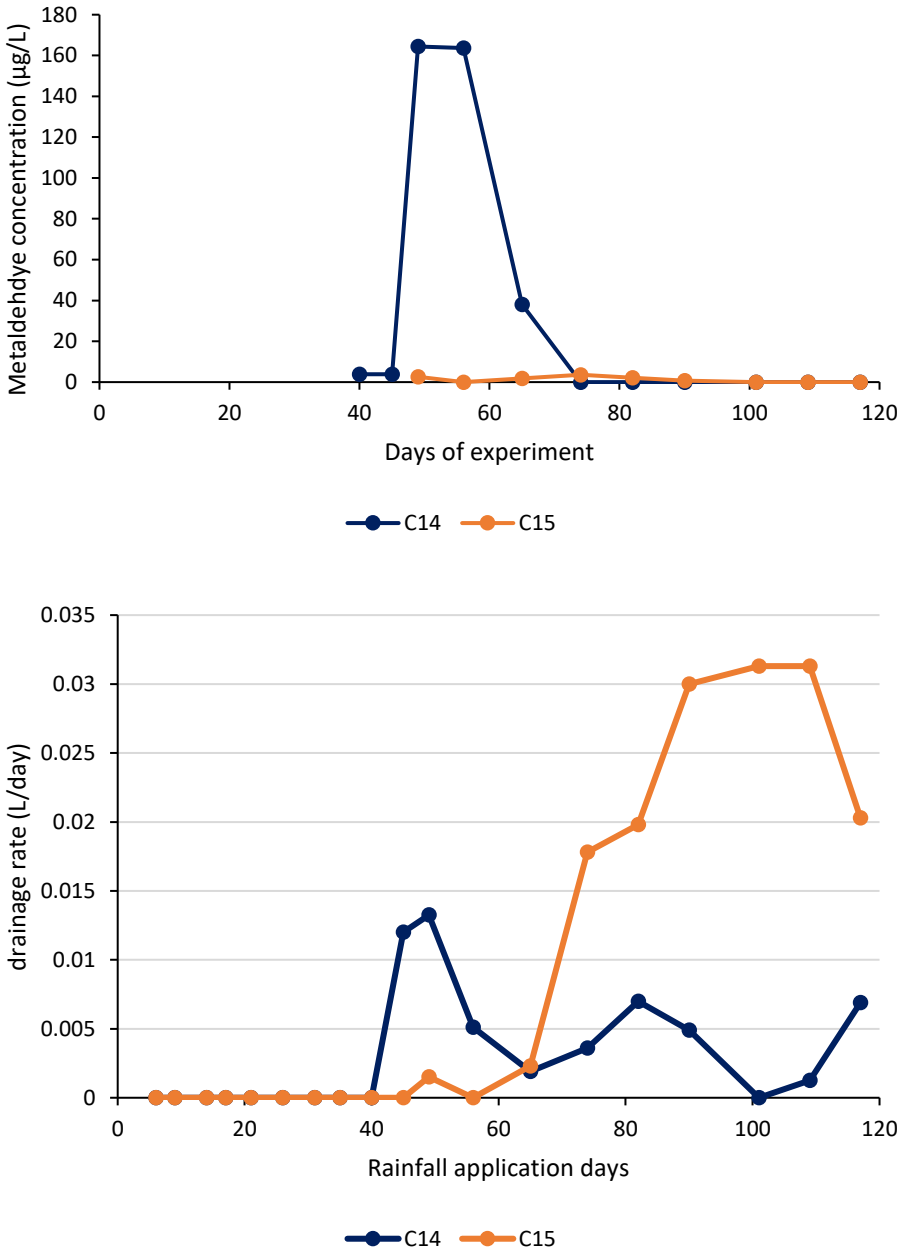

Figure 18: Breakthrough curve for metaldehyde in leachate from the silty clay loam uncultivated soil columns, with average drainage rate between the collection points shown by the chart below.

The silty clay loam columns were the least well-draining, hence the rainfall application had to be reduced. However, reduced rainfall provided an opportunity for the columns to dry, and desiccation cracks appeared on the surface of columns 14 and 15, and preferential flow enabled drainage to proceed, and leachate was first collected on day 45. Metaldehyde leached from column 14 in a band of high concentration (up to 164.4 µg L^{-1}). Column 15 had the greatest cracking on the surface and

drained with a much greater velocity. This meant that metaldehyde retained in the column was washed out at a much lower concentration.

Metaldehyde concentrations in drain water in the field scenario, as indicated from the lysimeter experiments, will depend on how free draining the soil is. In well drained fields, drain flow might be expected to contain lower concentrations of metaldehyde for a longer period. For fields where rainwater less readily drains, a higher concentration of metaldehyde might be expected for a short period as the drains begin to run and wash out residues of metaldehyde retained in the soil. This idiosyncrasy was demonstrated by the field excursion to intake lane, where metaldehyde was detected in drain water at high concentration when the drains started to run and was then observed to decline a short time after, as the drain flow increased velocity. This result was repeated for the same silty clay loam soil during the lysimeter experiment, also demonstrating a "washout" of metaldehyde.

4.3.3 Metaldehyde concentration profile in soil columns

Soil column fractions were extracted for residual metaldehyde and demonstrated a range of concentration distributions through the different depths of the soil profile. For the clay loam columns (Figure 19), most of the retained metaldehyde was present in the top cultivation horizon (0-15 cm). In the sandy loam columns (Figure 20), metaldehyde was present mostly in the range 0-30 cm depth, while in the silty clay loam columns, metaldehyde concentrations were distributed throughout the soil profile.

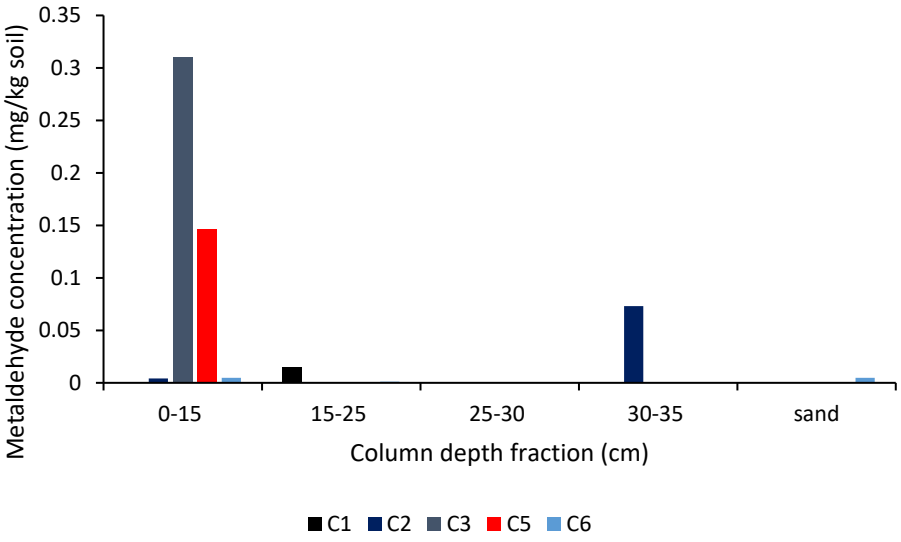

Figure 19: Metaldehyde concentration profile with soil depth for the clay loam columns

The clay loam columns had some of the highest concentrations of retained metaldehyde in the soil, with column 3 containing up to 0.31 mg kg^{-1} soil. The greatest proportion of the retained metaldehyde was present in the cultivated horizon (0-15 cm depth) and may be attributable to an adsorbed fraction in the soil which resulted from the binding to organic matter and soil particles shortly after application of metaldehyde to the soil.

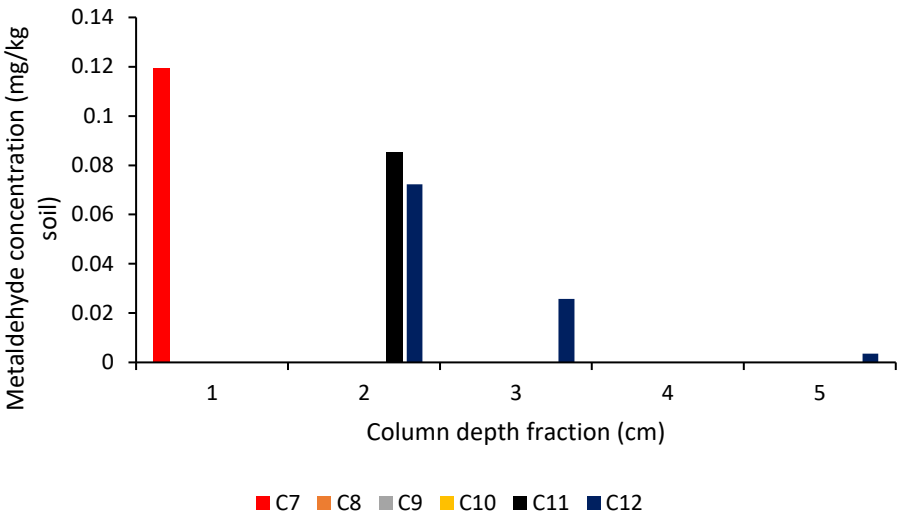

Figure 20: Metaldehyde concentration profile with soil depth for the sandy loam columns

Residual metaldehyde in the sandy loam soil columns was generally distributed over a greater range of the soil profile from 0-30 cm, at lower soil concentrations than those measured for the clay loam columns. The highest measured concentration was, again, present in the cultivated horizon (0-15 cm depth) and measured 0.12 mg kg^{-1} for column 7, which was uncultivated and only released a low concentration of metaldehyde in the leachate, hence it was expected that more would be retained in the soil or had been biodegraded. The other replicates, C8 and C9, which did release a greater quantity of metaldehyde in the leachate, did not have detectable levels of residual metaldehyde present in the soil. For the cultivated sandy loam soil columns, C11 and C12, metaldehyde residues appear to have leached and are mostly retained at 15-25 cm depth at concentrations of 0.085 mg kg^{-1} and 0.072 mg kg^{-1}. Column 10, which yielded more metaldehyde in the leachate, had no detectable levels of metaldehyde remaining.

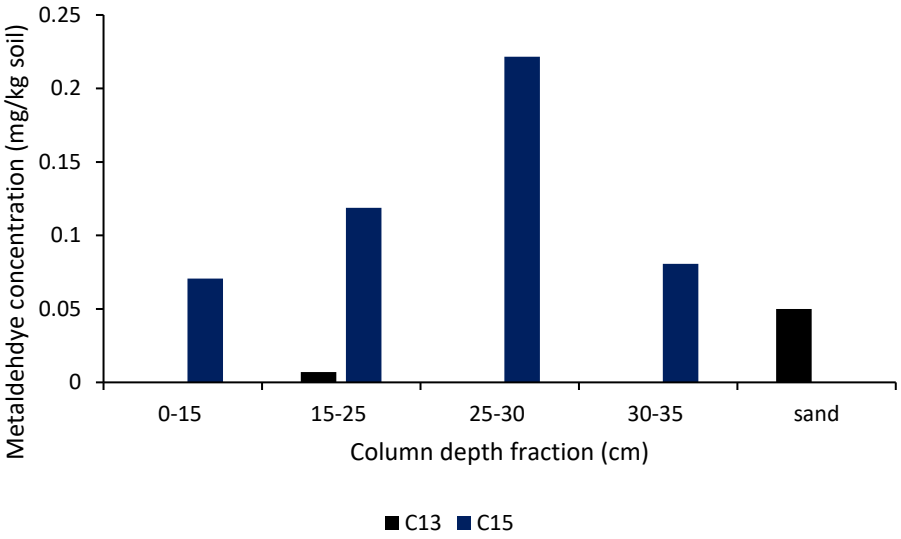

Figure 21: Metaldehyde concentration profile with soil depth for the silty clay loam columns

Metaldehyde residues in the silty clay loam columns were distributed throughout the soil profile. The highest concentration was detected at 20-30 cm depth for column 15, at 0.22 mg kg^{-1}. Significant concentrations were present in all the other depth fractions across both columns 13 and

15 also. A possible reason for the broader distribution of metaldehyde throughout the SCL columns is the slow drainage, which would give the chemical the opportunity to slowly leach through the soil, rather than being quickly washed out. If degradation rates were slow in the soil, this could lead to the potential for metaldehyde to accumulate in these heavier soils with subsequent applications, creating legacy pools.

Summing the metaldehyde collected in leachate and the amount extracted from the soil revealed that there was less metaldehyde dissipation, for example from biodegradation, than would be expected based upon the results from the batch biodegradation assessments in chapter 3. With reference to Figure 22, for many of the columns, a significant amount of the applied metaldehyde remains after the 120 days of the experiment. This may not have been expected, given that metaldehyde at a comparable fortification concentration was shown to degrade completely within the 60-day batch experiments.

High residue concentrations of metaldehyde were detected in topsoil as well as in subsoil, depending on the column type and so leaching to subsoil is not necessarily the cause for metaldehyde persistence in the soil, as suggested in the research hypothesis. Rather it could be that greater heterogeneity and less ideal contact of metaldehyde with the entire soil matrix and associated microbial community could explain why high residue levels were detected in the soil after 120 days, compared to the complete degradation in 60 days observed in the same soils during the batch degradation experiments.

The persistence of metaldehyde in soil would be less influential to surface water pollution during circumstances where rapid draining leads to the fast leaching of metaldehyde within a time frame where there is little opportunity for degradation to occur. The persistence in soil only becomes important when field drainage is poor and metaldehyde is retained in soil and the potential for legacy pools to build up with seasonal application of the pesticide could lead to a longer-term environmental contamination by metaldehyde, which could lead to gradual leaching from soil into surface waters.

At least one column from each soil type had a replicate where there was <50% dissipation. Column 9 was the most conspicuous result, where 90.1% of applied metaldehyde was recovered. From Figure 16, it is possible that metaldehyde accumulated on the surface of the column, as drainage was limited up until the surface dried and allowed water to pass, washing the metaldehyde straight through the column. This could be an explanation for no metaldehyde being recovered from the soil fractions (Figure 20). The second most persistent case for metaldehyde was in column 15, with 65.6% remaining. The feature that these columns have in common is poor drainage, meaning that the soil is likely to be saturated for a greater length of time after rainfall application, producing an anaerobic setting in which metaldehyde could not easily be degraded in the soil. There is no clear trend between soil type and persistence. This was also true for the batch degradation experiments. Instead, persistence could be related to soil conditions; it was indicated that moisture content might affect metaldehyde biodegradation rates during the batch experiments, and it is shown from the lysimeters that metaldehyde was most persistent in the water-logged columns. Furthermore, poorly draining soils benefited from both drainage and metaldehyde biodegradation, which mirrors the observations for the pasture and arable soils in the batch study.

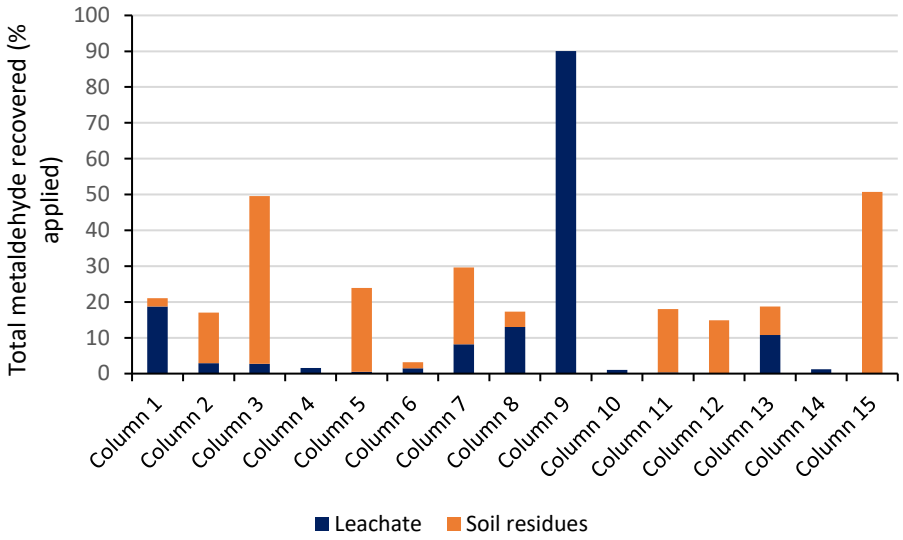

Figure 22: The amount of metaldehyde recovered from leachate and residues from the soil columns within the lysimeter experiment to illustrate the quantity dissipated during the course of the 120-day experiment when 1.26 mg of metaldehyde was originally applied.

4.3.4 General discussion

The standard OECD 312 (2004) guideline for soil column leaching experiments suggest using disturbed soil, which has been thoroughly homogenised by sieving 2 mm with uniform packing. The soil is collected from the top 20 cm of the field, so that the column consists entirely of surface soil. The advantage of this is that the homogeneity benefits the reproducibility of the results from the leaching assessments. However, although the guideline serves as a relative comparison of leaching in different soil types, these conditions are not realistic of field conditions. For specific compounds that are identified as an environmental concern, the use of intact soil cores for leaching studies are likely to give a more representative assessment of the compound's leaching behaviour in the field.

Experiments of this kind could be more useful to conduct a soil depth risk assessment if undisturbed field soils are used. Given that metaldehyde has been shown to degrade relatively quickly in aerobic batch experiments (e.g., Balashova et al., 2020; Bond, 2018; EFSA 2010), leaching to lower soil where conditions are expected to be more anaerobic might be an important parameter to factor into environmental fate models. Moreover, during this experiment, water ponding on the surface of the columns will have created and anaerobic environment even in the topsoil, which have also contributed towards the lack of metaldehyde degradation.

Standard practices perform leaching columns to identify how much of the compound is immediately washed through the soil. Assessment of aged residues, where metaldehyde is present in the column over time might be useful for assessing the amount of the compound that could leach in periods after initial application of slug pellets, and to what depth in the soil that the highest residue concentrations might be found. This could help to identify legacy pools of metaldehyde in the field. This will also relate to application rates in terms of number of pellets on the surface, type of pellet etc. (Bond 2018) and could be used to predict the washing of metaldehyde into field drains, whereby it will ultimately enter surface water.

The experiments done in this study encompassed different soil types of varying OC, pH and PSD. Required information of the field sample cores included: the history of the field site; application of agrochemicals, inorganic fertilisers, cropping, and previous test substance application.

Measurements were taken of the leachate volumes with time, and the concentrations of metaldehyde within the leachate. The concentration of metaldehyde within different segments of the soil column were also determined and used to create a graphical plot of concentration vs depth of the soil segment. A statistical analysis was performed to identify trends between the leaching behaviour of metaldehyde and the properties of the soils. These data could then be used for modelling purposes and the derivation of a risk assessment for surface water exposed through field drains.

The effect of cultivation on leaching is not widely examined in the literature. Despite this, it could potentially be an important aspect of risk assessment and for model building. Particularly in recently disturbed soil, as when seed beds are being prepared, water travel and chemical leaching could be expected to be greater at this time, compared to the leaching that occurs on established seed beds. This is also when metaldehyde slug pellets tend to be applied, when the crop is immature and vulnerable to slugs. The extent of leaching occurring from cultivated soil will likely be influenced by rainfall amount and frequency. Therefore, examining these environmental parameters might be of importance to exposure assessments for metaldehyde specifically in the UK scenario.

4.3.5 Model predictions and risk assessment

Metaldehyde was shown to degrade rapidly during the batch degradation experiments. Therefore, persistence in the environment would not be expected. Consequently, the DT_{50} values selected for the modelling inputs represent realistic environmental scenarios where metaldehyde was shown to remain for a longer period of time in the soil under conditions of high soil moisture content, which would be expected for autumn conditions in the UK. The longest measured DT_{50} value of 4150 days, from experiment A(3.1) was chosen for maximum effect in order to identify how the PEC might be affected in a situation where metaldehyde showed limited degradation. Specifically, in a worst-case scenario where pasture grass land is converted to arable use, and under high soil moisture conditions. These experiments were compared with the experiment A(1.0) with DT_{50} of 46 days, to serve as a point of reference. The results of the modelling are shown in Table 17.

Table 17: Results for the predicted environmental concentrations of metaldehyde in drain flow from scenarios with varied input DT_{50} parameters for the model.

Simulation	Scenario	Max concentration (µg L^{-1})	Lost to drains (mg m^{-2})	Dissipated (mg m^{-2})
1	Oilseed rape, winter (DT_{50} 46 d); (D2) clay, rainfall 642 mm yr^{-1}	2.62	0.38	83.02
2	Oilseed rape, winter (DT_{50} 46 d); (D4) loam over sandy loam, rainfall 659 mm yr^{-1}	47.3	7.82	67.14
3	Oilseed rape, winter (DT_{50} 46 d); (D5) loam over clay loam, rainfall 651 mm yr^{-1}	25	5.02	79.31
4	Cereal, winter (DT_{50} 46 d); (D1) silty clay over clay, rainfall 556 mm yr^{-1}	1.02	0.14	91.77
5	Cereal, winter (DT_{50} 46 d); (D3) sand, rainfall 747 mm yr^{-1}	10.9	4.43	90.67
6	Cereal, winter (DT_{50} 46 d); (D4) loam over sandy loam, rainfall 659 mm yr^{-1}	52.5	8.18	71.14
7	Cereal, winter (DT_{50} 46 d); (D5) loam over clay loam, rainfall 651 mm yr^{-1}	42.1	6.44	80.64
8	Cereal, winter (DT_{50} 46 d); (D6) clay loam over clay, rainfall 683 mm yr^{-1}	79.6	11.14	85.57
9	Cereal, spring (DT_{50} 46 d); (D1) silty clay over clay, rainfall 556 mm yr^{-1}	0.43	0.06	76.76
10	Cereal, spring (DT50 46 d) (D4) loam over sandy loam, rainfall 659 mm yr-1	17.4	3.65	68.06
11	Cereal, spring (DT_{50} 46 d); (D5) loam over clay loam, rainfall 651 mm yr^{-1}	4.42	1.24	70.29
12	Oilseed rape, winter (DT_{50} 4150 d); (D2) clay, rainfall 642 mm yr^{-1}	10.4	3.26	10.04
13	Oilseed rape, winter (DT_{50} 4150 d); (D3) sand, rainfall 747 mm yr^{-1}	258	102.94	-6.92
14	Oilseed rape, winter (DT_{50} 4150 d); (D4) loam over sandy loam, rainfall 659 mm yr^{-1}	314	70.76	-7.95
15	Oilseed rape, winter (DT_{50} 4150 d); (D5) loam over clay loam, rainfall 651 mm yr^{-1}	270	91.38	-1.96
16	Cereal, winter (DT_{50} 4150 d); (D1) silty clay over clay, rainfall 556 mm yr^{-1}	4	0.68	7.38
17	Cereal, winter (DT_{50} 4150 d); (D3) sand, rainfall 747 mm yr^{-1}	203	96.72	-2.1
18	Cereal, winter (DT_{50} 4150 d); (D4) loam over sandy loam, rainfall 659 mm yr^{-1}	293	72.31	0.22
19	Cereal, winter (DT_{50} 4150 d); (D5) loam over clay loam, rainfall 651 mm yr^{-1}	263	89.19	-0.54
20	Cereal, winter (DT_{50} 4150 d); (D6) clay loam over clay, rainfall 683 mm yr^{-1}	210	73.8	10.87
21	Cereal, spring (DT_{50} 4150 d); (D1) silty clay over clay, rainfall 556 mm yr^{-1}	4.47	0.9	8.26
22	Cereal, spring (DT_{50} 4150 d); (D4) loam over sandy loam, rainfall 659 mm yr^{-1}	265	74.58	0
23	Cereal, spring (DT_{50} 4150 d); (D5) loam over clay loam, rainfall 651 mm yr^{-1}	257	95.07	-1.07

NB: negative dissipation values from possible model inaccuracies.

From these results, which are shown graphically in Figure 23, a range of metaldehyde concentrations could be expected in drain flow waters for different scenarios. This was true for the simulations and the leachate collected from the lysimeter experiment in chapter 4. The highest predicted concentrations, above 200 µg L^{-1}, for the simulations stem from the model input with the worst-case DT_{50} of 4150 days. This trend would be expected, as less degradation occurs, so more metaldehyde remains over time, which may be washed into drain flow. Interestingly, some of the column drain flow results from chapter 4 have comparable metaldehyde concentrations in the leachate, suggesting that degradation rates under more environmentally realistic conditions might be slower than those measured during the batch incubation experiments. This would support the hypothesis that metaldehyde degradation rates are overestimated from the regulatory process.

Not all the simulations that use the input parameter DT_{50} = 4150 days demonstrate high metaldehyde concentrations in drain flow. Simulations S12, S16 and S21 are among the lowest predicted concentrations at 10-4 µg L^{-1}, despite the incredibly long dissipation time. These simulations have a lower rainfall, and the scenarios involve heavy clay/silty clay soils. In these scenarios, water movement through soil, and therefore leaching of chemicals, would be slow. In the lysimeter experiments, columns which did not drain freely tended to give the highest peak concentrations, e.g., C8, C9 and C14. However, these measurements were taken after maximum flow velocity ensued, and prior to this minimal concentration of metaldehyde could be detected. This idiosyncrasy fits with the model predictions and supports the conclusions that drainage rates in the field will have a significant effect on the concentration of metaldehyde leaving the field via drain flow. However, the models will assume a constant hydraulic performance for a certain soil type, whereas in reality that might not be the case, as observed for the clay soils during the lysimeter experiments.

For the simulations that used the moderate reference point DT_{50} = 46 days, peak concentrations of metaldehyde predicted in drain flow were lower (<80 µg L^{-1}), as expected. The same trend of greater rainfall and lighter soil (coarser texture) produced the highest concentrations. This reinforces the conclusion that a seasonal “flush” is a governing factor in peak metaldehyde concentration in surface water. One disparity between the simulations and the lysimeter experiment is that, in the simulations, metaldehyde is applied before the drain flow event, whereas in the lysimeter experiment, the columns are already draining. This allows the metaldehyde to be washed out gradually, as demonstrated in columns C1-C6, which were free draining due to abundant preferential flow pathways. In contrast, columns which did not flow readily initially allowed for accumulation of metaldehyde residues, which washed out in high concentrations once drain flow ensued, which is more representative of the model.

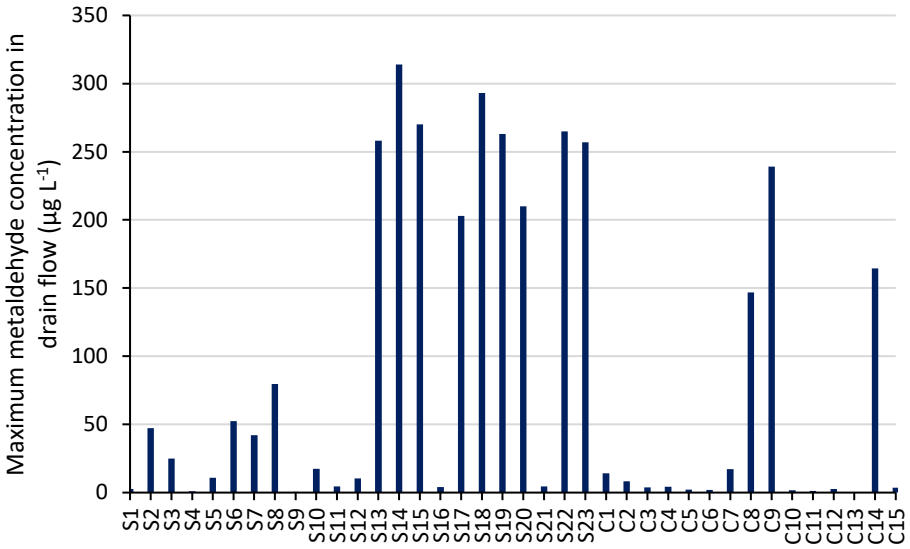

Figure 23: Chart showing the maximum concentration of metaldehyde in drain flow predicted from MACRO simulations (S1-23) and measured values from soil columns (C1-15) in the lysimeter experiment.

As discussed, drain flow is presumed to a determining factor for metaldehyde wash out and resultant concentration in surface water over a given time frame. However, for a complete assessment of pollution risk, it is necessary to consider residues of metaldehyde that remain in soil as "legacy pools". Figure 24 shows the proportion of metaldehyde lost to drains relative to the amount that is dissipated. The trend between the amounts of metaldehyde lost to drains versus the amount dissipated reflects the data outlined in Figure 23, as expected. These scenarios demonstrate high levels of metaldehyde lost to drain flow. The amount of metaldehyde lost in leachate from the soil columns is broadly in agreement with the model predictions. For the simulations where less metaldehyde is lost to drains, greater amounts are dissipated. This is logical, as more degradations leaves less metaldehyde available to wash out in drain flow.

Indeed, some of the simulations suggest that metaldehyde residues may accumulate over time, indicated by a negative value for the amount dissipated e.g., S13, S14 S15 and S17. This striking mass imbalance could result from a calculation error in the model itself as inaccuracies may arise in the model scenario, where physical processes are not well accounted for, such as the variability in hydraulic properties of the soil over time, which is not well accounted for in models. The model can only calculate a value based on the input parameters and there are often limitations to a model's capacity to predict outcomes due to the boundaries of the calculations.

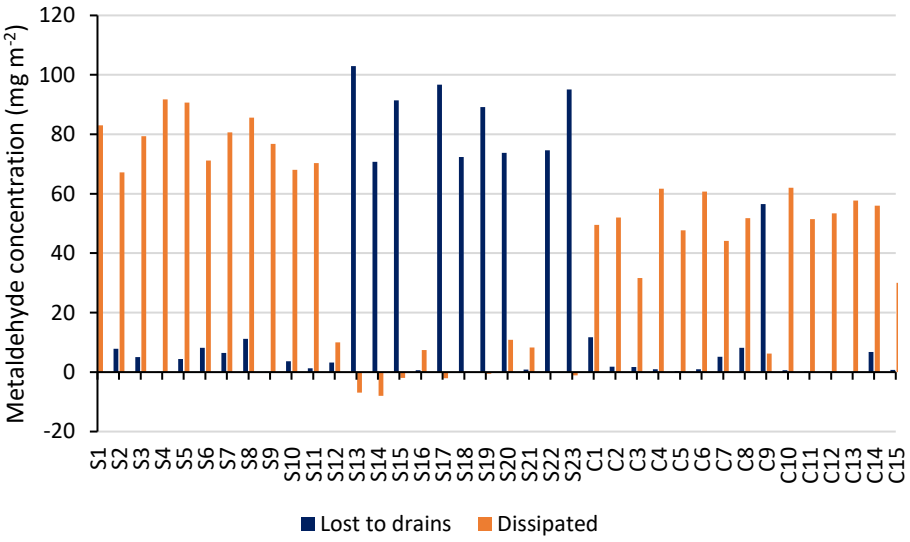

Figure 24: Chart showing the proportion of metaldehyde per square meter lost to drains or dissipated, flow predicted from MACRO simulations (S1-23) and measured values from soil columns (C1-15) in the lysimeter experiment.

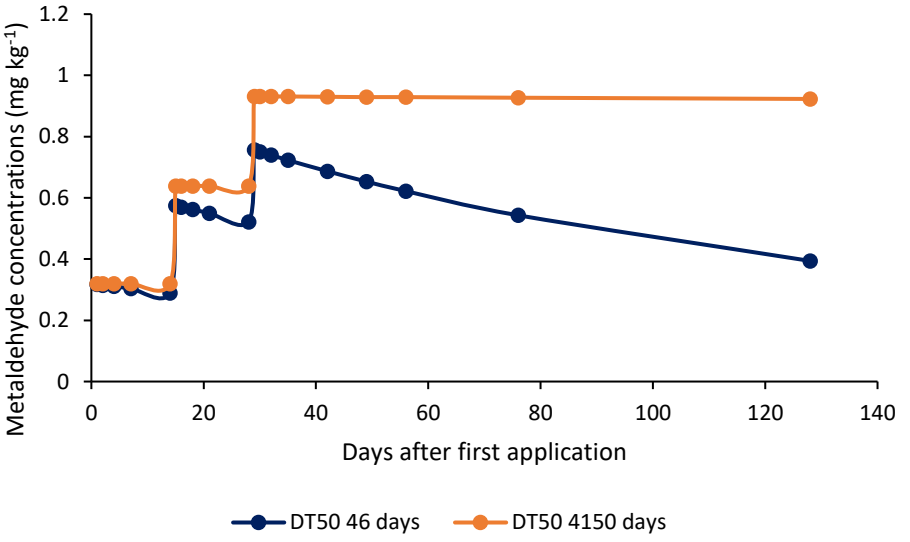

Figure 25: The fluctuation of metaldehyde concentration in soil over a period of 4 months, predicted from the excel model, PEC calculator, Version 1, October 2015, available at HSE.GOV.UK, using two laboratory-derived DT_{50} values.

Figure 25 shows that, over a period of 120 days, concentrations of metaldehyde in soil fluctuate over time from the first application. The pattern of changing concentration in the soil, shows that a certain amount of metaldehyde degradation takes place between the 14-day application intervals, but is followed by a sudden spike in concentration following dosing. This results in an overall accumulation in metaldehyde soil concentration over the application period. From this point forward, with no further application of metaldehyde, biodegradation decreases the concentration of metaldehyde in soil from its peak value in accordance with the degradation rate used in the model. It can be clearly seen that the greater measured DT_{50}, representative of soil under high moisture conditions, allows for accumulation of metaldehyde to a greater peak concentration, as less degradation occurs between application intervals, and from this point, the metaldehyde

concentration in soil does not decrease appreciable over the 4-month period. This demonstrates how metaldehyde could be persistent and accumulative in soil. The reference point experiment, with the smaller measured DT_{50}, by contrast, exemplifies how metaldehyde concentration in soil may decrease over time without subsequent applications. In the 4-month period, the concentration was shown to decrease to a level similar to the initial metaldehyde soil concentration observed after the first application. By increasing the application interval, this would allow for more degradation to occur between applications and therefore result in a lower peak concentration and possibly reduce the risk of accumulation of metaldehyde in soil. However, this would be difficult to manage, as slug pellets are applied based on the risk of slug damage to crops and weather conditions.

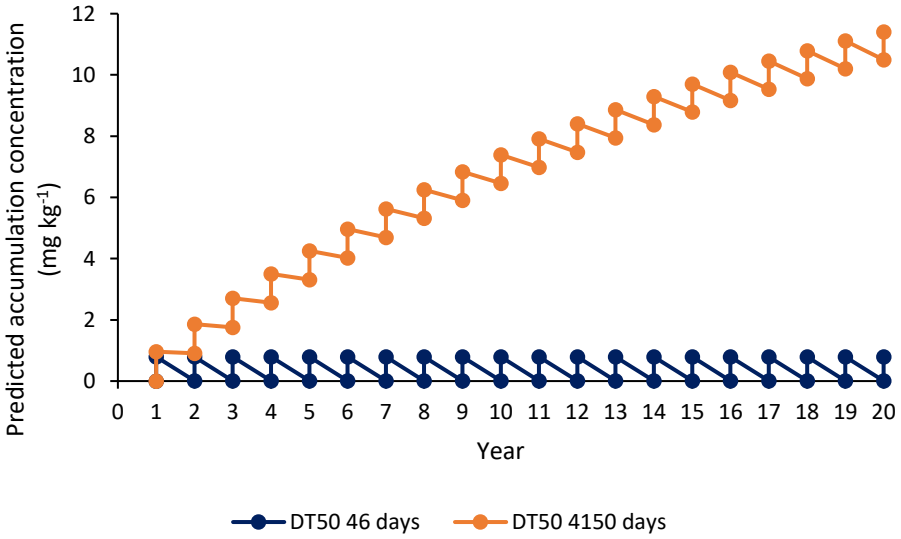

Figure 26: The predicted accumulation concentration of metaldehyde in soil over a period of 20 years, predicted from the excel model, PEC calculator, Version 1, October 2015, available at HSE.GOV.UK, using two laboratory-derived DT_{50} values.

The potential for metaldehyde accumulation in the environment is illustrated in Figure 26. For the largest measured DT_{50}, representing "worst-case" conditions, it can be clearly seen that metaldehyde concentrations in soil can increase continuously, as residual levels are added to with each year's application. This explicitly demonstrates how metaldehyde could become a problematic chemical with respect to pollution. In the case of the smaller DT_{50}, the steady state decline in concentration after application season is more significant, and so peak concentrations (estimated to be around 0.8 mg kg^{-1}) do not vary as significantly from year to year.

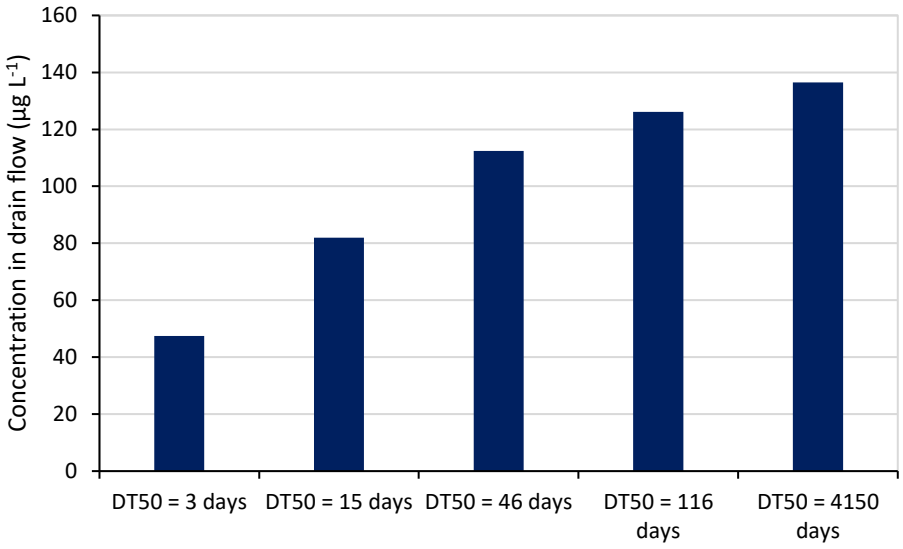

Figure 27: The concentration of metaldehyde in drain flow calculated for a range of laboratory derived DT_{50} values, predicted from the excel model, PEC calculator, Version 1, October 2015, available at HSE.GOV.UK.

Metaldehyde concentrations in drain flow were predicted by the excel model vary from 47 µg L^{-1} to 136 µg L^{-1}, depending on the DT_{50} values used as input parameters. Theses DT_{50} values represent a selection of the experimentally measured values, as discussed in chapter 3, ranging from "low-risk" to "high-risk". It can be seen from Figure 27 that, as DT_{50} becomes greater in value, metaldehyde concentrations expected in drain flow increase. This trend is logical; if less metaldehyde is degraded in the soil, indicated by a greater DT_{50} value, more will be present and washed out in drain flow. Figure 27 demonstrates that the expected concentrations in drain flow do not dramatically increase along with increasing DT_{50}. The difference in predicted drain flow concentration between DT_{50} measured at 116 days and DT_{50} measured at 4150 days is around 10 µg L^{-1}, and the predicted drain flow concentration of metaldehyde, with a DT_{50} of 3 days is around one third the value predicted for metaldehyde with a DT_{50} of 4150 days, even though this value for DT_{50} is over 1000 times greater. This is likely because metaldehyde is a mobile compound, with a low adsorption coefficient, so large amounts will wash out of soil before enough time has elapsed for degradation to occur.

To assess the accuracy of these models, these results can be compared to the findings from the lysimeter experiments. With reference to Figures 14, 15 and 17, the concentrations of metaldehyde measured in leachate from the soil columns are mostly <10 µg L^{-1}, while some of the columns, e.g. C8, C9 and C14, recorded metaldehyde peak concentrations in the approximate range 150-250 µg L^{-1}. This range of measured peak concentrations expands beyond the range of predicted values for the spectrum of DT_{50} values used as input parameters for the excel model. The excel models predict that metaldehyde concentrations in drain flow will be broadly similar, despite large differences in degradation rate. The predicted values from the excel model are broadly in the range of the measured values and those predicted by MACRO, so may serve as a useful guideline to estimate surface water exposure to metaldehyde. Based on the better agreement between the measured data and the predictions from MACRO, as compared to the excel model, MACRO is a superior tool for risk assessment purposes.

4.3.6 General discussion of metaldehyde risk assessment

Utilisation of environmental fate modelling based on information derived from batch incubation experiments and the lysimeters offers a two-tiered approach to risk assessment. This is beneficial because it improves the reliability and robustness of the models, which in turn, enhances the accuracy of PECs of pesticides. This is important for industry, which relies on the application of these models, such as regulatory bodies involved in pesticide registration and water companies, who must manage the quality of drinking water. Metaldehyde has been a challenge to water companies (UKWIR, 2015), so accurate assessment of its long-term prevalence in surface water is critical. This study served to outline any discrepancies between model predictions based on batch incubation tests and measured concentrations for drain water and soil from the lysimeter trials.

The results showed that metaldehyde concentrations in drain water are greatly affected by the drainage freedom of the soil. This was observed explicitly during the lysimeter experiments. Columns which drained freely, e.g., C1-C6 over a longer period of the experiment produced lower concentrations of metaldehyde in leachate, but over a longer time frame, than columns which drained less well. Metaldehyde breakthrough in these free-draining columns occurred as three peaks, mirroring the application regime. For the columns that did not initially drain freely, such as C8, C9, C14 and C15, metaldehyde was able to accumulate in between applications, resulting in higher concentrations being released into the leachate when drainage ensued. This produced broad breakthrough curves during the later stage of the experiment. However, in the field, lack of infiltration would result in a greater amount of surface run-off, which could be an alternative pathway for metaldehyde pollutions of surface water, that is not accounted for in a lysimeter experiment. Application of metaldehyde pellets to recently cultivated soils will lead to improved drainage and thereby metaldehyde dissipation by avoiding waterlogging.

Across the suite of MACRO simulations, peak metaldehyde concentrations were shown to correlate with rainfall quantity, which will relate proportionally to drain flow rate. The simulations with the lowest rainfall, and presumably least drain flow, demonstrated the lowest peak metaldehyde concentrations, particularly simulation with heavy clay-type soils, where drainage would be expected to be slower. This is expected, as hydraulic properties, along with sorption and biodegradation, are the main governing factors for pesticide transport (Børgesen et al., 2015; Alletto et al., 2010; Reichenberger, 2007).This trend is in fitting with the soil column results, up to the point at which the less well draining columns begin to drain freely, after which high metaldehyde concentrations were measured. A limitation of the MACRO model could be that it does not capture the “wash-out” scenario where metaldehyde accumulated in soil is expelled in drain flow once significant drain flow occurs. This would be a result of the fact that the model does not capture the change in hydraulic properties that occurs in the soil after drying out.

The peak metaldehyde concentrations in leachate, measured from the lysimeter experiment fit within the range of peak concentrations predicted from the MACRO simulations. This overlap supports the reliability of environmental fate assessments and the notion that leaching is among the most sensitive parameters in modelling (Farenhorst 2006). The most significant cause of variation in metaldehyde peak concentration in the simulations was the input DT_{50}. Two contrasting DT_{50} values were used in the simulations: a reference value of 46 days and a worst-case from the batch degradation experiments of 4150 days, derived under high soil moisture (100 % MWHC) conditions. This yielded highly diverging results, which is in line with the variability of the experimental results. For the reference point, most peak concentrations were <50 µg L^{-1}, while for the worst-case scenario, most peak concentrations were >250 µg L^{-1}. This suggested that a range of possible metaldehyde concentrations could be expected in drain flow, depending on how persistent the

pesticide is in soil, which in turn depends on the properties of the soil. Indeed, this was reflected in the lysimeter results. However, it appeared that trends in peak metaldehyde concentrations in leachate were more dependent on the column hydrodynamics than metaldehyde dissipation, when examining the mass balance for each column.

The excel model predicted peak metaldehyde concentrations in the same range but suggested that there is less sensitivity between peak concentration and degradation rate. This could suggest that drain flow processes are more important to pollution of surface water by mobile chemicals, such as metaldehyde than persistence in soil. This at least could be true in the short term. However, metaldehyde has been a pollution concern for several years in the UK and model predictions may not accurately assess risk from legacy pools in the field if indeed the compound is less disposed to degradation than the batch incubation tests would suggest. This point was exemplified in the excel model prediction for accumulation in soil, when using the worst-case DT_{50} value for metaldehyde (Figure 26). Residues were detected in soil after the 120 days of the lysimeter experiment. This is incongruous with the findings from the batch dissipation assessments, which showed that metaldehyde concentrations in soil had declined below the LOQ within 60 days for all the arable clay loam and sandy loam experiments; the same soils used in the lysimeters. This exemplifies the potential for the accumulation of legacy pools, and hence the need for refined risk assessment.

In addition to longevity in soil, another significant factor that contributes to the abundance of metaldehyde in surface waters is its widespread use (DEFRA pesticide usage survey, 2016). Metaldehyde-based slug pellets are the most widely adopted molluscicide by farmers. Application of one product for slug control results in lots of the active ingredient being applied to land. This would be less severe if a greater variation of molluscicide products were in general use. Consequently, because so much of the product has been used, it is inevitable that large concentration would appear in the environment.

Due to the prevalence of metaldehyde in drain flow, the resulting pollution of surface water necessitates stringent water monitoring initiatives. For surface waters intended for drinking water abstractions, it is crucial that metaldehyde concentrations are below the statutory limit, otherwise water companies are liable to penalties. Understanding drainage discharge rates for different parts of a catchment area and being able to ascertain when concentrations of metaldehyde above the regulatory limit could be expected, and for how long, could facilitate monitoring programmes, allowing them to be more targeted. This would improve the time and cost-effectiveness of these initiatives.

4.4 Conclusions

In terms of leaching, this study exemplified how drain flow waters could be exposed to a mobile compound such as metaldehyde. Metaldehyde leached quickly and was present in leachate early in the experiment in most cases. However, under circumstances where leaching was retarded by soil compaction, breakthrough of metaldehyde was much later in the experiment and occurred in a much higher concentration in the leachate. This might illustrate how the occurrence of metaldehyde pollution in surface water might vary under different field settings depending on soil conditions. Metaldehyde residues were detected after the 120-day experiment, suggesting that the compound is more persistent than the batch incubation experiments suggest. This could be evidence for the accumulation of metaldehyde to create legacy pools in the field, which could lead to contamination of surface water for periods after the cessation of use of this molluscicide.

Generally, there was good agreement between the model predictions and the measured contamination of leachate from the lysimeters. Where there were disparities, rationale could offer an explanation, for example, the high concentrations of metaldehyde in leachate that arise from heavy soil only after free drainage ensued. The agreement is largely determined as a result of the high mobility of metaldehyde, so the transport into drainage water can be reasonably well forecast. However, uncertainty arises for making predictions in the longer term. Current risk assessments assume that metaldehyde will degrade readily, whereas evidence from this study would suggest otherwise, depending on certain soil conditions, such as compacted soils, or high soil moisture contents. If residues that remain after the seasonal flush, when drains are flowing, are persistent in the soil, then there is the potential for these residues to accumulate each season. This could ultimately lead to legacy pools of metaldehyde in the field that could contribute to the contamination of surface water beyond the cessation of use of metaldehyde-based molluscicides in the UK.

Chapter 5: Molecular Microbiology for Assessing Metaldehyde Degradation in Soil

5.1 Introduction: the biological aspects of metaldehyde degradation

Measurements of metaldehyde degradation have been conducted during studies present in the literature, however, the nature of the microorganisms involved in its degradation are seldom considered. An understanding of the microbiology associated with metaldehyde degradation would facilitate in understanding the variability that is observed in the degradation rates. Studies in the literature (Thomas et al., 2016; Gutierrez et al., 2020) have identified a small number of microorganisms with the ability to degrade metaldehyde and these were found to be relatively rare taxa. It is likely that there are other species that possess genes that allow for metaldehyde utilisation, and this study represents continued work to identify and characterise these organisms.

5.2 Materials and Methods

5.2.1 Selection of soil samples

Soil samples were saved from the metaldehyde batch degradation experiments to determine how the microbial community changes over time in response to metaldehyde application under different soil types and soil moisture contents. Triplicate soil samples from each of the metaldehyde degradation experiment time points were saved and stored in a freezer at -18 °C prior to DNA extraction of a 0.5 g representative subsample of the pooled and homogenised samples, in triplicate, for the chosen time points.

The study sought to examine the effect of soil moisture content on the composition and functions of the microbial communities in three agricultural soils: the permanent pasture soil, which had no previous exposure to metaldehyde, and the arable clay loam and arable sandy loam. These were chosen because the results from the batch degradation experiments showed that lack of previous exposure had the greatest effect on degradation rate. For greatest effect, experiments conducted at the highest soil moisture content of 100% MWHC were chosen, along with the corresponding reference experiment at 60 % MWHC. These experiments produced a varied range of results for DT_{50} values during the batch experiments, which varied from 3 days to over 4000 days in these three soils.

Sequencing was used to determine the relative abundance of different microorganisms at the family/genus/species level to help attribute community distributions in relation to the observed degradation rates. These experiments were supported with qPCR by quantifying the known degrader genes identified from the literatures. The samples chosen for sequencing (Table 18) included day 0 time points before metaldehyde was applied to the soil in order to gauge the population composition prior to exposure to the chemical and time points 2 weeks after metaldehyde application to see how the population composition might have changed in response to metaldehyde application under different soil moisture conditions. These were compared to the reference experiment 1.0 for each of the soil types. DNA was extracted in triplicate soil samples.

Table 18: Shortlist of selected samples for DNA extraction from agricultural soils used during batch degradation experiments selected for 16S rRNA gene sequencing.

Labels	Experiment number	Time points (days)	Soil moisture content (% MWHC)	Measured DT_{50} (days)
M2, M9	(A)1.0	0, 14	60	56.8
M57	(A)3.1	14	100	4150
M63, M64	(B)1.0	0, 14	60	2.97
M68	(B)3.1	14	100	5.6
M97	(C)1.0	0	60	10.8
M110	(C)3.1	14	100	7.65

Soil samples were also saved from the intact soil cores used for lysimeter studies to assess metaldehyde leaching. The aim of this aspect of the study was to examine the soil microbial communities at different depths of the soil profile after a prolonged exposure to metaldehyde. After 120 days, the columns were destructively sampled into separate depth fractions and samples were set aside and stored in the freezer at -18 ^{o}C for molecular analyses. These samples represented different soil depths and different cultivation preparations for the three soils used during the leaching experiment (Table 19). DNA was extracted in triplicate for these samples. Selection of these samples was at different depths of the soil profile including where metaldehyde had been demonstrated to persist and therefore attempt to deduce an understanding of why the persistence is observed.

Table 19: Soil samples for molecular analysis from the lysimeter experiments comprising different depths of the soil profile.

Column	Sample description
C3	Clay loam intact cultivated horizon (0-15 cm)
C5	Clay loam simulated cultivated horizon (0-15 cm)
C2	Clay loam subsoil (30-35 cm)
C7	Sandy loam intact cultivated horizon (0-15 cm)
C12	Sandy loam simulated cultivated horizon (0-15 cm)
C12	Sandy loam subsoil (25-30 cm)
C15	Silty clay loam cultivated horizon (0-15 cm)
C15	Silty clay loam subsoil (30-35 cm)

5.2.2 Molecular analyses: MinION sequencing of 16S rRNA gene amplicons and quantitative PCR protocol to amplify metaldehyde-degrading genes

The DNA extractions were performed in triplicate to demonstrate the repeatability in results. Extraction of DNA was done following the procedure given with the FastDNA® Spin Kit for soil from MP Biomedicals™. The yield of DNA in the raw extracts was determined using a nanodrop DS-11 series spectrophotometer/fluorometer (Denovix®, Delaware, USA).

The 16S rRNA genes, which is also a proxy for total bacteria, were quantified using qPCR for all samples on a BioRad CFX C1000 system (BioRad, Hercules, CA USA). For quantification of the target genes, 2 µl template DNA (5 ng/uL) was used in a reaction mixture containing 5 µL 2 × SsoAdvanced Universal SYBR Green Supermix (Bio-Rad), 500 nmol L^{-1} of each forward and reverse primer, and molecular grade H_2O (Invitrogen, Life Technologies, Paisley, UK) to a final volume of 10 µL. Reaction

conditions for quantification of each target gene were 98 °C for 3 min (1x), then 98 °C for 15 s, and the Primer Annealing Temperature (Ta) for 60 s (40 cycles). Standard curves were produced for every qPCR assay. All the samples, standards and negative controls (molecular grade water replaced the DNA template) were run in duplicates, and the DNA samples were diluted to a working solution of 10 ng uL^{-1} using molecular grade water to reduce inhibitor effects.

Amplification of marker genes for metaldehyde degradation was attempted by PCR using the OXY gene isolated from *Acinetobacter* E1 (Thomas 2018, Gutierrez 2020). This gene was identified as coding for the enzyme being responsible for the initial and rate-determining lysis of metaldehyde during the degradation pathway, hence, due to its importance to the degradation mechanism, it was selected for analysis. Polymerase activation was done at 95 °C for 5 min. Stage 1 of the amplification programme used at temperature or 96 °C for 5 sec, 60 °C for 5 sec, and 68 °C for 10 sec, repeated over 35 cycles. Stage 2 of the amplification used a temperature of 72 °C for a single 1 min cycle. PCR amplification of mahS genes isolated from metaldehyde degrading *Sphingobium sp*., analogous to mahX isolated from *Rhodococccus globerulus* (Gutierrez et al., 2020) was also done. Stage 1 of the amplification programme used a temperature of 98 °C for 3 min, then 98 °C for a further 0.15 min, then 57 °C for 0.30 min for 39 cycles in stage 2. Then 65 °C for 0.05 min, 95 °C for 0.05 min. The primer sequences used for PCR are given in Table 19. Gel electrophoresis was done to assess whether amplification of any genes was successful. Analysis of amplifying samples was done using qPCR also, following the protocol used by Gutierrez et al. (2020).

A sub portion of the extracted DNA samples was then also prepared for 16S rRNA gene MinION sequencing. Library preparation was done using the 16S barcoding kit (SQK-16S024) from Oxford Nanopore Technologies, which was used for PCR amplification of the 16S rRNA gene with barcoded primers for the multiplex sequencing of up to 24 samples on a single flow cell in accordance with the protocol provided by Oxford Nanopore Technologies. The PCR products were assessed by gel electrophoresis. Afterwards the libraries were cleaned using Ampure® beads, in accordance with the product protocol. Quantification of each of the cleaned libraries was done using a Qubit fluorimeter. The library was then pooled in preparation for loading on to the flow cell for 16S rRNA amplicon sequencing using the MinION kit from oxford nanopore technologies.

Table 20: Primer sequences used during the PCR and qPCR assays.

	Primer sequences
PCR of ribosomal 16S sequences	8F: AGA GTT TGA TCC TGG CTC AG 785R: GGA TTA GAT ACC CTG GTA GTC C
PCR amplification of OXY gene	10F: GAG CTT GAG TCC GCC GTG AA 253R: TGC GTC TTT CCG CGA TCT CC
PCR amplification of mahS gene	mahS 117F: GCT TGA GCT TAA CGG CTAC T mahS 760R: GATA CCA CTC CTG CGG AAA C
qPCR amplification of mahS gene	mahS 187F: CGA GAG GTA TTA CTG CGG ATT T mahS: 310R: CGT CCA GCA GAA TCT GAT ACA T

5.2.3 Enrichment experiments to isolate and characterise metaldehyde degraders from agricultural soil

Attempts were made to isolate metaldehyde degraders from the same arable clay loam and sandy loam soils that were used in the degradation experiments, in which rapid degradation of

metaldehyde was observed. These soils were collected from the same field locations on 15/9/2020. The method to isolate metaldehyde degraders involved an enrichment experiment using a liquid culture medium inoculated with these soils, with metaldehyde being the sole source of organic carbon.

Soil microcosms were prepared for enrichment experiments to enhance the proliferation of metaldehyde degraders in the two soil types. The soils were stored in a refrigerator in the dark at 4 °C for a period of 2 weeks prior to experimental set-up. Soil samples (20 g dry weight) were weighed into 50 ml plastic tubes and left to equilibrate for 2 weeks before application of metaldehyde. Metaldehyde was applied as 0.33 ml from a 100 mg L^{-1} aqueous stock solution, in accordance with the method used for the previous batch degradation experiments. Enough samples were prepared to monitor the degradation of metaldehyde in the soil over 60 days, to attempt to reproduce the degradation observed in these soils during the Chapter 2 batch degradation assessments. The soil moisture content was maintained at 60% MWHC for the duration of the incubation, with weekly moisture adjustments. On day 28, a subsample of each soil (1 g) was taken in triplicate and used to inoculate the liquid culture media.

A minimal salts medium (MSM) was prepared containing the reagents listed in Table 18, in accordance with the method described by Thomas (2016). The MSM (2 L) contained aqueous metaldehyde (200 mg L^{-1} or 20 mg L^{-1}). From this stock, 100 ml was dispensed into 250 ml sterilised plastic bottles and was autoclaved. There was also a control in place with no added metaldehyde. The fortified MSM were inoculated with soil (1 g) from each microcosm experiment, done in duplicate. The liquid culture media were incubated with mild agitation at 25 °C for 72 hrs. Fresh MSM (100 ml) was inoculated with an aliquot (1 ml) of the first-generation enrichment and incubated for a period of 72 hrs. This process was then repeated a third time and the final enrichment was used for inoculating culture plates to isolate microbial strains. A 40 µl aliquot was removed from each culture medium on days 0, 1, 3, 7 and 14 of the incubation to measure the consumption of metaldehyde in the liquid culture medium. These samples were stored in vials in a freezer at -18 °C prior to analysis by LC-MS.

Table 21: The concentrations of salts used to make up the minimal salt media.

Reagent	Concentration mM
Na_2HPO_4	55
KH_2PO_4	11
NH_4Cl	6
$MgSO_4$	0.4
Metaldehyde	17600 or 1760

The final liquid culture medium was used to inoculate solid phase agar culture plates to grow single colonies of metaldehyde degrading organisms. There was no additional carbon source added besides the metaldehyde contained within the MSM. Aliquots (10 µL) of the MSM were scraped on to agar plates. The plates contained no additional metaldehyde to that present in the MSM. The plates were incubated for 72 hr at 25 °C in the dark. Isolates were collected and preserved by freezing at -20 °C in 50% glycerol in the period prior to analysis. The plates were also stored in the freezer at -20 °C for this time.

Extraction of DNA from the saved culture isolates was done using a water boil at 100 °C for 20 minutes. The samples were then cleaned up using EXOSAP reagent (©Thermo Fisher inc.), following the product protocol. The extracted DNA samples were used for 16S rRNA and mahS gene

amplifications using the aforementioned PCR protocols. These amplicons were submitted to Durham University for Sanger sequencing.

5.3 Results and Discussion

5.3.1 The microbial abundance in agricultural soils

Metaldehyde was hypothesised to become persistent under soil conditions where the diffusion of air is restricted, such as at high soil moisture content and at greater depth in the soil profile. The batch incubation experiments showed that high soil moisture content could result in reduced metaldehyde degradation. The lysimeters study showed that metaldehyde can remain in soil for longer periods when field conditions were replicated. Molecular microbiology was used to attempt to understand how these conditions influenced soil bacterial community composition and its functions, including metaldehyde degradation. Initially, DNA was extracted from these soil samples and the number of 16S rRNA gene copies was quantified as a measure for the overall abundance of bacteria and archaea (Figure 28). The overall abundance would be likely to relate to the overall microbial activity, including metaldehyde degradation.

To provide context, the theoretical microbial growth resulting from the applied 2 mg metaldehyde per kg of soil, serving as a source of carbon (1.09 $x10^{-3}$ g per kg soil) for bacterial growth, would be approximately 1.07 $x10^{9}$ cells per kg, assuming 33% utilisation (Elazhari-ali et al., 2013). If every bacterium contains 4 16S rRNA genes (averaged) resulting in 4.3 $x10^{6}$ gene copies per gram of soil, then it could be expected that metaldehyde exposure could lead to as much as 50% growth in the bacterial population based on the measurements in Figure 28. However, sampling of time points 2 weeks after metaldehyde exposure e.g., samples M2-M9 and samples M63-M64 show a reduction in the number of 16S gene copies from day 0 to 14 days after application, suggesting that metaldehyde is not a suitably viable fuel source to support longer term augmentation of the soil bacteria population, which was instead controlled by any other confounding factors that contributed towards diminished populations.

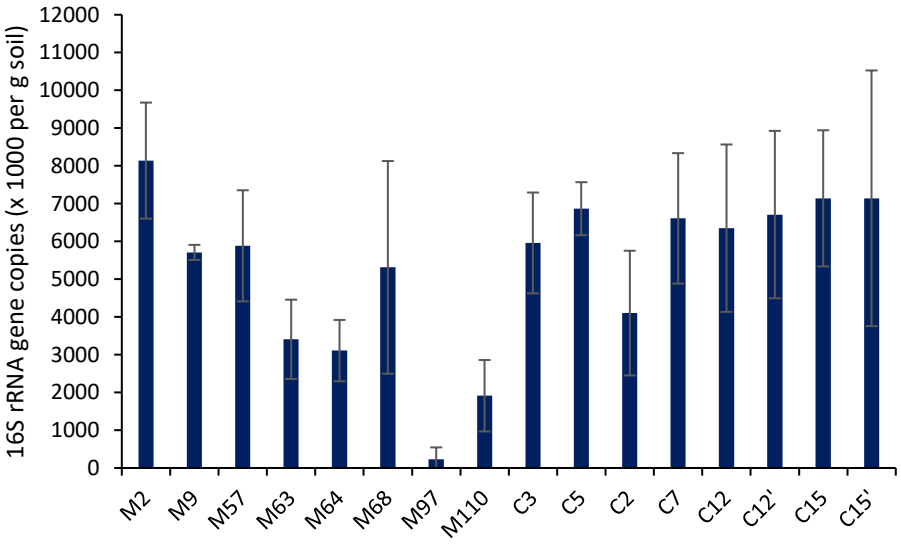

Figure 28: Number of 16S rRNA gene copies per gram of soil in the samples selected for molecular analyses.

In terms of the degradation characteristics, the pasture clay loam soil and arable clay loam soil yielded very different results, with degradation occurring much faster in the arable soil. It was

assumed that this would be attributable to significant differences in this soil microbial community. However, with reference to Figure 28, the total microbial abundance in the pasture soil was greater than that of the comparable arable soil. This would indicate that metaldehyde degradation is due to some rare taxa (Thomas et al., 2016; Gutierrez et al., 2020) as degradation rates do not correlate with total microbial abundance. This is also apparent for the lysimeter soil, which had a greater total microbial abundance than the incubated soils, yet metaldehyde residues remained in the soil for a longer period.

5.3.2 Putative metaldehyde degraders in the sequencing libraries of the batch incubation experiments

By quantifying bacterial taxa of interest (Figure 29), a more robust appraisal of the trends between microbial population and measured degradation rates can be made. Absolute abundances of 16S rRNA genes from individual taxa were estimated by multiplying the relative abundance of 16S rRNA genes attributed to the taxa in the sequencing libraries, with the total number of 16S rRNA genes quantified by qPCR. The most notable result is that, of the handful of putative metaldehyde degraders that have previously been identified (Gutierrez et al., 2020), (*Acinetobacter calcoaceticus*, *Pseudomonas vancouverensis*, *Rhodococcus globerulus, Caballeronia jianguensis*), only *Caballeronia jianguensis* is present in these soil samples in significant abundance. The greatest abundance of this particular species did not correlate with samples, which demonstrated fast metaldehyde degradation. On the other hand, there were no samples absent of *Caballeronia jianguensis* that demonstrated fast degradation, suggesting that, in the soils used for these experiments, *Caballeronia jianguensis* could have been responsible for degrading metaldehyde. Comparing these results with the abundance of 16S rRNA gene copies in Figure 29, reveals that the sandy loam soil samples, M97 and M110 were much lower in microbial abundance than the other samples. This indicates that the sample M110 soil was relatively enriched with *Caballeronia jianguensis* as compared to the other soil microbial communities in response to amended high soil moisture content and metaldehyde application.

Assessment of the numbers of degrading species was done to examine the abundance of microorganisms in the soil which have the capacity to degrade metaldehyde. There is no strong correlation shown on Figure 29 between the number of *Caballeronia jianguensis* 16S rRNA genes and the measured degradation rate. For example, samples M2, M9 and M57 from the permanent pasture soil had a higher relative abundance of the characterised metaldehyde degraders than the arable clay loam samples, despite consistently slower biodegradation of metaldehyde. Furthermore, sample M110, which contained the greatest numbers of *Caballeronia jianguensis* 16S rRNA genes, measured degradation rates were slower than in the arable soils. There are only a small number of genera containing putative metaldehyde degrading bacteria, predominantly *Caballeronia jianguensis,* present in samples M64 and M68. These samples correspond to degradation experiments (B)1.0 and (B)3.1, with measured DT_{50} values of 2.97 and 7.65 days, respectively, which were among the fasted measured degradation rates. This suggests that the abundance of microorganisms capable of degrading metaldehyde might be less important than the conditions of the soil that encourage aerobic mineralisation processes.

In soils with a high moisture content, the abundance of genera containing putative metaldehyde degrading bacteria are lower in both the pasture clay loam and arable clay loam (Figure 29), which suggests that metaldehyde-degraders do not thrive under these conditions. However, in the sandy loam soil, at high moisture content in sample M110, the abundance of degrading genera is greater than that of the reference experiment, suggesting that in the sandier soil, these types of microorganisms can proliferate at higher soil moisture. This could be because the coarser grains

provide a looser matrix to enable more diffusion of air for aerobic mineralisation of metaldehyde to occur. The number of degrading species also appear to decline at later time points, suggesting than the proliferation of these microorganisms subsides over the course of the experiment as metaldehyde concentrations are exhausted and become a less viable substrate for metabolism.

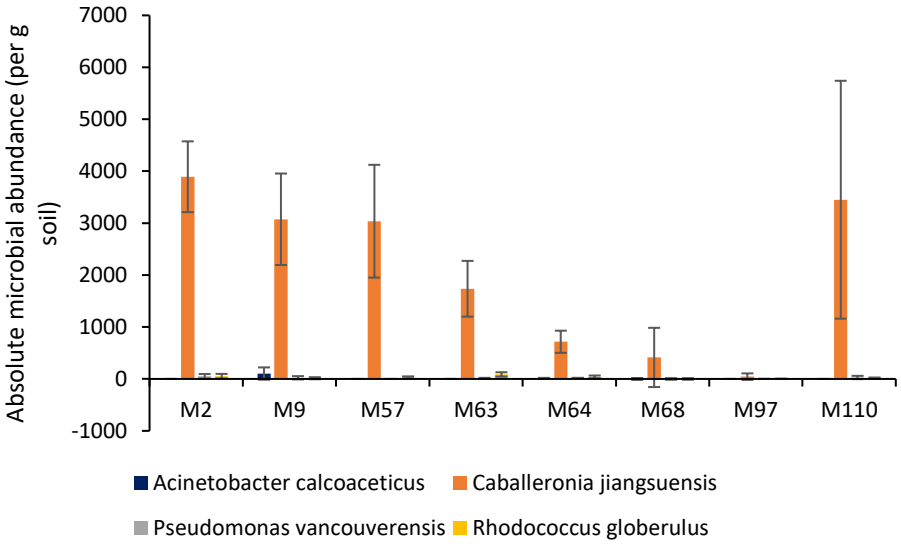

Figure 29: Taxa of interest identified from sequencing analysis of the soils used in the batch incubation experiments using the MinION

5.3.3 Putative metaldehyde degraders in the sequencing libraries of the column studies as a function of soil depth

The soil samples taken from columns C3 and C5 contained some of the highest concentrations of metaldehyde (0.32 mg kg^{-1} and 0.15 mg kg^{-1}, respectively) extracted from the deconstructed lysimeter columns experiment. This residual metaldehyde was present in the top cultivated horizon (0-15 cm depth). To analyse the microbial population in the subsoil, a representative sample was taken from C2, which of the clay loam columns, demonstrated the highest concentration of metaldehyde in the subsoil (0.1 mg kg^{-1}). This was to assess how the potential for metaldehyde degradation might change with leaching to lower soil horizons. Column 7 was the only replicate of the intact sandy loam columns to demonstrate a significant concentration of residual metaldehyde. This was present in the surface (0-15 cm) horizon. Column 12, which was the sandy loam soil subjected to simulated cultivation, demonstrated residual metaldehyde concentrations in surface soil and subsoil, so was selected for analyses to compare the microbial population in each of these horizons. Metaldehyde residual concentrations were more evenly distributed throughout the soil profile in the silty clay loam soil columns, so sampling for microbial analysis selected samples from the surface cultivated horizon (0-15 cm) and the subsoil (30-35 cm) from column 15 to examine how the microbial communities varies between these horizons and assess the risk for metaldehyde becoming persistent at different depths of the soil profile in this heavy soil.

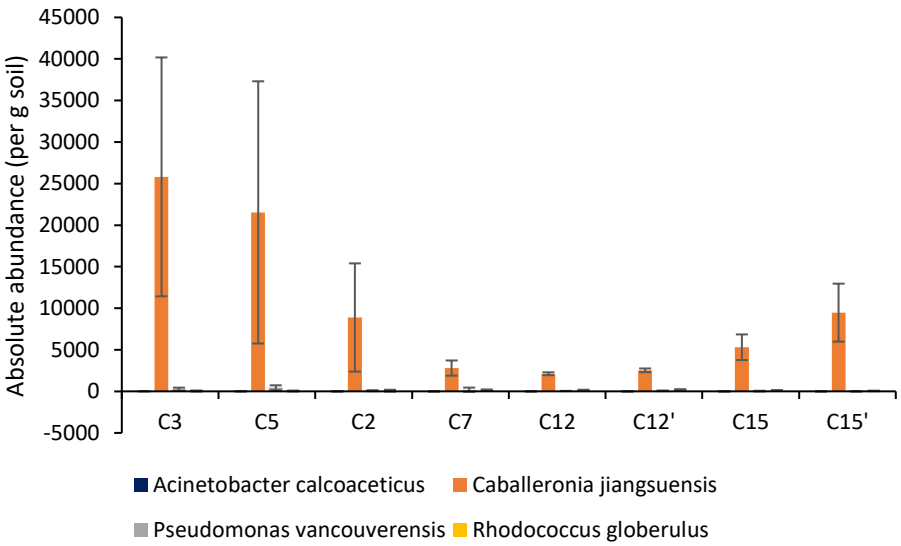

Figure 30: Taxa of interest identified from the 16S rRNA gene sequencing analysis of the soils used during the intact soil lysimeter experiments

In accordance with the results observed for these same soils in the batch experiments, the only significant taxa previously identified as a metaldehyde degrader present in the column study soils was *Caballeronia jianguensis*. Generally, there was a much higher abundance of *Caballeronia jianguensis* in these intact soils (2117-25811 16S rRNA genes from this species per g soil) than there was in the soil used during the batch incubation (414-3075 16S rRNA genes from this species per g soil). From the results in Figure 30, it can be clearly seen that samples from columns C3 and C5 contained the greatest abundance of *Caballeronia jianguensis*. These were the arable clay loam surface soil, and the abundance of this microorganism declines in these soils at greater depth, indicated from the subsoil sample, C2. The loam columns C7 and C12 had the lowest abundance of *Caballeronia jianguensis*, and this was slightly higher in the silty clay loam soil. In these samples, there was less difference in the abundance of *Caballeronia jianguensis* between the surface soil and subsoil (samples 15, 15', Figure 30).

Metaldehyde concentrations in the soil columns could reflect the presence of metaldehyde degraders in the soil profile. These results are summarised in Figure 31. The abundance and activity together, or independently might influence the concentrations of metaldehyde that remained in the intact soil after the end of the lysimeter experiment. It is worth noting, however, that this will also be largely influenced by the mechanical processes of water movement. The highest residual metaldehyde concentration was found in column 3, in the surface horizon. The high abundance of *Caballeronia jianguensis* was in sample C3, which would suggest that the presence of residual metaldehyde is not through lack of degradation potential. This result shows that less degradation occurred in the intact arable clay loam soil than during the batch incubation experiment, even though the abundance of *Caballeronia jianguensis* was greater. This could be because the conditions such as oxygen availability within intact soil versus sieved soil prepared for the laboratory tests are less amenable for degradation processes. There was no significant difference between the abundance of *Caballeronia jianguensis* between column replicates with simulated cultivation or preserved packing.

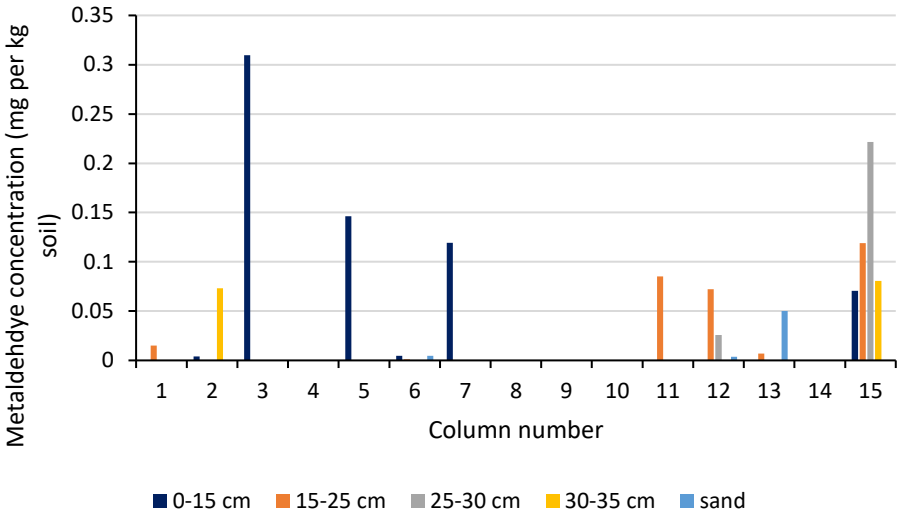

Figure 31: Residual metaldehyde concentrations in soil

In the sandy loam soil columns, C7 and C12, the abundance of *Caballeronia jianguensis* was much lower than it was in the clay loam. However, the amount of metaldehyde recovered from these columns was lower (15-30%, Figure 31). The sandy loam columns were compacted and leaching of metaldehyde was much slower. This may have allowed for more metaldehyde to be degraded before it escaped into leachate. Similarly, in the silty clay loam soil, leaching was slow. Appreciable amounts of metaldehyde were recovered (12-53%, Figure 31).

This experiment showed that the presence of identified metaldehyde degraders, or at least a subset of them, does not necessarily correlate with fast metaldehyde degradation in soil. This was illustrated in the molecular analyses of samples from the arable clay loam, in which metaldehyde degradation was fast, and yet the microbial population of identified degraders was lower than those of other samples with slower degradation. Additionally, the abundance of degraders was much greater in the intact soil columns of the lysimeter, and yet metaldehyde remained in the soil for a much longer period of time. These results suggest that metaldehyde was used less efficiently as a substrate for metabolism in the intact soil. Overall, these finding indicate that there are other factors that govern how readily metaldehyde is degraded besides the population of identified degraders and the conditions that might be shown to encourage them. Instead, conditions need to be suitable to ameliorate metaldehyde degradation. Conditions need to not only encourage the proliferation of these identified microorganism, but also lend themselves towards facilitating the biochemical processes involved in the degradation. This might include the ideal soil moisture content and aeration necessary to facilitate aerobic mineralisation processes. It should also be acknowledged, that MinION derived classifications to species level are not always 100% reliable (Acharya et al., 2019), that different strains within a species of bacteria may still have different attributes, and also that the 16S rRNA gene is useful for taxonomic classification, but not directly involved in metaldehyde metabolism. Further experimentation thus sought to detect and quantify two marker genes coding for enzymes directly involved in metaldehyde metabolism according to previous reports (Thomas et al., 2016; Gutierrez et al., 2020).

5.3.4 Amplification, characterisation and quantification of marker genes for metaldehyde degradation

To support the results from the 16S rRNA gene sequencing analyses of the soil samples collected during this study, attempts were made to amplify and quantify the genes known to be involved in

metaldehyde degradation by using qPCR analysis. Gel electrophoresis was done to assess the success of the amplification of the OXY gene. The result of the PCR was that the OXY gene could not be amplified in any of the samples from either the batch incubations, or the soil lysimeters, as shown from the gel in Figure 31. However, the PCR method was shown to work, as amplification was achieved in the positive control. It was concluded that microorganisms with the gene that was isolated from *Acinetobacter* E1 (Thomas et al., 2017) were absent from these samples.

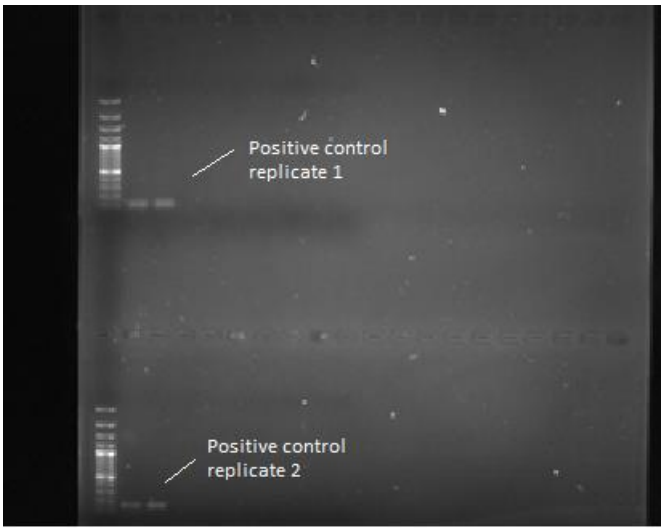

Figure 31: Gel image for the PCR amplicons of the OXY gene

In further work to quantify metaldehyde-degrading genes, DNA was sourced from another characterised degrader, namely *Sphingobium chlorophenolicum*, and primers were used to amplify its mahS gene (Gutierrez et al., 2020). Amplification was successful in two of the samples (Figure 33) producing faint bands in the gel, indicating that the gene was a rare occurrence in the DNA extracted from some of the soil samples used in this study.

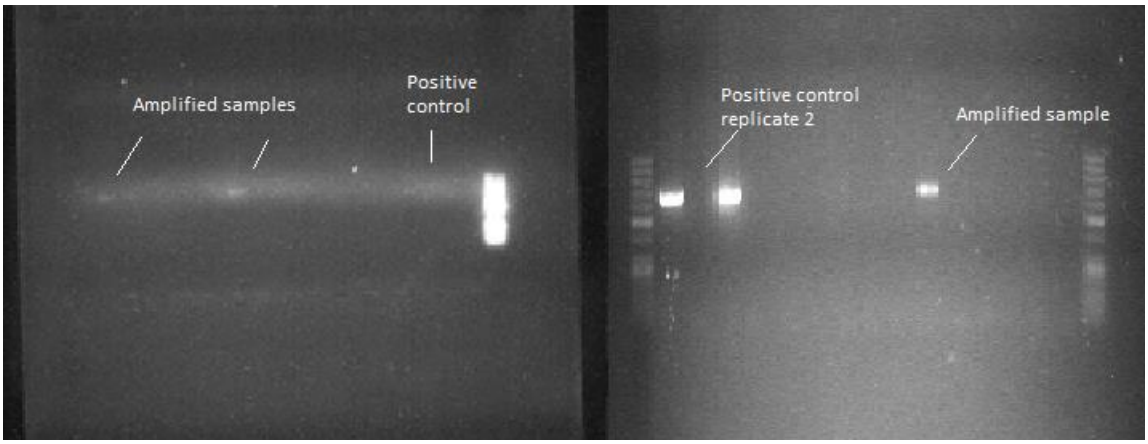

Figure 32: Gel image for the PCR amplicons of the mahS gene

Because the mahS gene was shown to be present in at least two of the samples, qPCR analysis was performed with primers for this gene. The qPCR result mirrored the finding from the gel, with the gene only being present in a few samples that had shown positive amplification in the gel. This is shown in Figure 33. The samples that contained the mahS gene were a mixture of soils used in this study, so there was no relation made to soil type. The gene was present at around 500 genes copies

per gram on soil in the silty clay loam subsoil fraction from the columns and 1500 gene copies per gram soil in samples taken from the clay loam and sandy loams columns. None of the batch incubation soil samples contained the gene, however the total microbial abundance in these samples was lower, so the rarity of this gene could mean that it was not present in sufficient quantity to be amplified. Also, other taxa that have not yet been characterised may also be degrading metaldehyde in the soils. Further experimentation to isolate degraders from these soils were therefore conducted to examine this hypothesis.

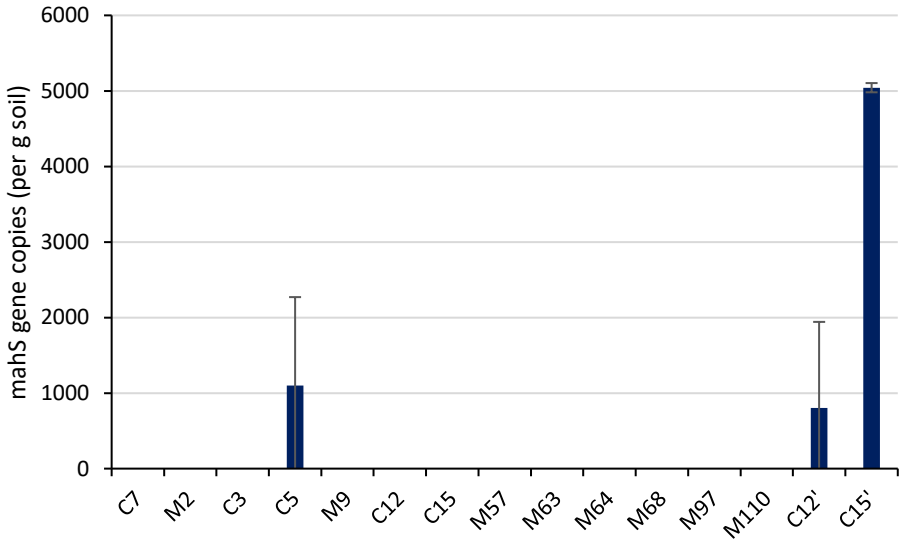

Figure 33: mahS gene copies quantified by qPCR

5.3.5 Biodegradation of metaldehyde in replicated soil incubation experiments

Enrichment experiments were conducted to understand whether *Caballeronia jianguensis* and/or *Sphingobium chlorophenolicum* were indeed the predominant microbes involved in metaldehyde degradation in these soil samples and to elucidate whether any other species could be isolated and identified as having a role in this process. Isolation and characterisation of metaldehyde degraders can be of beneficial uses for genetic screening, development of primers for PCR assays, and enzyme synthesis for decontamination. Hence these lines of research have several beneficial applications.

The repeatability of the results from the batch degradation experiments in chapter 2 was assessed during this replicated experimental set-up. It was found that metaldehyde degradation did proceed again quickly, as expected. In the soils that were used throughout this study, metaldehyde degradation proceeded with a DT_{50} of 5.01 days in the sandy loam soil, derived with Single First Order kinetics. In the clay loam soil, metaldehyde degradation proceeded with a DT_{50} of 5.5 days, also derived with Single First Order kinetics. These results are in keeping with those from the previous run of degradation experiments in these same soils at 20 °C, namely experiment (B)1.0 with a DT_{50} of 4.77 days, and experiment (C)1.0 with a DT_{50} of 4.74 days. The degradation profiles are shown in Figures 34 and 35. These soils were collected from the same field locations, and although the soils in the Chapter 3 experiment were collected in February, and those in the replicated experiment were collected in September, the reproducibility of the results might suggest that seasonality might not have a greater effect on metaldehyde degradation as hypothesised during the Chapter 3 discussion.

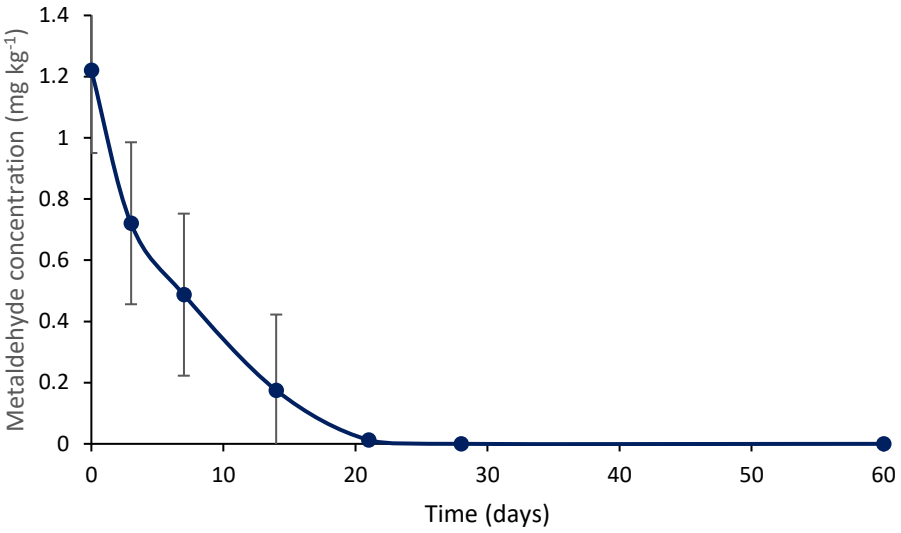

Figure 34: Degradation profile for metaldehyde in the clay loam soil used to inoculate liquid culture media.

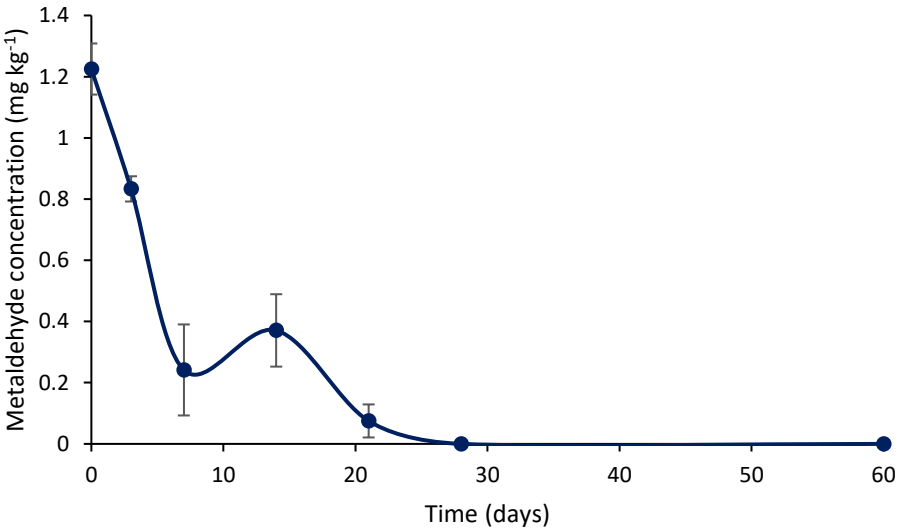

Figure 35: Degradation profile for metaldehyde in the loam soil used to inoculate liquid culture media.

5.3.6 Dissipation in liquid culture media

In the liquid culture media, the results from time-point sampling suggested that there was a concentration-dependency of metaldehyde dissipation. Generally, metaldehyde dissipation proceeded quickly in the 200 mg L^{-1} MSM, whereas concentrations remain more-or-less static in the 20 mg L^{-1} the measured degradation rates are shown in Table 22. It is possible that the higher concentration MSM allowed for more biomass growth, leading to increased degradation being observed.

Table 22: Dissipation rates of metaldehyde in the MSM

Culture medium	Rate constant k (s^{-1})	DT_{50} (days)	Error (chi^2)
G1 L 20 mg L^{-1}	1.71E-13	>10,000	5.19
G1 L 200 mg L^{-1}	0.02818	24.6	19.5
G1 CL 20 mg L^{-1}	1.63E-12	>10,000	7.91
G1 CL 200 mg L^{-1}	0.1365	5.08	16
G2 L 20 mg L^{-1}	0.00454	153	6.87
G2 L 200 mg L^{-1}	0.00751	92.3	0.336
G2 CL 20 mg L^{-1}	0.01064	65.1	3.08
G2 CL 200 mg L^{-1}	0.1792	3.87	32.5
G3 L 20 mg L^{-1}	0.01147	60.4	15.5
G3 L 200 mg L^{-2}	0.2822	2.46	24.4
G3 CL 20 mg L^{-1}	0.005883	118	3.61
G3 CL 200 mg L^{-1}	0.2822	2.46	24.4

5.3.7 Growth on plates

The MSM used to inoculate the agar plates produced dense colonies, suggesting that the liquid culture media were enriched. Only minimal growth was observed on the plates dosed with the no-metaldehyde soil MSM control. This supports the conclusion that growth was due to microorganisms that were using metaldehyde as a carbon source, but this needed to be confirmed with molecular analyses. The colonies produced on the plates were homogeneous in appearance and morphology. The colonies were a yellowish green, with clearly defined boundaries between them (Figure 36). This would suggest that the growth was from one species of microorganism.

This result would indicate that there were no other species of metaldehyde degraders present in the soils used in this investigation, as these would be expected to produce distinctly different colonies. However, not all soil bacteria can be isolated by culturing, and this needed conforming with molecular analyses. There could also be other factors at play, such as the predominance of one species over others through artificial selection, for example, if a certain species is better adapted at using metaldehyde as the sole source of organic carbon when grown in liquid cultures and on plates, it would have the advantage over other species that were less well-suited to the in-vitro conditions, regardless of the starting population distribution in the soil. For example, a species that proliferates in vitro may outcompete other degraders that thrive better in the soil environment, such as *Caballeronia jianguensis*. Also, growth might even be due to non-metaldehyde degrading organisms which feed on the biomass produced from the metaldehyde degrading organisms. There could be the case that the population in the liquid culture medium shows a dynamic change over the course of the incubation, as metaldehyde degraders grow, then other species adapt to feed on their biomass. Therefore, sampling for metaldehyde degraders would need to be taken at the correct time of the experiment. Sanger sequencing was used to identify the cultured isolates and see if they would conform to organisms with known capability for degrading metaldehyde.

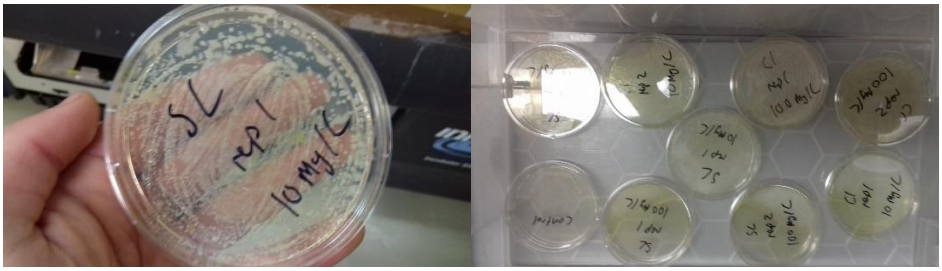

Figure 36: Growth of metaldehyde degraders on plates; SL plate left (close) and collection of the plates right

5.3.8 Results from Sanger sequencing

Sequencing of the isolates gave an ID for *Sphingobium sp.* Summarised in Table 23. This was an expected result, given that the mahS primer was developed from a *Sphingobium* isolate (Gutierrez et al., 2020), and mahS genes had been detected, albeit at low levels, in some of the soils. However, sequencing with the mahS primers yielded no district similarities with any gene sequence in the database. But alignment of the isolated degrading gene from the positive control with the Sanger sequencing result proved that the isolates contained the mahS gene. Hence, the genome of this species is not yet present in the database. The lack of similarity with other microorganisms might indicate that the metaldehyde degrading *Sphingobium sp* are rare, or the gene is quite novel. The sequences produced using the mahS primer all appeared to be identical, suggesting that the isolates were all the same organism cultured during this experiment. It was decided that more advanced studies to characterise this species, such as sequencing the whole genome and screening it for degrading genes was beyond the scope of this project.

Table 23: Inventory of the isolate samples selected for sanger sequencing and the genomic ID.

Sample number	Sample code	genomic ID with 16S primer
1	Plate 1/SL/a	*Sphingobium sp.*
2	Plate 1/SL/b	*Sphingobium sp.*
3	Plate 1/SL/c	*Sphingobium sp.*
4	Plate 2/SL/a	*Sphingobium sp.*
5	Plate 2/SL/b	*Sphingobium sp.*
6	Plate 2/SL/c	*Sphingobium sp.*
7	Plate 3/CL/a	*Sphingobium sp.*
8	Plate 3/CL/b	*Sphingobium sp.*
9	Plate 3/CL/c	*Sphingobium sp.*
D6	C5/CL/a	No significant similarity found
E7	C12/SL/a	No significant similarity found
F7	C15/SCL/b	No significant similarity found
F8	C15/SCL/c	No significant similarity found

5.4 Conclusions

From the results of this study, it has been shown that the genes that have been previously identified to be responsible for degrading metaldehyde are rare in the investigated soils. Of the organisms characterised as metaldehyde degraders, only *Caballeronia jianguensis* was present in appreciable abundance in 16S rRNA gene libraries, but *Caballeronia jianguensis* abundances were not well correlated with metaldehyde degradation rates. Attempts to quantify two marker genes for

metaldehyde degradation again revealed that these genes are rare, with a low occurrence of the mahS gene being detectable in a few of the samples.

However, enrichment studies isolated *Sphingobium* isolates that contained the mahS gene but were not notable in the 16S rRNA sequencing libraries of DNA isolated from the soils. Since metaldehyde has been shown to degrade quickly in many of the soil samples, it is possible that there are other microorganisms present in the soil that can utilise metaldehyde as a carbon substrate, which are difficult to isolate and have yet to be identified and characterised. This study did not conclusively explain why metaldehyde remained for a longer period of time in the intact soil columns versus the batch incubation experiments because the intact soil had a greater total microbial abundance and abundance of *Caballeronia jianguensis*, as well as presence of mahS genes, which would expectantly lead to faster degradation, but this was not the case. Perhaps the conditions of the soil with respect to moisture content and compaction, relating to aeration, has a greater influence on the amenability for the aerobic mineralisation of pesticides. Moreover, there is no evidence that *Caballeronia jianguensis* was responsible for metaldehyde degradation in these soils, as the only species isolated from the soil that exhibited rapid metaldehyde degradation using metaldehyde as sole carbon source was a *Sphingobium* species. From these results, it could be speculated that *Sphingobium* species are capable of degrading metaldehyde quickly at low levels of biomass, or alternatively there are other species present in the soil that are responsible for the mineralisation of metaldehyde.

Chapter 6: Conclusion and outlook

The experiments conducted to isolate and culture metaldehyde degraders reinforced the conclusion that microorganisms with the ability to degrade metaldehyde are likely rare, as it appeared that only one species was present in the cultured isolates during this study, but this species was not abundantly present in the 16S rRNA gene sequencing libraries generated from the DNA extracted from the soils. However, it could also be the case that these organisms do not grow particularly well in artificial media, hence only a handful have been successfully isolated and characterised (Thomas et al., 2017; Gutierrez et al., 2020). The abundance of metaldehyde-degrading organisms could be greater than the experimental data suggest, based on inferences from rapid degradation in batch incubation tests. Equally, the degraders that have been identified could be highly active under certain soil conditions, where degradation is observed to be fast, and these mineralisation processes may become suppressed under conditions that do not ameliorate aerobic biodegradation processes, such as soil under high moisture contents, or within compacted subsoil. It is noteworthy that metaldehyde degrading bacteria have been isolated from different soil types, and more generally metaldehyde degradation was observed in all of the soil types investigated if the conditions were conducive.

This study demonstrated that metaldehyde biodegradation can be highly variable, despite of the presence of metaldehyde degrading bacteria in soil. The worst-case conditions for metaldehyde persistence in soil was found to be for scenarios where the soil has a high moisture content, and the temperature is low, more typical of the application season for metaldehyde slug pellets in the UK. These conditions are likely to result in lower activity of aerobic microbes in the soil. Hence, the biodegradation of metaldehyde would be reduced, as high soil moisture will reduce the diffusion of air into soil for respiring microbes and the lower temperature will reduce the rate of enzyme-catalysed reactions. Additionally, the application of metaldehyde as pellets results in a heterogeneous distribution of the compound in the soil, with localised high concentrations around the pellet.

During the batch incubation tests, it was found that microbes have a finite capacity to degrade metaldehyde, resulting in an apparent reduction in the rate of biodegradation. In the undisturbed column experiments, the build-up of metaldehyde residuals was observed in some cases which could also be because of heterogeneity in the distribution of metaldehyde, degrading organisms, moisture content and oxygen availability. Metaldehyde biodegradation was particularly slow in the pasture soil used during the batch degradation tests. This could be an example of where microbial populations that have not been pre-conditioned to exposure of xenobiotics have a lesser ability to degrade pesticides, as compared to the arable soils tested during this study. A scenario can be envisaged where pastureland repurposed for arable crop production is subjected to metaldehyde application during cool, moist conditions in the autumn may result in a high risk of metaldehyde pollution.

During the risk assessments generated using modelling software, such as MACRO, scenarios generated using the data from the pasture clay soil produced the highest risk of metaldehyde contamination in drain flow waters. This would be an expected result under circumstances where biodegradation is suppressed. However, it was also revealed that rainfall patterns had a high influence on the exposure that surface waters had to metaldehyde. This is a consequence of the low sorption coefficient of metaldehyde with soil organic carbon, meaning that it is a highly mobile

compound. When rainfall was high in the models, high concentrations of metaldehyde were washed out from soil into drain flow water.

The leaching of metaldehyde from soil was also influenced by the soil type; both in the model prediction and during the lysimeter experiments. Typically, water movement in heavy clayey soils is slower (note that preferential flow in the clay loam lysimeters meant that water transport was faster) which resulted in lower concentrations of metaldehyde leaching into drain flow waters both in the model predictions and in the silty clay loam lysimeters. However, once preferential flow ensued in the lysimeters, this led to the highest concentration of metaldehyde in leachate. Presumably, metaldehyde had accumulated in the soil profile over the course of the experiment, then was released during the period of rapid draining. This idiosyncrasy is not accounted for in the model predictions. The same observation was made with the compacted sandy columns, which initially did not drain. These conditions: a wet period followed by drying to produce desiccation cracks to allow preferential water flow might present a window where surface waters are at high risk of metaldehyde contamination.

The amount of metaldehyde accumulating in soil prior to leaching will depend on the real degradation rates in the field. Based on the findings from the lysimeter experiment, where metaldehyde residues were detected in soil after 120 days, indications are that biodegradation rates in the field might be lower than those measured during the batch incubation tests. This presents limitations regarding how representative these standard test paradigms may be. This experiment highlighted the possibility for the accumulation of metaldehyde in legacy pools in fields where metaldehyde had been applied year on year. This could lead to metaldehyde pollution being observed for a time span beyond the cessation of use on agriculture fields in 2020 (DEFRA, 2020).

However, the major reasoning for metaldehyde becoming a prominent source of pollution is likely to be because of its widespread use. There are few other molluscicide products in mainstream use; metaldehyde slug pellets have traditionally been the main product of choice. Hence, with such large quantities of the product being applied to UK fields (FERA 2016), it could be expected that it would appear in the environment, when conditions occur locally and/or temporarily which promote persistence. Once the ban is enforced and farmers stop using metaldehyde products, it is likely that contamination of surface waters will subside. In the meantime, water companies must be able to predict the quantities and timings of legacy pollution from metaldehyde in order to manage water abstraction times. This research has aimed to highlight the disparity between the standard environmental fate assessments predicting a low pollution risk for metaldehyde and the observed persistence in the environment. This research could be built upon in the future to improve the reliability of model predictions in order to support drinking water abstraction during the period that metaldehyde pollution continues to present a concern in drinking water.

The findings from this project broadly agreed with the original research hypotheses. It was originally postulated that "*the biodegradation of metaldehyde in UK scenarios is over-estimated by current risk assessment models*." Indeed, it was discovered that the biodegradation of metaldehyde displayed a high degree of variability, and in some incubation experiments became highly persistent (DT_{50} up to 4150 days in one instance). However, this was highly subjective to the conditions of the incubation test. Lower incubation temperature, high soil moisture content and a high concentration of metaldehyde led to persistence, in agreement with the original hypotheses. While in some cases, metaldehyde was readily biodegradable with the lowest measured DT_{50} of 3 days.

Field assessments typically have an even greater degree of variability than laboratory tests regarding persistence assessment and therefore are only useful for supporting laboratory findings e.g. in a

Weight of Evidence approach to risk assessment. However, to examine the disparity between the observed pollution of surface water by metaldehyde and apparent ready biodegradability under the standard test paradigm, it was hypothesised that "*leaching to lower soil horizons will reduce the rate of metaldehyde degradation therefore metaldehyde will be measured in high recovery in leachate and deconstructed lysimeters"*.

Under the "realistic" conditions of the intact soil cores, metaldehyde was observed to be more persistent than might be expected from certain degradation tests where complete degradation was measured within 60 days and was detected in the intact soil cores after a period of 120 days. This meant that metaldehyde could be detected in leachate for the duration of the 120-day experiment, in accordance with the hypothesis. However, the spread of metaldehyde in the soil column was heterogenous, whereas it was hypothesised to remain in legacy pools within the subsoil. This was not that case. Metaldehyde was sometimes present in the cultivated horizon, contrary to the original hypothesis. This might suggest that a fraction became bound and less mobile.

The accompaniment of microbial characterisation can sometimes help to explain observations of varied biodegradability of chemicals. It was hypothesised that "*the degradation of metaldehyde in soil will be dependent on the presence of microorganisms with the capacity to utilise metaldehyde as an energy source. The abundance of these microbes will influence the degradation rate of metaldehyde*". However, for the case of metaldehyde, it was difficult to find trends between the quantity and nature of identified degraders and observed trends in degradation rate. This is not surprising, as soil microbiology is a highly complex system and poorly understood. It is likely that only a fraction of the microorganisms with the ability to degrade metaldehyde have ever been characterised and therefore there is not a complete picture for the processes that are occurring in the soil when metaldehyde is degraded, making it difficult to draw conclusions.

This study highlighted the intrinsic variability of laboratory-derived biodegradation data, which is recognised in the industry. However, standard test paradigms serve as a pragmatic means to compare the degradation rates of chemicals and serve as benchmarks for persistence assessment. For this purpose, standard test guidelines function well and having more case-specific tests, analogous to the ones conducted here, would broadly add unnecessary complication to chemical risk assessment. Metaldehyde has served as an excellent case study to highlight the potential limitations of the chemicals risk assessment process. For several years, metaldehyde was a chemical of concern for water companies, being a major pollutant in surface waters. This is despite been deemed low risk under the standard chemicals risk assessment procedure. The aim of this study was to draw attention to the potential for certain chemicals to fall through the net of standard persistence assessment and become pollutants of concern in the environment. Future directions of research around metaldehyde pollution in UK surface water will likely become less important in light of the ban on its use on agricultural fields. However, the question of reliability of chemicals risk assessment based on key studies versus weight of evidence remains an important and evolving topic area in environmental risk assessment and this study will remain as an important illustration of these challenges.

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