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Probe-based qPCR assay enables the rapid and specific detection of bacterial degrading genes for the pesticide metaldehyde in soil

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Abstract

Metaldehyde, a molluscicide pesticide, has been identified as a pollutant of concern due to its repeated detection in drinking water, thereby generating numerous compliance failures for water utilities. Biological degradation potential for metaldehyde is widespread in soils, occurring at different rates, but to date, no molecular methods for its assessment have been reported. Here, three genes belonging to a shared metaldehyde-degrading gene cluster present in bacteria were used as candidates for development of a quantitative PCR (qPCR) assay for assessing the metaldehyde-degrading potential in soil. Screening of gene targets, primer pairs and optimization of reaction conditions led to the development of a sensitive and specific probe-based qPCR method for quantifying the *mahY* metaldehyde-degrading gene from soil. The technique was tested across 8 soils with different compositions and origins. The degrading pathway was detected in 4/8 soils, in which a higher number of gene copies correlated with periods of greater metaldehyde removal. Additionally, swift elimination of the pesticide was observed in soils with an elevated initial number of *mahY* gene copies. The gene cluster was not detected in other soils, even though metaldehyde removal occurred, indicating that other biological degrading pathways are also important in nature. The method described here is the first one available to estimate the microbial metaldehyde degradation potential and

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activity in soils, and can also be used to detect degrading microorganisms in systems such as sand filters for water purification or to monitor degrading strains in engineered processes.

Keywords

qPCR; degrading gene; metaldehyde; soil; pesticides; bacteria

1. Introduction

The assessment and prediction of the fate of contaminants in the environment during bioremediation or natural attenuation can be carried out using different strategies: analysing the disappearance of the parent compound, detecting its transformation products or through evidence of biotransformation potential in a specific setting (Fenner et al., 2013). It is in this third approach in which knowledge regarding degrading genes can be used to detect and quantify their abundance or relative gene expression, which provides a measure of degradation activity.

Several approaches for detecting and quantifying xenobiotic degrading strains in environmental samples through marker (non-degrading) genes have been undertaken in the past (Widada et al., 2002). However, since pesticide degrading genes can be acquired by previously non-degrading microbes (DiGiovanni et al., 1996) or lost from degrading organisms (Changey et al., 2011), detecting and quantifying the specific degrading genes responsible for biodegradation directly links the monitoring approach to the pollutant degrading function of the microbial community. Utilising gene detection in environmental samples (soil, sediments, water) for pesticide degrading potential via molecular techniques has been used for different pesticides (Fuller et al., 2021), however no method has been described for metaldehyde.

Metaldehyde is a molluscicide widely used to control snail and slug populations that damage agricultural crops and domestic gardens (Eckert et al., 2012). Metaldehyde has widespread application, and pollutes water resources resulting in drinking water compliance

failures, therefore it has been identified as a pollutant of concern (Cooke et al., 2020; Cosgrove et al., 2019; Dillon et al., 2013).

Microbial activity is essential to metaldehyde degradation in the environment and has been found to be widespread in soil (Balashova et al., 2020; Simms et al., 2006). The compound degrades to acetaldehyde, subsequently to acetate, which can be modified and used as a carbon and energy source by microorganisms. Acetate is then mineralised to water and carbon dioxide, via common aerobic metabolic processes (Bieri, 2003; Thomas, 2016). Due to its physico-chemical properties, once metaldehyde enters an aquatic environment its degradation slows down, it becomes semi-persistent (Bieri, 2003) and it is very difficult to effectively remove using traditional drinking water treatment strategies (Rolph et al., 2019). These characteristics make the assessment of metaldehyde biodegradation in soil an important component to determine its environmental behaviour as this process is responsible for mitigating transport of the pesticide to water courses (Balashova et al., 2020).

A cluster of three functional genes (*mahX*, *mahY* and *aldH*) responsible for the first steps of metaldehyde degradation in bacteria from different taxonomic origins within the phylum Proteobacteria has been recently described (Castro-Gutiérrez et al., 2020) which are shown in Figure 1. Other, still undescribed, prokaryotic metaldehyde-degrading pathways exist in nature (Castro-Gutiérrez et al., 2020; Thomas et al., 2017). Based on the information of the known shared pathway, we have screened these gene candidates and optimized a qPCR assay for detection of the *mahY* gene. The performance of this assay was then tested in several soil samples. Here we describe the first molecular method for estimating the metaldehyde biodegradation potential of environmental samples through detection of a horizontally-transmitted gene cluster shared between taxonomically distinct bacterial taxa.

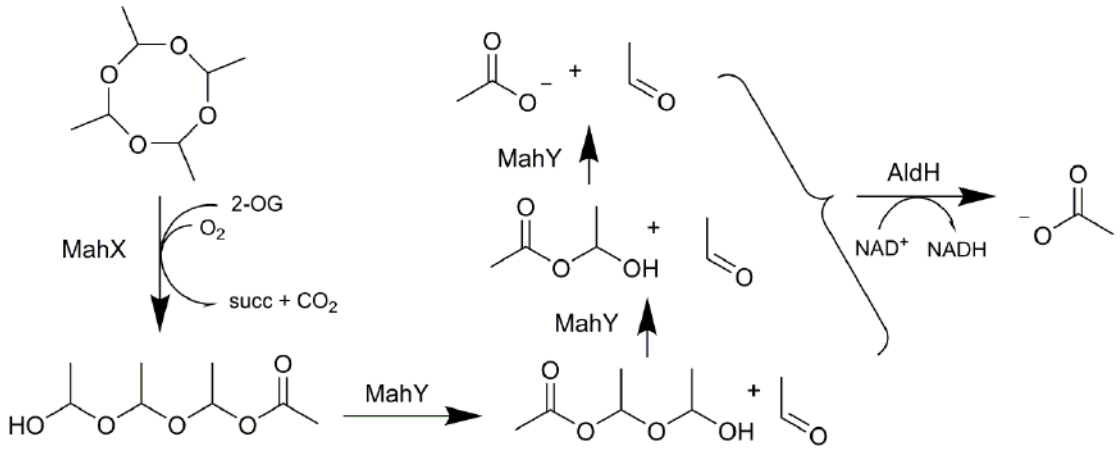

Figure 1. Predicted pathway for metaldehyde biodegradation. The first enzyme, MahX, is related to 2-oxoglutarate (2-OG)-dependent oxygenases that generate succinate (succ) and CO_2. It oxygenates the metaldehyde molecule to release a linear hemiacetal that is iteratively cleaved into acetaldehyde plus a shorter chain hemiacetal, and eventually acetate. AldH oxidises acetaldehyde to acetate in an NAD^+-dependent reaction.

2. Materials and Methods

2.1. Bacterial strains

Metaldehyde-degrading strains isolated from soil: *Acinetobacter calcoaceticus* E1 (Thomas et al., 2017), *Acinetobacter bohemicus* JMET-C, *Acinetobacter lwoffii* SMET-C, *Pseudomonas vancouverensis* SMET-B and *Caballeronia jiangsuensis* SNO-D, all of which share the same metaldehyde-degrading pathway (based on *mahX*, *mahY* and *aldH* genes), *Rhodococcus globerulus* HNO-A and *Sphingobium* CMET-H, which degrade metaldehyde using a different, still undescribed, pathway (Castro-Gutiérrez et al., 2020), and closely related type strains that do not degrade metaldehyde, *Acinetobacter calcoaceticus* RUH2202, *Sphingobium chlorophenolicum* NBRC 16172 and *Rhodococcus globerulus* NBRC 14531 (DSMZ, Braunschweig, Germany) were used to test all primer pairs. Genomic DNA was extracted from these strains using the Powersoil DNA isolation kit (Qiagen, Venlo, Netherlands) following the manufacturer's instructions.

2.2. Soil sampling and characterization

Eight soil samples were collected in Northern England, UK, from allotment and agricultural plots either exposed or unexposed to metaldehyde applications (Table 1). For each soil sampling event, triplicate 300 g sub-samples were taken from the top 10 cm of the soil and stored in sterile plastic bags. These were combined into a composite sample, stored in loosely tied plastic bags and stored in a temperature-controlled room at 23 °C until analysed one week later. Soil samples were air dried overnight and sieved (2 mm mesh) to remove coarse material. Physical and chemical parameters for soils were determined (YARA, UK) using accredited methods (Supplementary Table S1). Eight different soils in total were used for the analyses.

Table 1. Details for the soil collections performed for metaldehyde removal and degrading gene analysis.

Soil code	Collection date	Location	Coordinates	Type of plot	Crop	Metaldehyde use history
SME	October 2017	Scarcroft Allotments, York, North Yorkshire	53.950840, -1.092036	Allotment	Strawberry	Applied for three months before sampling
SNO	October 2017	Scarcroft Allotments, York, North Yorkshire	53.950840, -1.092036	Allotment	Strawberry	No metaldehyde application history
HME	October 2017	Hob Moor Allotments, York, North Yorkshire	53.946113, -1.103654	Allotment	Strawberry	Applied for three months before sampling
HNO	October 2017	Hob Moor Allotments, York, North Yorkshire	53.946113, -1.103654	Allotment	Strawberry	No metaldehyde application history
STA	June 2019	Urlay Nook Road Allotments, Eaglescliffe, Stockton on Tees, County Durham	54.520417, -1.369611	Allotment	Broad beans	Applied for three months before sampling only
PAW	September 2019	Nafferton Farm, Stocksfield, Northumberland	54.981343, -1.901597	Agricultural	Wheat	Metaldehyde applied one year before sampling
ING	September 2019	Nafferton Farm, Stocksfield, Northumberland	54.986730, -1.911462	Agricultural	Oilseed rape	Metaldehyde applied one year before sampling
LEE	September 2019	Leeds Farm, Tadcaster, Leeds, West Yorkshire	53.868944, -1.326139	Agricultural	Grass-clover mix	No metaldehyde application history

2.3. Metaldehyde degradation profiles and DNA extraction from soil microcosms

Metaldehyde concentrations were monitored in laboratory microcosms for soils STA, PAW, ING, and LEE after a single metaldehyde application at 15 mg kg^{-1}. Before the start of the assay, soil moisture was adjusted to 40% of the maximum water holding capacity. Three different sets of samples per soil were prepared. The first set was treated with metaldehyde and used for pesticide quantification analyses. The second set was also treated with metaldehyde but used for genomic DNA extraction. DNA was also extracted from the third set, which was not treated with metaldehyde, and was used as a control. For each set and time point, 10 g of soil were added to triplicate 50 mL glass Falcon tubes. A 1.5 mg mL^{-1} solution of metaldehyde in methanol was prepared and 100 µL were added to soil tubes used for pesticide analyses and for genomic DNA extraction. For the controls, only methanol was added. Methanol was allowed to evaporate from all systems for 3.5 h. Soil in each tube was then manually homogenized and the mass for all systems was recorded. Incubation was carried out in the dark at 25±2°C and 100% relative humidity for 64 days. Constant soil moisture was maintained by additions of sterile deionized water, when necessary, as determined by mass measurements. At time points 0, 4, 8, 16, 32, 48 and 64 d, triplicate systems were stored at -20°C for metaldehyde quantification and DNA extraction to determine changes in degrading gene concentrations in response to metaldehyde addition and incubation. DNA extractions were performed at the end of the assay using the NucleoSpin Soil DNA extraction kit (Macherey-Nagel, Düren, Germany), according to the manufacturer's instructions. The quality of nucleic acid extractions for strains and soils was verified in SybrSafe-stained (Thermo Fisher Scientific Paisley, UK) 1.0% agarose gels. DNA concentration and purity were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific).

Metaldehyde was quantified using gas chromatography as described elsewhere (Castro-Gutiérrez et al., 2020). The data was subjected to regression analysis and curve fit was performed using CAKE version 3.3.6 (Computer Assisted Kinetic Evaluation, Syngenta, UK). The

regression model was chosen according to FOCUS work group guidelines (FOCUS, 2006). In this study, Single First Order or Modified Hockey stick models were chosen from visual inspection of fit of model to data and from lowest residual values. The degradation profiles shown here for soils SME SNO, HME and HNO had been reported previously and did not include a 48 d time point (Castro-Gutiérrez et al., 2020).

2.4. Sequence alignment and primer design

Sequences for the genes *mahX*, *mahY*, and *aldH* were compared between the metaldehyde-degrading strains *A. calcoaceticus* E1 (Thomas et al., 2017), *A. bohemicus* JMET-C, *A. lwoffii* SMET-C, *P. vancouverensis* SMET-B and *C. jiangsuensis* SNO-D, all of which share the same metaldehyde-degrading pathway (Castro-Gutiérrez et al., 2020). These sequences represent all *mahX-mahY*-aldH cluster-containing strains known to date. Whole-genome sequencing data for the metaldehyde-degrading strains is available in the European Nucleotide Archive under study PRJEB30540. Coding regions for each one of these genes were aligned using MUSCLE (Edgar, 2004) in MEGA7 (Kumar et al., 2015). Conserved regions were identified and selected as templates for primer design. Base substitution quantification was conducted using the Maximum Composite Likelihood model (Tamura et al., 2004). Primers for qPCR analysis were designed using the PrimerQuest online tool (Integrated DNA Technologies) with optimal parameters set to Tm = 62 °C, GC% = 50, primer size = 22 bp, amplicon size = 100 bp. Primer pairs that showed probable secondary structure formation or primer dimers were discarded. The specificity of the remaining primer candidates was validated *in silico* through BLAST searches against the NCBI nucleotide collection. There were no target sequences with significant similarity to both the forward and reverse primers which could generate an amplicon. All oligonucleotides were synthesized by Merck Life Science (Dorset, UK).

2.5. Initial testing through endpoint PCR

Primer pairs were initially tested using endpoint PCR, and the products were visualised on 1.0% agarose gels. Different number of cycles (30-40) and an annealing temperature gradient (50-65 °C) were tested. Extracted DNA from a collection of metaldehyde-degrading strains, from closely related non-degrading type strains and from selected soils pre-exposed to metaldehyde (SME and HME) were used for this purpose. The oligonucleotides used in this study are presented in Table 2. Oligonucleotides for *aldH* gene amplification, 5F (5'-ACGGAGATCGTGGTTTGAC-3') and 102R (5'-CTTCGCAGCAACCCATTTG-3') were only used for the screening step and not used further in the study.

Table 2. Oligonucleotides used in this study for metaldehyde degrader quantification*

Purpose	Name	Sequence (5'-3')	Location†	Amplicon length (bp)
mahX std.	mahX-10F	GAGCTTGAGTCCGCCGTGAA	10-29	244
	mahX-253R	TGCGTCTTTCCGCGATCTCC	234-253	
mahX qPCR	mahX-86F	GTCAGCCGGACACTGATTT	86-104	98
	mahX-183-R	CGGAGGAACCACGCAATAG	165-183	
mahY std.	mahY-69F	GATCTTCGCCGTCCCTTTC	69-87	260
	mahY-328R	AAGAGAAGGTCGGTCCGTAT	309-328	
mahY qPCR	mahY-143F	GGCTCGGTGTCGAACTTATT	143-162	145
	mahY-287R	AGGTGCTCGATGTCAGATTTC	267-287	
	mahY-P177	[6FAM]CAGCGACAGCCAGTTTGCTCAGGA[BHQ1]	177-200	-

*All oligonucleotides were synthesized by Merck Life Science (Dorset, UK) and were purified by desalting
†Nucleotide position in reference to *A. calcoaceticus* E1 genes

The optimal cycling conditions for both primer pairs mahX-86F/183R and mahY-143F/287R in endpoint PCR were: 95 °C/3 min; 95 °C/30 s, 60 °C/30 s and 72 °C/30 s (35 cycles), and 72 °C/5 min. PCR reaction mixture (50 µL) contained 2 mM $MgCl_2$, 200 µM each dNTP, 1.25 U Taq-polymerase (Thermo Fisher Scientific), 400 nM each primer, 20 µg bovine serum albumin (BSA) (Promega, UK) and 1 ng of template DNA (from strains) or 40 ng of template DNA (from soils). DNA amplification was conducted using a T-100 Thermal Cycler (Bio-Rad, Hercules, CA, USA).

2.6. qPCR optimization

2.6.1. Quantification standards

To generate quantification standards for qPCR, primers were designed to amplify a broader region of genes *mahX* and *mahY*. Primer design was undertaken as described above with optimal amplicon size set to 250 bp. The primer sequences for generating quantification standards are shown in Table 2.

The optimal cycling conditions for primer pair mahX-10F/253R were: 95 °C/3 min; 95 °C/30 s, 64 °C/30 s and 72 °C/30 s (30 cycles), and 72 °C/5 min. For mahY-69F/328R these were: 95 °C/3 min; 95 °C/30 s, 58 °C/30 s and 72 °C/30 s (30 cycles), and 72 °C/5 min. PCR reaction mixture (50 µL) contained 2 mM $MgCl_2$, 200 µM each dNTP, 1.25 U Taq-polymerase (Thermo Fisher Scientific), 400 nM each primer, 20 µg BSA (Promega, UK) and 1 ng of template DNA, for which genomic DNA from *A. calcoaceticus* E1 was used. PCR products were purified using Qiaquick PCR purification kit (Qiagen) and quantified using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific) according to manufacturer's protocol to generate the quantification standards for qPCR. Standard curves using each of the purified standards were generated from 10^1 to 10^6 target copies per reaction. Serial dilutions were performed in loBind DNA tubes (Eppendorf, Hamburg, Germany) to minimize DNA adhesion to tube walls. For further studies, synthetic gene fragments could be utilised for this purpose based on the specific sequences for

metaldehyde-degrading genes in *A. calcoaceticus* E1, available in the literature (Castro-Gutiérrez et al., 2020).

2.6.2. Intercalating dye-based qPCR

Intercalating dye-based qPCR was tested for genes *mahX* and *mahY* in 20 µL reactions which contained 10 µL FastSybr Green Master Mix (2x) (Thermo Fisher Scientific), 0.4 µL BSA (20 mg mL^{-1}) (Promega, UK), 1 µL each primer (10 µM), 7.8 µL nuclease free water and 1 µL template DNA. All standard levels and samples were tested in triplicate. qPCR was performed in a QuantStudio 3 thermal cycler (Thermo Fisher Scientific). Cycling conditions were 95 °C/20 s; 95 °C/1 s, 60 °C/20 s (40 cycles). A melting curve was used to check that unspecific amplification had not occurred: 95 °C/1 s, 60 °C/20 s, and increasing to 95 °C at a rate of 0.1 °C/s. After the qPCR, amplification products were subjected to electrophoresis in agarose gels to verify the melting curve results.

Cycle thresholds (Ct) where sample fluorescence exceeds background fluorescence were recorded for the samples and quantification standards. The copy numbers of *mahX* or *mahY* gene targets were interpolated from the standard curve generated from the quantification standards in relation to their Ct. qPCR data analysis was performed using the Thermo Fisher Scientific Design and Analysis Software version 2.6.1 using the automatic Ct calling method. Sensitivity (limit of detection) was defined as the lowest standard concentration of template DNA that produced at least 95% positive replicates. Analysis of the results showed that even though the intercalating dye assay had a high efficiency, linearity was lost below 100 gene copies per reaction. Therefore, subsequent qPCR analyses were undertaken using probe-based assays as opposed to intercalating dye qPCR.

2.6.3. Probe-based qPCR

To increase the specificity and sensitivity of the assay, a qPCR method using a nucleotide probe targeting the *mahY* gene was designed based on the same alignment previously described for the *mahY* gene sequences, using the PrimerQuest tool with optimal parameters set to Tm = 68 °C, GC% = 50, oligonucleotide size = 24 bp. The probe presented no mismatches with the *mahY* sequences and is presented in Table 2. The same *mahY*-targeting primers used in the intercalating dye qPCR were also used for the probe-based qPCR.

For probe-based qPCR, each 20 µL reaction contained 10 µL inhibitor-resistant KAPA Probe Force Master Mix (2x) (Kapa Biosystems - Roche, Cape Town), 1 uL mahY-P177 (10 uM), 0.5 µL BSA (20 mg mL^{-1}), 1.8 µL each primer (10 µM), 3.9 µL nuclease free water, 1 µL template DNA. All standard levels and samples were tested in triplicate. qPCR was performed in a QuantStudio 3 thermal cycler (Thermo Fisher Scientific). Cycling conditions were 98 °C/3 min; 95 °C/10 s, 60 °C/20 s (45 cycles). ROX was used as passive reference. Ct variation at lower limit was 1.11 cycles. No amplification was present in any of the no template controls. The sensitivity (limit of detection) of the probe-based *mahY* qPCR assay was tested by performing serial dilutions of the *mahY* standard to nominal concentrations of 40, 20, 10 and 5 gene copies per reaction. Ten replicates of each dilution were subjected to qPCR amplification, and the limit of detection was determined as the lowest concentration where all ten replicates were positive.

2.6.4. Quantification of the metaldehyde-degrading gene mahY in soils

DNA from all eight soils and their respective controls with no metaldehyde added in the laboratory were subjected to *mahY* gene quantification using the probe-based qPCR protocol. For some soils, *mahY* gene copies were below the detection limit of the technique. These samples were tested for inhibitors by combining DNA from a positive sample of a different soil with DNA from the negative samples at ratios of 2:1, 1:1, and 0.1:1 and checking if a reduction in the amplification occurred. Even though an inhibitor-resistant mastermix was

used for probe-based qPCR, certain inhibitors or enhancers might still influence the reported concentrations for the positive samples. All analyses, except the physical and chemical characterization of the soils were carried out at the Biology Department laboratories of the University of York. Compliance with the MIQE guidelines is presented in Supplementary Table S2 (Bustin et al., 2009).

3. Results and Discussion

3.1. Primer design and initial testing with pure cultures

Two of the different sets of primers which were designed for the *aldH* gene did not generate strong bands on the gels or produced unspecific amplifications (additional bands of different molecular weight, data not shown) which could not be eliminated by increasing the annealing temperature. Therefore, the *aldH* gene was not selected as a suitable candidate for the metaldehyde-degrading gene cluster detection. Aldehyde dehydrogenases are widely distributed in all domains of life; for instance, in an analysis of 258 strains of the genus *Pseudomonas* there was a median of 24 ALDH-coding genes per strain (Riveros-Rosas et al., 2019), which poses a serious challenge to the specificity of the primers to be designed for use in environmental samples, even if *in silico* screening suggests no issues. Also, the *aldH*-encoded protein from *A. calcoaceticus* E1 showed a high similarity to other proteins present in the NCBI database (up to 78%), which therefore carries an increased chance of cross-reactions. Subsequent analyses were focused on *mahX* and *mahY* genes only.

Nucleotide sequences for genes *mahX* and *mahY* were aligned using MUSCLE (Edgar, 2004). For gene *mahX*, 9/945 positions showed a substitution in at least one of the five different sequences (Supplementary Figure S1). For gene *mahY* this number was only 1/450 (Supplementary Figure S2). The number of base substitutions per site was calculated using the Maximum Composite Likelihood model after Tamura et al. (2004) (Supplementary Table S3). All the genes in the cluster are identical for strains *A. calcoaceticus* E1 and *A. bohemicus* JMET-

C. Overall, these genes showed a high degree of conservation, which facilitated primer design in shared regions. Data indicate that the metaldehyde-degrading genes in this cluster are being spread through horizontal gene transfer through plasmids and insertion sequences (Castro-Gutiérrez, 2020) and therefore a high degree of sequence conservation in metaldehyde-degrading isolates identified in the future is expected. The resulting screened oligonucleotides are presented in Table 2. Primer candidates were initially tested using endpoint PCR with pure isolates. The DNA from all the metaldehyde-degrading strains positive for the *mahY* pathway produced positive amplifications, while no amplification was detected for non metaldehyde-degrading strains or those strains that degrade metaldehyde using still undescribed pathways.

3.2. Metaldehyde removal profiles in soil

Metaldehyde removal profiles for soils after a single metaldehyde application at 15 mg kg^{-1} are shown in Figure 2. To quantify the persistence of metaldehyde in the soil samples, regression analysis was performed using Single First Order or modified Hockey-stick models (Table 3) (FOCUS, 2006). With half-lives of 0.65 d and 3.9 d, removal was much faster for the soils STA and SME than the other soils (31.7 - 47.7 d).

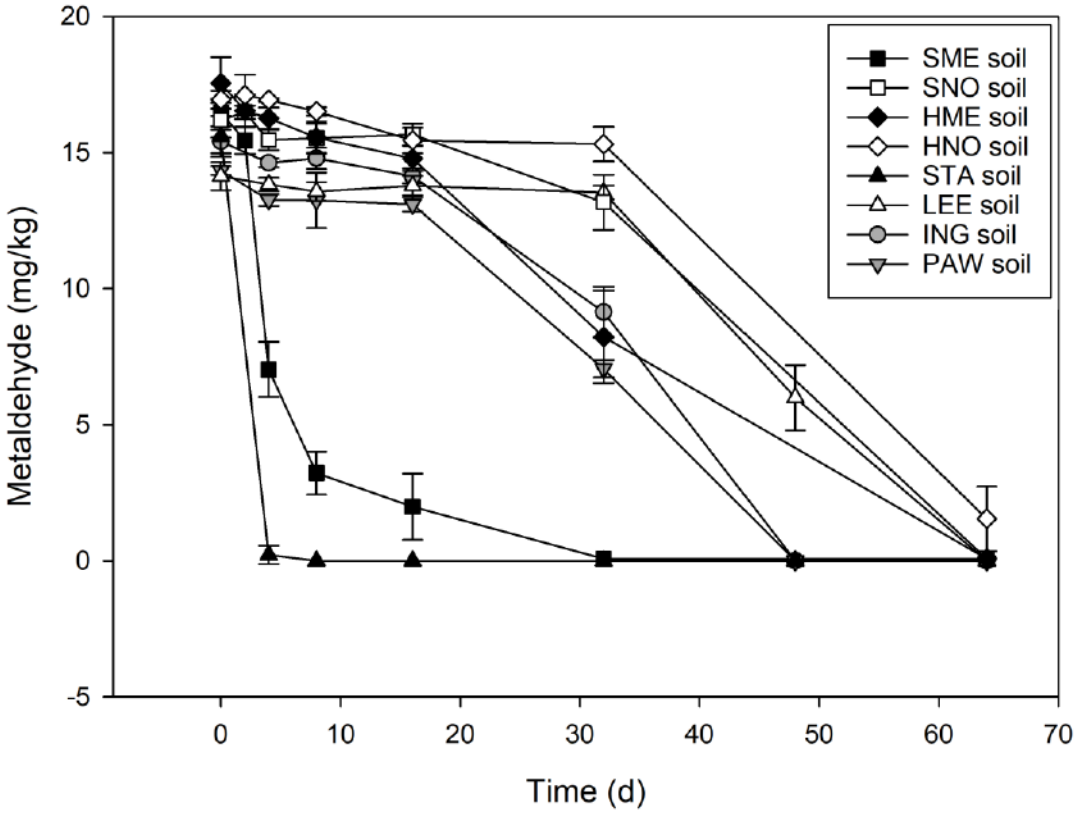

Figure 2. Metaldehyde removal profiles in freshly collected soils after an initial application of 15 mg kg^{-1} soil. Black filled symbols represent soils with recent metaldehyde applications (during the last three months before sampling). Open symbols represent soils with no known metaldehyde applications. Grey filled symbols represent soils with older metaldehyde applications (one year before sampling). Error bars represent standard deviation for three replicates. Profiles for soils SME, SNO, HME and HNO had been reported previously (Castro-Gutiérrez et al., 2020).

Table 3. Regression statistics for Single First Order and modified Hockey-stick fittings of metaldehyde removal in laboratory-incubated allotment soil samples.

Soil	Storage	Metaldehyde application	Regression model	k_1 (d^{-1})	k_2 (d^{-1})	Half-life (d)	r^2
SME*	Fresh	Single	SFO†	-	0.1776	3.9	0.9373
SNO*	Fresh	Single	MHS‡	0.002523	0.1615	35.1	0.9974
HME*	Fresh	Single	MHS‡	0.010070	0.1468	31.7	0.9984
HNO*	Fresh	Single	MHS‡	0.003852	0.0735	40.7	0.9976
STA	Fresh	Single	SFO†	-	1.0670	0.65	1.0000
PA	Fresh	Single	MHS‡	0.004890	0.6378	32.0	0.9984
ING	Fresh	Single	MHS‡	0.004759	0.6992	32.3	0.9995
LE	Fresh	Single	MHS‡	9.98E-004	0.5660	47.7	0.9994

* Statistics from Castro-Gutiérrez et al. (2020)
† Single First Order
‡ Modified Hockey-stick

3.3. Endpoint PCR testing in soil samples

Endpoint PCR amplifications for the genes *mahX* and *mahY* (98 and 145 bp fragments respectively) from soils SME and HME during metaldehyde removal and their respective unexposed controls are shown in Figure 3. Similar patterns of DNA bands were obtained for both genes, which was not surprising since both genes are present in the same cluster. *mahY* gene amplifications for soils SNO and HNO are shown in Supplementary Figure S3.

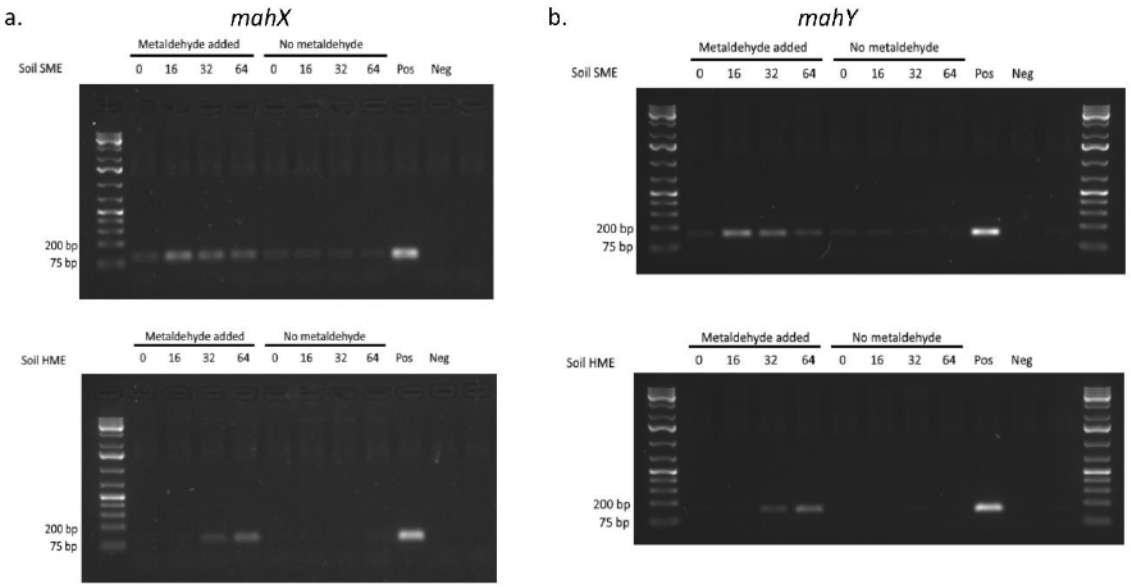

Figure 3. Endpoint PCR for soil samples SME and HME throughout a 64 d incubation using primer pairs: **a.** mahX-86F/183R and **b.** mahY-143F/287R.

Amplification patterns for soil SME, in which metaldehyde had a half-life of just 3.9 d (Table 3), showed a weak reaction at time 0 d, indicating that the number of gene copies was already detectable from the start of the incubation (Figure 3). Sixteen days after metaldehyde exposure the sample exhibited a stronger amplicon band, which declined in subsequent time points (Figure 3). Therefore, stronger amplifications coincided with periods of greater metaldehyde removal (Figure 2). On the other hand, the unexposed control maintained only the initial amplification level throughout the entire assay. For soil HME, in which metaldehyde had a much longer half-life (31.7 d), no initial amplification was detected, however, 32 d after metaldehyde addition, a positive amplification was observed, again coinciding with metaldehyde removal (Figure 2). Contrastingly, the unexposed control had no evidence of amplification. Overall, positive reactions for both soils coincided with the theoretical expected increases in the number of metaldehyde degraders through time, based on decreasing metaldehyde concentrations.

3.4. Intercalating-dye qPCR testing

Initial assessment of the intercalating dye qPCR method for genes *mahX* and *mahY* showed efficiencies of 66% and 77% respectively, with R^2 values of 0.99 in both cases. The method could detect the *mahX* gene down to 10^3 copies per reaction without losing linearity; on the other hand, the performance of *mahY* was better, detecting 10^2 copies per reaction without losing linearity. Given these results, gene *mahY* was chosen for further qPCR optimization. For *mahY*, an increase in primer concentration resulted in an improved qPCR efficiency of 91%, with an R^2 value of 0.98. The assay still remained linear at 10^2 gene copies per qPCR reaction. The calibration and melt curves for these conditions are presented in Supplementary Figure S4. Melt curves from soil samples and standards showed a single peak and agarose gel electrophoresis after the qPCR run resulted in single bands of the desired size (145 bp) with no other amplicons, therefore, the assay was considered specific.

3.5. Probe-based qPCR

The initial tests with soil samples suggested that it was necessary to be able to quantify gene copies for most soils below 10^2 per qPCR reaction. This is a consequence of metaldehyde degraders making up a very low percentage of the overall bacterial population in soil (Castro-Gutiérrez et al., 2020). To achieve quantification below this level, a nucleotide probe for use with the primer pair mahY-143F/287R was designed and used with the inhibitor-resistant Kapa Probe Force qPCR Master Mix for quantifying the degrading genes.

The fluorescent nucleotide probe was coupled with an increased concentration of primers and BSA, which led to the improvement in the lower linear range of the assay from 10^2 copies per reaction (12 500 copies g^{-1} soil) with the SybrGreen chemistry, to 10^1 copies per reaction (1 250 copies g^{-1} soil) with the probe-based assay. The mean efficiency (4 runs) for this technique was calculated at 93.4% (±3.0%), and the mean R^2 value was 0.989 (±0.07). Figure 4 shows the standard curve for a representative run. Sensitivity was determined to be 10^1 copies per reaction through replicate standard dilution series (Supplementary Table S4). Given these results, gene detection using probe-based qPCR was considered sufficiently optimized and used for subsequent efforts to characterise the degrading activity in soils.

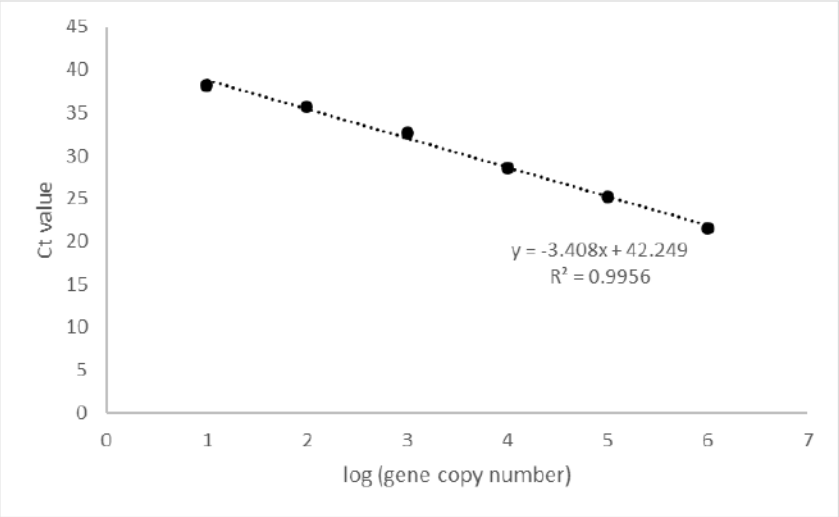

Figure 4. Gene copy number vs. Ct values for *mahY* gene standards at different concentrations for a single, representative, qPCR run using probe-based PCR.

3.6. Quantification of the metaldehyde-degrading gene *mahY* in soils

Quantification of *mahY* gene copies was carried out for eight different soils after a single metaldehyde application at 15 mg kg^{-1} soil. In four soils (SME, SNO, HME, STA) *mahY* gene copies were successfully quantified, while for the other four soils (HNO, PA, ING, LE), *mahY* gene copies were undetectable. The *mahY* gene copy numbers during metaldehyde removal in the former soils compared to their respective controls is shown in Figure 5. The peaks in *mahY* gene copy numbers coincided with moments of active metaldehyde removal in the soils. It is also evident that control soils with no metaldehyde addition in the laboratory showed lower, relatively invariable, or undetectable levels of gene copies for degradation. These data confirm that metaldehyde degraders are proliferating in the soil in response to metaldehyde addition. Also, soils with a higher initial number of degrading gene copies (SME and STA), showed faster removal (shorter half-lives) than soils with a lower initial number (HME and SNO), which indicates that this parameter effectively provides an indication of metaldehyde degradation potential and activity in soils in which this degradation pathway is present.

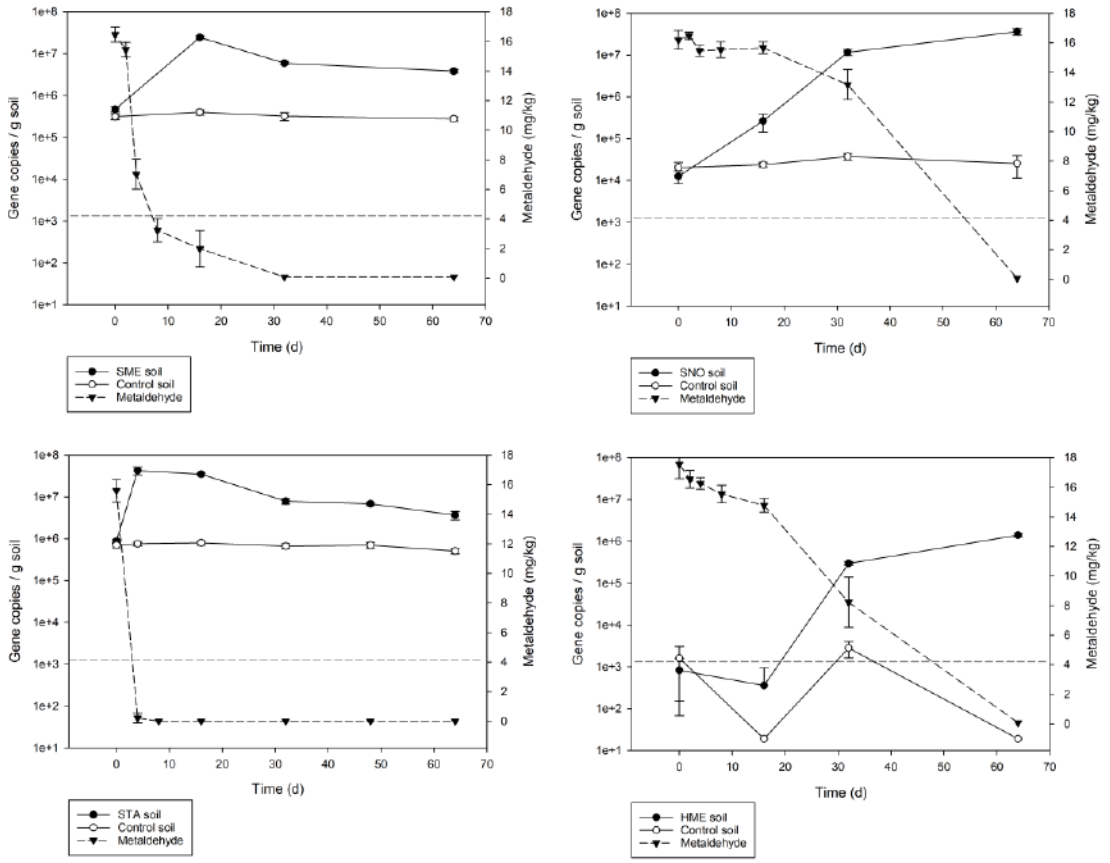

Figure 5. *mahY* gene copy number and metaldehyde concentrations during metaldehyde removal in different soils after an initial application of 15 mg kg^{-1} soil. Dashed horizontal line indicates the estimated limit of detection of the qPCR technique (1250 gene copies / g soil) for accurate quantification of gene copies. Error bars represent standard deviation for three technical replicates (average CV=14.49% in samples above limit of detection).

Several studies have found a correlation between degrading gene copy number and pesticide degradation/mineralization activity (Bælum et al., 2012; Rousidou et al., 2017; Sagarkar et al., 2013), highlighting the value of this approach for biodegradation monitoring. In these experiments, pre-exposed soils usually harboured a higher amount of degrading gene copies than non-exposed soils (Lal *et al.*, 2015; Castillo *et al.*, 2016). However, one limitation is that soils with similar genetic degrading potential can, in some instances, exhibit different pesticide-degrading activities, depending on factors such as soil type (Martin-Laurent et al., 2004) and pH (Yale et al., 2017). This emphasizes the value of assessing potential of active biodegradation using several different strategies simultaneously.

In this study, for the rest of the soils (HNO, PA, ING, LE), *mahY* gene copy number was undetectable even when metaldehyde removal from the soil was actively occurring (data not shown). Inhibitory effects on PCR amplification from substances in the genomic DNA extracts from these specific soils were ruled out as a cause of non-detection, as addition of these extracts in different amounts did not supress previously positive reactions (Supplementary Figure S5). Very low or undetectable numbers of degrading genes have been observed in instances where elimination is negligible (Nousiainen et al., 2014), others in which degradation was mainly through abiotic processes (Ben Salem et al., 2018), or when degradation was presumably occurring through alternative biological pathways, such as for the pesticide diuron (Pesce *et al.*, 2013). In this case, metaldehyde elimination invariably occurred in these soils and modelling of metaldehyde degradation kinetics showed a marked lag phase before the onset of fast elimination (modified hockey-stick model), a hallmark that biological degradation is occurring (FOCUS, 2006). Therefore, it is posited that metaldehyde removal was occurring through an alternative biological pathway in these soils. The fact that other strains are being isolated, which use different, still undescribed, metaldehyde degrading pathways (Castro-Gutiérrez et al., 2020; Thomas et al., 2017) supports this idea. Elucidation of these alternative

pathways will provide new opportunities for development of complementary tests for metaldehyde degradation potential in soils.

4. Conclusions

Here we describe the development of the first molecular assay for metaldehyde-degrading gene detection. The technique was successfully tested, found to be sensitive and useful to estimate the metaldehyde-degrading potential in a variety of soils from different origins using a single primer probe set. This method can be used to monitor *mahY*-mediated metaldehyde degrading potential and activity in various environmental and artificial settings during natural attenuation or bioremediation. Upon elucidation of alternative metaldehyde degradation pathways that undoubtedly exist in nature, the development of complementary assays for degrading gene detection in soil could be pursued.

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Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Highlights

- Metaldehyde-degrading genes *mahX*, *mahY* and *aldH* were screened as qPCR targets
- A sensitive and specific probe-based qPCR assay was designed for gene *mahY*
- The method was tested across 8 soils with different compositions and origins

- The specific metaldehyde-degrading pathway was detected in 4/8 soils
- Increased *mahY* gene copies correlated with periods of greater metaldehyde removal

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Probe-based qPCR assay enables the rapid and specific detection of bacterial degrading genes for the pesticide metaldehyde in soil

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