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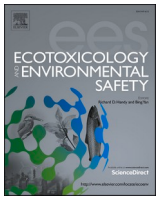

Differential responses of Type I and Type II methanotrophs to nonanoic (pelargonic) acid and glyphosate

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ABSTRACT

Glyphosate (GLY) is the most widely used herbicide worldwide. However, in organic and conventional farming systems, a natural alternative — nonanoic (pelargonic) acid (PA) — is gaining importance. PA is a non-selective herbicide whose mode of action is through disruption of the cellular membranes. PA is also added to commercial glyphosate formulations as surfactant, thereby increasing the effectiveness of this herbicide. Through agricultural application, both compounds enter the soil environment, which serves as a habitat for methanotrophic bacteria (MB). Methane (CH_4) contributes to approximately 20% of the greenhouse effect, and by limiting its net emissions from the environment, MB play a crucial role in mitigating global warming. Therefore, potential disruption of MB activity by herbicides is of great concern. This is the first study to examine the effects of PA, GLY, and their combinations on MB. We demonstrate the negative impact of field-relevant concentration of PA (but not GLY) on *Methylomonas methanica* and methanotrophic soil enrichment cultures (ECs) dominated by Type I (γ-proteobacteria). Type II (*α-proteobacteria*) MB were more resilient to PA exposure than Type I MB. The underlying mechanism of this differential response was the variable susceptibility of cellular membranes to disintegration by PA.

1. Introduction

Nonanoic (pelargonic) acid (PA) is a naturally occurring, medium-chain C9 fatty acid (FA) that is increasingly used in agriculture. Its primary application is as a non-selective, contact herbicide for weed control in both conventional and organic systems, offering an alternative to synthetic herbicides (Álvarez et al., 2025; Loddo et al., 2023; Torres-Pagán et al., 2024). Pelargonic acid is effective against a broad spectrum of weeds, particularly in post-emergence applications, and employed as a desiccant to aid in pre-harvest crop management (Havstad et al., 2022; Soltani et al., 2024). The rapid action and natural origin of PA make it attractive for integrated weed management, especially as regulatory pressures increase against synthetic herbicides (Álvarez et al., 2025; Cabrera-Pérez et al., 2022). In some EU countries, pelargonic acid is the only natural herbicide authorized for professional use, as for example, in Spain for vineyards (Álvarez et al., 2025). PA is also used as an adjuvant (surfactant) to enhance herbicidal efficacy and reduce the environmental impact of chemical pesticides such as glyphosate (Marcinkowska et al., 2018; Wehtje et al., 2009). In addition to its agricultural use, PA has been proposed for removing plants from railway tracks (Poiger et al., 2024) and has been studied for its potential to control algal blooms, particularly those caused by cyanobacteria like *Microcystis aeruginosa* (Hu et al., 2025; Shao et al., 2009)

PA efficacy is often lower than that of synthetic herbicides (e.g. glyphosate or glufosinate), particularly against perennial and grass weeds, and it may require higher application rates or repeated treatments (Kanatas et al., 2020; Loddo et al., 2023; Pannacci et al., 2022; Soltani et al., 2024; Webber et al., 2014). PA is characterised by rapid and complete degradation and a low leaching potential (Poiger et al., 2024). It has been reported that in *in vitro* conditions, PA and its

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derivatives are readily biodegradable, with 63–78% mineralization within 10 days, leaving no residual activity (Syguda et al., 2023). However, it is worth noting that upon direct contact, PA causes the breakdown of cell membranes and rapid loss of cell function. Therefore, given the need for application of elevated concentrations and repeated use, the question arises regarding its impact on non-target organisms, especially microorganisms, whose response to PA has so far been poorly studied.

PA is subject to the European Union's pesticide regulations. It has undergone peer review and risk assessment by the European Food Safety Authority (EFSA), as required by Commission Implementing Regulation (EU) No 844/2012 and its amendments. The recently updated document (Álvarez et al., 2025) states that due to the rapid biodegradability of PA, soil microorganisms are at low risk. However, it should be noted that the assessment was based on a limited set of data. Following general guidance, PA was tested for mutagenicity (using *Salmonella* and *Escherichia* strains), as well as for its effects on soil nitrogen transformation and soil microbial respiration. The risk assessment does not identify microorganisms as a primary target of PA, but rather as a factor contributing to its removal from the environment and thereby limiting its impact on other organisms.

Meanwhile, it is known that PA can have a negative effect on microorganisms, although its mode of action is not typically toxic, but rather mechanical. PA disrupts bacterial cell membranes, leading to decreased growth rates, extended lag phases, and morphological changes such as filamentation and biofilm formation in some bacteria (Dev Kumar et al., 2020; Kumar et al., 2019). PA exhibits broad antimicrobial activity, affecting both Gram-positive and Gram-negative bacteria, as well as fungi (Chadeganipour and Haims, 2001; Dev Kumar et al., 2020; Lade et al., 2023) Recent studies have even demonstrated PA's efficacy as a sanitizer for reducing foodborne pathogens such as *Salmonella* sp. and *Escherichia coli* on produce surfaces, outperforming traditional sanitizers like chlorine and peroxyacetic acid in some cases (Cimowsky et al., 2022; White et al., 2021).

Based on the above, it cannot be ruled out that application of PA may affect important microorganisms or environmental processes that were not considered in the original risk assessment. One such group is methanotrophic bacteria (MB) — microorganisms commonly found in both terrestrial and aquatic environments. MB play a crucial role in maintaining global homeostasis by removing methane (CH_4), a major greenhouse gas, directly from the atmosphere and acting as a biological filter, thereby preventing its release from anaerobic environments. Methanotrophic activity is highest in natural, undisturbed ecosystems. Anthropogenic pressures — such as physical disturbance (e.g. soil management practices) or chemical habitat alternation (e.g. the introduction of fertilizers) — lead to a reduction in the abundance of MB and a decline in their activity (Lim et al., 2024).

The antimicrobial activity of PA is non-selective (Álvarez et al., 2025). It integrates with cellular membranes, increasing their fluidity and permeability. For this reason, we hypothesize that the effects of PA may vary among different MB. Within this group, two types are distinguished: Type I and Type II. These differ in taxonomy (Type I is affiliated with *γ-proteobacteria*, whereas Type II is affiliated with *α-proteobacteria*), physiological characteristics (such as carbon assimilation pathway and the formation of resting stages), and importantly, in the composition, structure and organization of their outer cell membranes (OCM) and intracellular membranes (ICM) (Guerrero-Cruz et al., 2021). The consequences of these variable cellular membrane (CM) arrangements for MB responses to PA are not yet known. Since the cell membrane is the first point of contact between micro-organisms and PA, differences in ICM structure may result in varying susceptibility to the destructive effects of this compound.

PA is often used as adjuvant in commercial formulations (Supplementary Material A) of GLY, the most widely used vegetation control agent globally (Maggi et al., 2020). To date, only one recent study has investigated the effect of GLY on soil methanotrophic bacteria (Furtak, 2026). In that experiment, the response of microbial community was clearly influenced by the adjuvant (in that case - ethoxylated isotridecyloxypropanamine (3-IPA)) which presence altered both the response of soil methane uptake and MB counts. This emphasises the importance of adjuvants in the studies of commercial herbicide formulations.

With the above in mind, this study aimed to assess the impact of pelargonic acid (PA) on MB. Two scenarios were considered: one in which PA is used as a standalone active substance (as in cases where PA is applied as a herbicide), and another where PA is combined with glyphosate (GLY) — reflecting situations in which PA is added as an adjuvant to a conventional synthetic herbicide.

2. Materials and methods

2.1. Research material

Actively growing cultures of *γ-proteobacteria* (Type I methanotroph) *Methylomonas methanica* (DSM 25384) and *α-proteobacteria* (Type II methanotroph) *Methylocystis bryophila* (DSM 21852) were obtained from DSMZ (German Collection of Microorganisms and Cell Cultures) and maintained according to the provided protocols. The protocol for soil sampling and the establishment of soil-delivered enrichment cultures (ECs) is described in Supplementary Text B.1.

2.2. Experiment design

The experiments consisted of two stages. In stage I, the effect of a wide range of nonanoic (pelargonic) acid (PA) concentrations (Angene Chemical, London, UK, 98% purity) 0, 0.5, 2, 10, 50 and 100 μg cm^{-3}) was studied to assess toxicity levels towards MB. Stage II aimed to evaluate the impact of the selected PA concentrations (Table 1) co-administered with glyphosate (GLY) (Merck KGaA, Darmstadt, Germany, $\geq$ 98% purity).

In the case of pure cultures, the effect was measured by bacterial growth rate and methane oxidation. In the case of ECs, community composition and MB numbers were also evaluated. The PA and GLY concentrations used in the second stage corresponded to the label doses of commercially used products which vary between 1,51 and 82,98 μg g^{-1} for PA (Supplementary Material A), and between 2 and 10 μg g^{-1} for GLY (Nguyen et al., 2016).

The response of *M. methanica*, *M. bryophila,* and ECs obtained from Gleysol (EC-G), Leptosol (EC-L), and Fluvisol (EC-F) was studied under controlled laboratory conditions for 48 h. Hermetically sealed 120 cm^{-3} vials containing 50 cm^{-3} of NMS medium and 10% $_{v/v}$ of CH_4 were used with constant orbital shaking at 180 rpm and 20°C. In order to prevent GLY adsorption, all glassware was coated with a hydrophobic layer using Sigmacote® (Merck KGaA, Darmstadt, Germany) prior to the experiment, in accordance with the manufacturer's protocol.

Daily measurements included optical density (OD_{600}, λ = 600 nm,

Table 1
Combinations of GLY and PA concentrations used in the second stage of the experiments.

Concentration	0 μg PA cm^{-3}	2 μg PA cm^{-3}	5 μg PA cm^{-3}	10 μg PA cm^{-3}
0 μg GLY cm^{-3}	0 PA + 0 GLY	2 PA + 0 GLY	5 PA + 0 GLY	10 PA + 0 GLY
10 μg GLY cm^{-3}	0 PA + 10 GLY	2 PA + 10 GLY	5 PA + 10 GLY	10 PA + 10 GLY

UV–VIS Spectrophotometer, Hach Lange GmbH, Düsseldorf, Germany) and CH_4 concentrations determined by gas chromatography (Perkin Elmer Clarus 500, Shelton, USA), equipped with a flame ionization detector (FID, 220 °C) and a Poraplot Q 0.53 mm column. In parallel, CO_2 (to confirm that the observed CH_4 decrease was due to methanotrophic activity rather than vial leakage) and O_2 (to ensure oxygen was not limiting for the MB) concentrations were also measured. CO_2 was determined using FID after methanization at 400°C (Perkin Elmer AutoSystem XL GC Auto-Ignite FID Catalytic Reactor Accessory, Shelton, USA), whereas O_2 was measured using a thermal conductivity detector (TCD) at 150 °C with a Molecular Seve 5 A (0.53 mm ID, 30 m). Helium was used as a carrier gas. The results of CO_2 and O_2 measurements are not shown.

Methanotrophic activity was expressed as the hourly decrease in CH_4 concentration, normalized to culture volume, and reported as mg CH_4 cm^{-3} h^{-1} (Szafranek-Nakonieczna et al., 2019). The experiment began immediately after the addition of herbicides and CH_4 to the cultures. After the final measurements, bacterial pellets from each culture were collected by centrifugation at 5000 g for 15 min and stored at −80°C for subsequent molecular analyses. The results of optical density and methanotrophic activity are presented as percentages relative to the control treatment (set at 100%).

2.3. Transmission electron microscopy

To visualize the effect of PA on Type I and Type II methanotrophs, pure strains of *M. bryophila* and *M. methanica* were selected. The cultures in the logarithmic growth phase were exposed to 10 μg cm^{-3} PA for 10 min. Unexposed cultures were used as a control.

After exposure, the cultures were washed twice with 50 cm^{-3} of the appropriate medium in order to remove any PA residues and then harvested by centrifugation at 3000 rpm for 10 min at 4°C. The resulting methanotroph pellets were fixed in 2% $_{w/v}$ glutaraldehyde in 0.05 M cacodylate buffer for 2 h at 4°C, followed by post-fixation in 1% w/v osmium tetroxide (OsO_4) in the same buffer for 4 h at 20°C.

After dehydration through a graded ethanol series, the samples were embedded in medium-hardness resin blocks (Agar Low Viscosity Resin). Ultrathin sections were prepared using a PowerTome XL ultramicrotome (RMC Boeckeler), mounted on copper grids, and contrasted with 3% $_{w/v}$ uranyl acetate in 70% $_{v/v}$ ethanol for 30 min, followed by staining with lead citrate (Dykstra and Reuss, 2011) at 20°C for 5 min. The preparations were examined using a Libra transmission electron microscope operated at an accelerating voltage of 80 kV.

2.4. Molecular analyses of methanotrophic communities

The selected EC samples were subjected to genomic DNA isolation using a PowerLyzer PowerSoil DNA Isolation Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The quantity of the extracted DNA was measured using a Quantus™ fluorometer with the QuantiFluor® dsDNA Dye System (Promega Corporation, Madison, USA). DNA samples were stored at −80°C for downstream analyses.

To quantify MB, a digital PCR (dPCR) approach was implemented. For this purpose, the QIAcuity ONE thermocycler (QIAcuity Software Suite v3.0.0) with a dedicated 24-well 8.5 K nanoplate and EvaGreen EG PCR KIT were used (Qiagen, Hilden, Germany). A primer set of A189f (5'-GGNGACTGGGACTTCTGG) and mb661 (5'-CCGGMGCAACGTCYTTACC) targeting the methane monooxygenase gene (*pmoA*) was selected (Costello and Lidstrom, 1999). Each dPCR assay was performed in triplicate. Based on Chiri et al. (2020), an average of two gene copies of *pmoA* per cell was assumed for methanotroph cell quantification. Detailed dPCR conditions are specified in Supplementary Table B.1.

MB community structure was determined by next-generation sequencing (NGS) of the V4 region of the 16S rRNA gene using amplicon sequencing with MiSeq Illumina technology and universal primers 515 F (5'-GTGYCAGCMGCCGCGGTAA) and 806 R (5'-GGACTACNVGGGTWTCTAAT) (Thompson et al., 2017). Detailed NGS conditions are specified in Supplementary Text B.2.

2.5. Statistical analysis

All experiments were conducted with three biological replicates, encompassing bacterial growth, methane oxidation, DNA isolation, dPCR and NGS. Statistical analyses were conducted in Statistica 13.3 (TIBCO Software Inc., Santa Clara, USA), and included the Shapiro-Wilk test for normality ($\alpha = 0.05$), the Brown-Forsythe test for homogeneity of variance ($\alpha = 0.05$), followed by one-way ANOVA ($\alpha = 0.05$) and Tukey HSD multiple comparison post-hoc test ($\alpha = 0.05$). Detailed information about the results of statistical analysis are available in Supplementary Material C.

3. Results

3.1. Response of M. methanica and M. bryophila to PA and a combination of PA and GLY

Stage I experiments conducted on pure cultures showed that the reference strains of type I and II methanotrophic bacteria exhibited different sensitivities to PA. In *M. methanica*, a large decline in both growth (by 60%) (Fig. 1A) and the methanotrophic activity (by 65%) (Fig. 1B) occurred already at 10 μg cm^{-3} PA. In comparison, the same PA dose resulted in only an approximate 10% reduction in growth (Fig. 1C) and a 30% decrease in methanotrophic activity (Fig. 1D) in *M. bryophila.* A decline in optical density in *M. bryophila* cultures was not observed until exposure to 100 μg cm^{-3} PA, compared to 10 μg cm^{-3} PA in *M. methanica*. Notably, *M. bryophila* and *M. methanica* displayed different response patterns. In *M. bryophila* (Type II), the effect was concentration-dependent, while *M. methanica* (Type I), the decline was rapid with no further reduction at higher PA concentrations. Such differential response not only provides evidence for their varying tolerance to PA, but also suggests that the two species possess distinct defence mechanisms against PA (Fig. 1A-D).

In Stage II experiments, which aimed to determine the combined effect of PA and GLY, it was found that the cell density and methanotrophic activity of *M. methanica* significantly decreased under exposure to PA at concentrations of 5 and 10 μg cm^{-3}, yet the addition of 10 μg cm^{-3} GLY stimulated its growth (Fig. 2A), but not methanotrophic activity (Fig. 2B). The cell density of *M. bryophila* was unaffected by either PA or GLY (Fig. 2C). Only a slight (and statistically insignificant) decrease in methanotrophic activity was observed with increasing PA concentrations (Fig. 2D).

3.1.1. Disintegrating effect of PA on cellular membranes of Type I and Type II MB – transmission electron microscopy (TEM) observation

TEM observations revealed that *M. methanica* cells underwent substantial damage after exposure to 10 μg cm^{-3} PA for 10 min (Fig. 3B), compared to untreated ones (Fig. 3A). In most cells, the OCM had visibly lost their integrity, and the contents of the cell had leaked out. Moreover, even in cells that remained structurally intact, the characteristic stacked ICM structures were deformed, indicating the PA had penetrated into the cytoplasm (Fig. 3B).

In contrast, *M. bryophila* cells, did not undergo disintegration following PA treatment. Control cells were pleomorphic (which is characteristic of *Methylocystis)* with visible PHB granules (commonly found in type II MB and absent in Type I MB) seen as white, electron-translucent spheres, and ICM tightly attached to OCM (Fig. 3C). *M. bryophila* cells treated with PA remained intact, although some exhibited altered cell shape and detachment of ICM from the OCM (Fig. 3D).

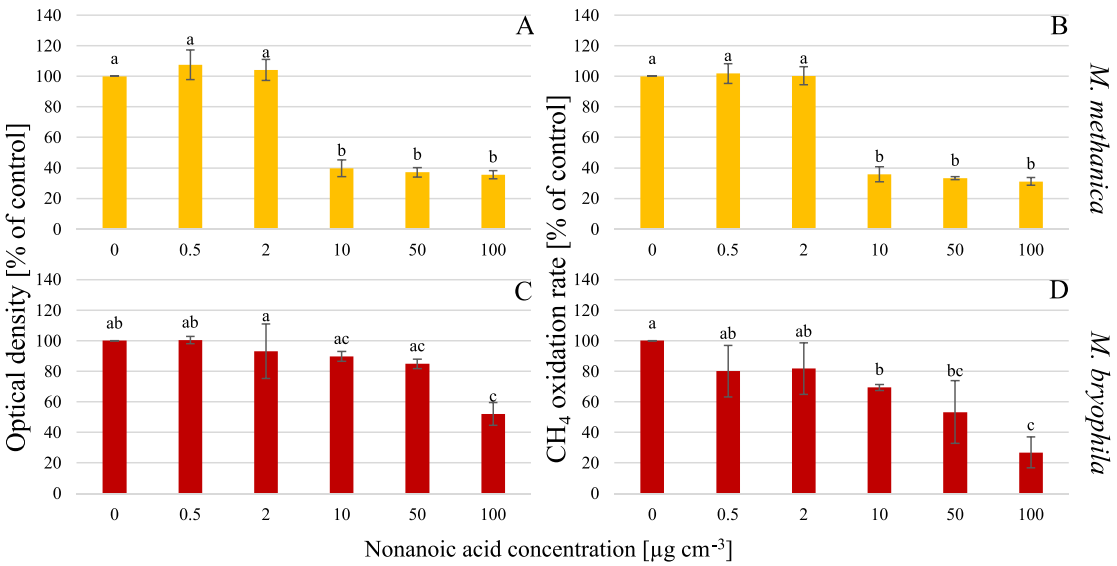

Fig. 1. Optical density (left, λ = 600 nm) and methane oxidation rate (right) of pure methanotrophic strains after 48-hour exposure to pelargonic acid (0, 0.5, 2, 10, 50 and 100 μg mL^{-1}). The figure shows parameter changes relative to the control treatment (0 μg mL^{-1} PA). Data are presented as mean ± SD for n = 3. Statistical analysis was performed on absolute values, separately for each strain and assay, and include Tukey HSD test at $p < 0.05$.

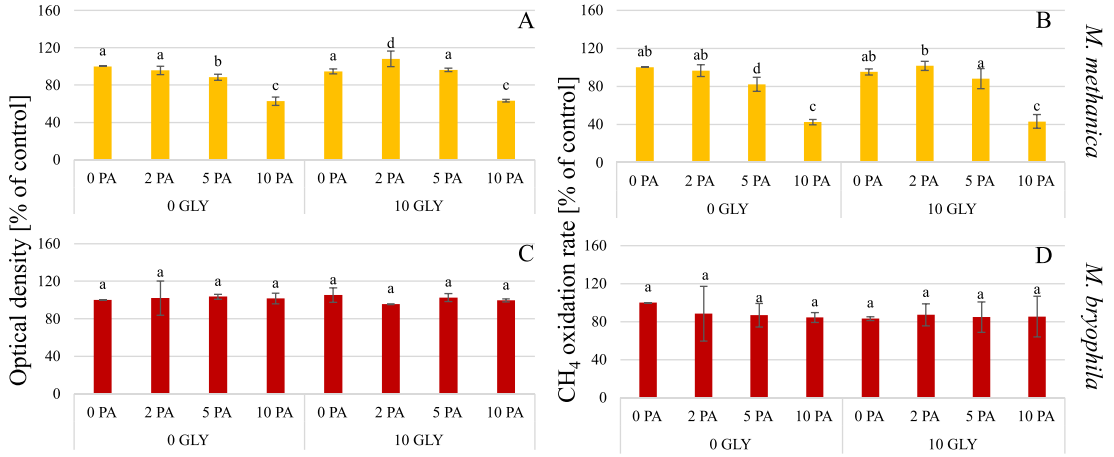

Fig. 2. Optical density (left, λ = 600 nm) and methane oxidation rate (right) of pure methanotrophic strains of pure methanotrophic strains after 48-hour exposure to glyphosate (0 and 10 μg mL^{-1}) and pelargonic acid (0, 2, 5 and 10 μg mL^{-1}). The figure shows parameter change relative to the control treatment (0 μg mL^{-1} PA + 0 μg mL^{-1} GFP). The dashed red line separates treatments with 0 and 10 μg mL^{-1} GFP. Data are presented as mean ± SD for n = 3. Statistical analysis was performed on absolute values, separately for each strain and parameter, and include Tukey HSD test at $p < 0.05$. The ns index indicates the absence of statistically significant differences between flagged treatments.

3.2. *Response of methanotrophic enrichment cultures to PA and GLY*

3.2.1. *CH_4 oxidation rate and cell density of ECs exposed to PA*

In Stage I of the experiments, the response of the different ECs to PA was highly variable (Fig. 4). In EC-G, growth rates were not significantly affected until exposure to 50 μg cm^{-3} PA (Fig. 4A). Methanotrophic activity began to decrease significantly at just 2 μg cm^{-3} PA (although only by 27.49% compared to the control) but a 50% reduction was not observed until 50 μg cm^{-3} PA (Fig. 4B). In contrast, both CH_4 oxidation and optical density in EC-L cultures decreased sharply at 10 μg cm^{-3} PA (Fig. 4C-D). Interestingly, despite the reduction in methanotrophic activity, further increases in PA concentration resulted in enhanced bacterial growth (an increase in optical density at concentrations above 10 μg cm^{-3}) (Fig. 4C), suggesting a shift in microbial community structure towards non-methanotrophic microorganisms capable of PA utilization. The pattern of methanotrophic activity change followed similar trend to that observed in *M. methanica* (Fig. 1B). Growth rates in the EC-F community were also significantly stimulated by exposure to 50 and 100 μg cm^{-3} PA (Fig. 4E), while methanotrophic activity was only marginally affected (Fig. 4F). The response of EC-F methanotrophic activity to PA was similar to that observed in *M. bryophila* (Fig. 1D).

In Stage II (PA and GLY cross experiment) (Fig. 5), comparing to control treatment, both cell density and methanotrophic activity in EC-G decreased under exposure to both PA and GLY (Fig. 5A,B). In this case, GLY did not provide any additional effect to CH_4 oxidation rates or cell density. In EC-L, both optical density and methanotrophic activity significantly decreased under exposure to 10 μg cm^{-3} PA; however, the addition of 10 μg cm^{-3} GLY stimulated growth and activity of MB (Fig. 5C,D). In EC-F, CH_4 oxidation significantly increased when exposed to GLY, with only a minimal effect on cell density (Fig. 5E,F).

3.2.2. *Microbial community structure and methanotrophic cell quantity as affected by PA and GLY*

The variable responses of the ECs to PA and GLY were a consequence

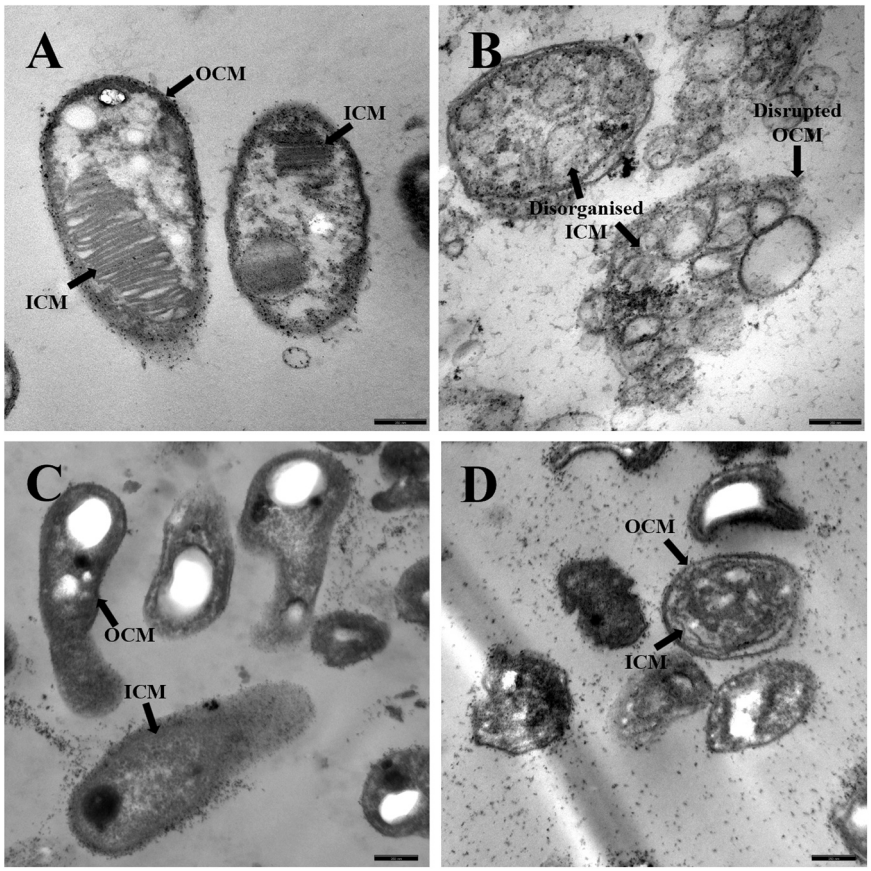

Fig. 3. Transmission electron micrographs (TEM) showing the effect of pelargonic acid on *M. methanica* (top) and *M. bryophila* (bottom). Control cells (A, C) after 10 min of exposure to 10 μg mL^{-1} PA (B, D). *Scale bar* = 250 nm. OCM – outer cellular membrane, ICM – intracellular membrane.

of differences in their microbial community structure (Fig. 6). EC-G was abundant in *Methylocystis* (78.31 ± 1.69%; Type II MB). With increasing PA concentration, the relative abundance of MB decreased to approximately 62%, while the addition of GLY increased it to around 73% (Fig. 6A). The EC-L community was dominated by *Methylobacter* (98.54 ± 0.39%; Type I MB) (Fig. 6B). Unlike EC-G, exposure of EC-L to 10 μg cm^{-3} PA led to a drastic decline in the relative abundance of MB, down to 8.50% of the total community. A similar reduction was observed under simultaneous exposure to both PA and GLY (8.24%).

In contrast to the MB-rich ECs, EC-F was abundant with heterotrophs (MB comprised only 36.51 ± 26.09%: *Methylocystis)* (Fig. 6C). Increasing PA concentrations further reduced the relative abundance of MB, reaching 17.52% (7.32% with GLY) and 1.74% (5.8% with GLY) at PA concentrations of 2 and 10 μg cm^{-3}, respectively. Notably, similar to EC-G (Fig. 6A), in EC-F community, in the treatment without PA but with GLY, the relative abundance of MB also decreased (to 13.88%). Detailed information on changes in the non-methanotrophic community following PA and GLY treatment can be found in Supplementary Figure B.1.

Results of the dPCR reactions quantifying the *pmoA* gene revealed that both PA and GLY have the potential to impact MB cell counts, although the effects differed among the communities (Fig. 7). In EC-G, MB cell count remained largely unchanged under PA exposure, while a significant increase was reported with combined PA and GLY treatment (Fig. 7A). In contrast, in EC-L, exposure to 10 μg cm^{-3} PA significantly reduced MB counts, and notably, GLY did not alleviate this adverse effect (Fig. 7B). In EC-F, the addition of both PA and GLY resulted in an increase in MB abundance. In this case, GLY alone resulted in a significant increase in MB quantity (Fig. 7C).

The decrease in MB cell count in EC-L (Fig. 7B) corresponds with a reduction in optical density (Fig. 5C), methanotrophic activity (Fig. 5D) and the relative abundance of MB (Fig. 6B). However, this pattern was not observed in EC-G and EC-F, where a simultaneous increase in MB cell count (Fig. 7A,C) and decrease in MB relative abundance (Fig. 6A,C) an may be explained by the growth stimulation of heterotrophic microorganisms in response to the addition of herbicides.

3.3. The response of Type I and II methanotrophs to PA and GLY

The response of methanotrophic activity and cell growth is directly linked to the composition of the methanotrophic community, particularly to the MB type they represent. In *M. methanica* and EC-L (*Methylobacter*-dominated) (Supplementary Figure B.2), which both represent Type I MB, cell growth (Figs. 2A and 5C), the CH_4 oxidation rate (Figs. 2B and 5D), and MB relative abundance (Fig. 6B) were all severely decreased upon exposure to PA at concentrations ≥ 10 μg cm^{-3}, and the addition of GLY did not alleviate the negative effects of PA.

In contrast, in *M. bryophila* and Type II MB-dominated ECs (EC-G and EC-F) (Supplementary Figure B.2), PA did not have negative effect on MB growth (Figs. 2C and 5A,E). Although, exposure to PA in EC-F resulted in a significant decrease in the MB ratio (Fig. 6C). This was not due to the inhibition of *Methylocystis* growth (Fig. 7C), but rather to the stimulation of the non-MB community (Supplementary Figure B.2). This effect was not observed in EC-G, where *Methylocystis* accounted for more than 60% of total microbial community (Fig. 6A).

4. Discussion

4.1. Effect of PA on activity and growth of methanotrophic bacteria

Despite extensive research on the antibacterial activity of the medium-chain FA (conducted mostly for medical research), to date, the

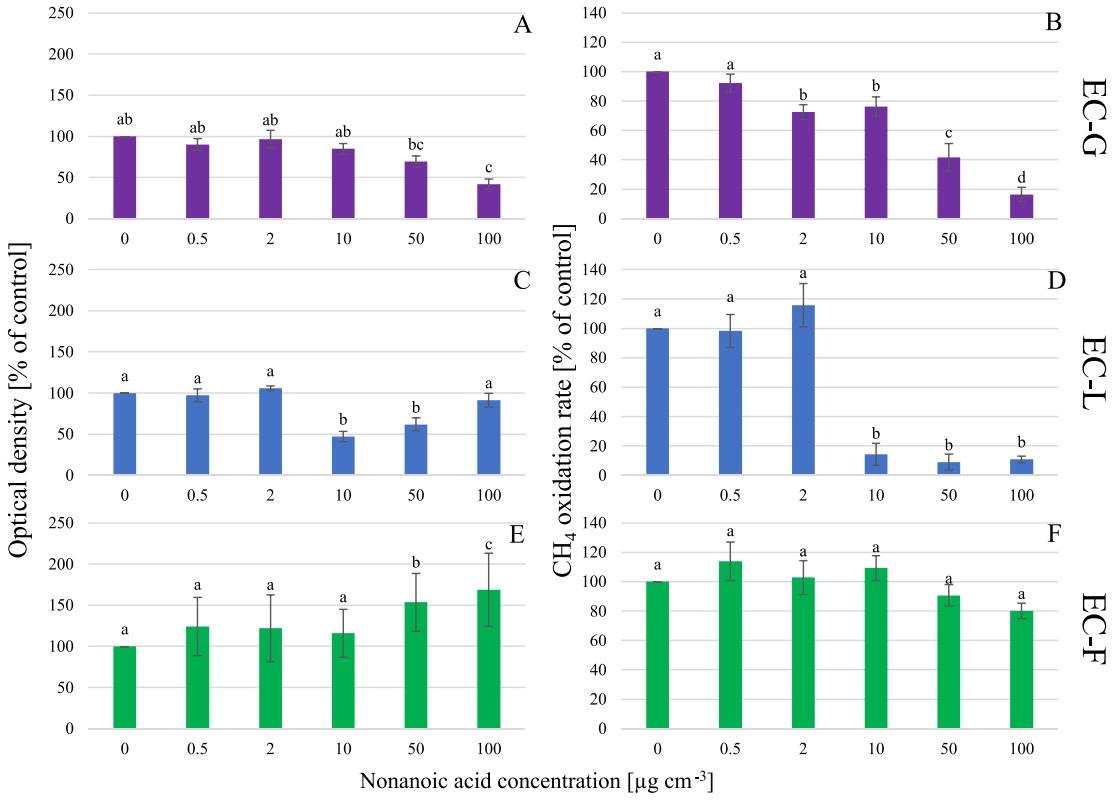

Fig. 4. Optical density (left, λ = 600 nm) and methane oxidation rate (right) of methanotrophic enriched cultures isolated from Gleysol (EC-G), Leptosol (EC-L) and Fluvisol (EC-F) after 48-hour exposure to pelargonic acid (0, 0.5, 2, 10, 50 and 100 µg mL^{-1}). The figure shows parameter changes relative to the control treatment (0 µg mL^{-1} PA). Data are presented as mean ± SD for n = 3. Statistical analysis was performed on absolute values, separately for each enriched culture and assay, and include Tukey HSD test at $p < 0.05$.

studies investigating the effect of PA on environmental bacteria are scarce or, in the case of MB – entirely absent. This knowledge gap is surprising, considering that PA is already used in agriculture (Supplementary Material A) and, due to its non-selective mechanism of action, targets a wide range of microorganisms (Ciriminna et al., 2019). The antibacterial properties of PA result from its ability to permeabilize, damage, and disrupt cellular membranes, also leading to cytoplasmic disorganisation (Bergsson et al., 2001a,b; Hu et al., 2025). The results presented in this study clearly show that the response of MB to PA differs between Type I and II, with the latter being more resistant to the disruptive action of PA. The key factor explaining this difference appears to be the durability of the CM (Fig. 3).

Type I and Type II MB synthesise different lipid structures. Regarding the main component of cell membranes – phospholipids – Type I MB are rich in phosphatidylethanolamine (PE) and phosphatidylglycerol (PG) and contain primarily saturated and monounsaturated C16 fatty acids (FA). In contrast, Type II MB are rich in phosphatidylmethylethanolamine (PME) and PG, and are dominated by C18 FA (Fang et al., 2000; Sundh et al., 2000). The cell membranes of MB also contain a range of bacteriohopanoids (BHPs) – pentacyclic triterpenoid lipids that contribute to the structural integrity of cell membranes and are known to protect against environmental stress by modulating membrane permeability (Osborne et al., 2017; Zeng et al., 2022). These vary between Type I (Rush et al., 2016) and II (Osborne et al., 2017) MB. In general, such cellular features make Type II MB more resilient to environmental factors than Type I MB (Graham et al., 1993; Ho and Frenzel, 2012; Ho et al., 2016). The findings of the current study are consistent with this understanding and confirm that the greater CM stability of Type II MB also applies to resistance to PA exposure (Fig. 3C, D).

Research conducted on environmentally abundant fungi *Candida albicans* revealed that PA strongly inhibited its optical density, virulency, hyphal growth, and cell aggregation in the 0.5 – 10 µg cm^{-3} concentration range (Lee et al., 2021). Importantly, these findings are in line with the results presented in the current study (Fig. 3A, B). This is likely because the CMs of PA-sensitive *C. albicans*, similar to Type I MB, are primarily composed of PE and contain a high proportion of C16 FA. In contrast, PME, which is abundant in Type II MB, is not present in *C. albicans* (Hitchcock et al., 1986; Mishra and Prasad, 1990; Prasad and Singh, 2013). These similarities in lipid composition between the CM of Type I MB (*M. methanica* and dominant MB in EC-L) and *C. albicans* may explain the increased sensitivity to PA, compared to Type II MB (*M. bryophila* and dominant MB in EC-G and EC-F).

Furthermore, the negative effect of PA has also been previously reported for the cyanobacterium *Microcystis aeruginosa*. In that case, growth inhibition was observed at PA concentrations as low as 0.5–0.75 µg cm^{-3} (Hu et al., 2025; Shao et al., 2009), which is approximately 13 – 20 times lower than those used in the current study on MB. According to (Hu et al., 2025), the negative impact of PA on *M. aeruginosa* resulted from disruption of the crucial membrane-associated process of photosynthesis. Exposure to 0.75 µg cm^{-3} PA led to reduction in chlorophyll *a* content and damage to the photosystem II (decrease of F_v/F_m parameter) (Hu et al., 2025). The thylakoids of *M. aeruginosa* are arranged in lamellar stacks (Hong et al., 2009), a structure similar to the intracellular membranes (ICM) in Type I MB such as *M. methanica* and *Methylobacter*. These stacked membrane structures contain the enzyme methane monooxygenase (MMO) and are essential for CH_4 metabolism in MB. As shown in Fig. 3A, their structural arrangement (analogously to thylakoids of *M. aeruginosa),* is highly disrupted following PA exposure, leading to a decline in the methane oxidation capacity of the cells (Fig. 1A, B). In

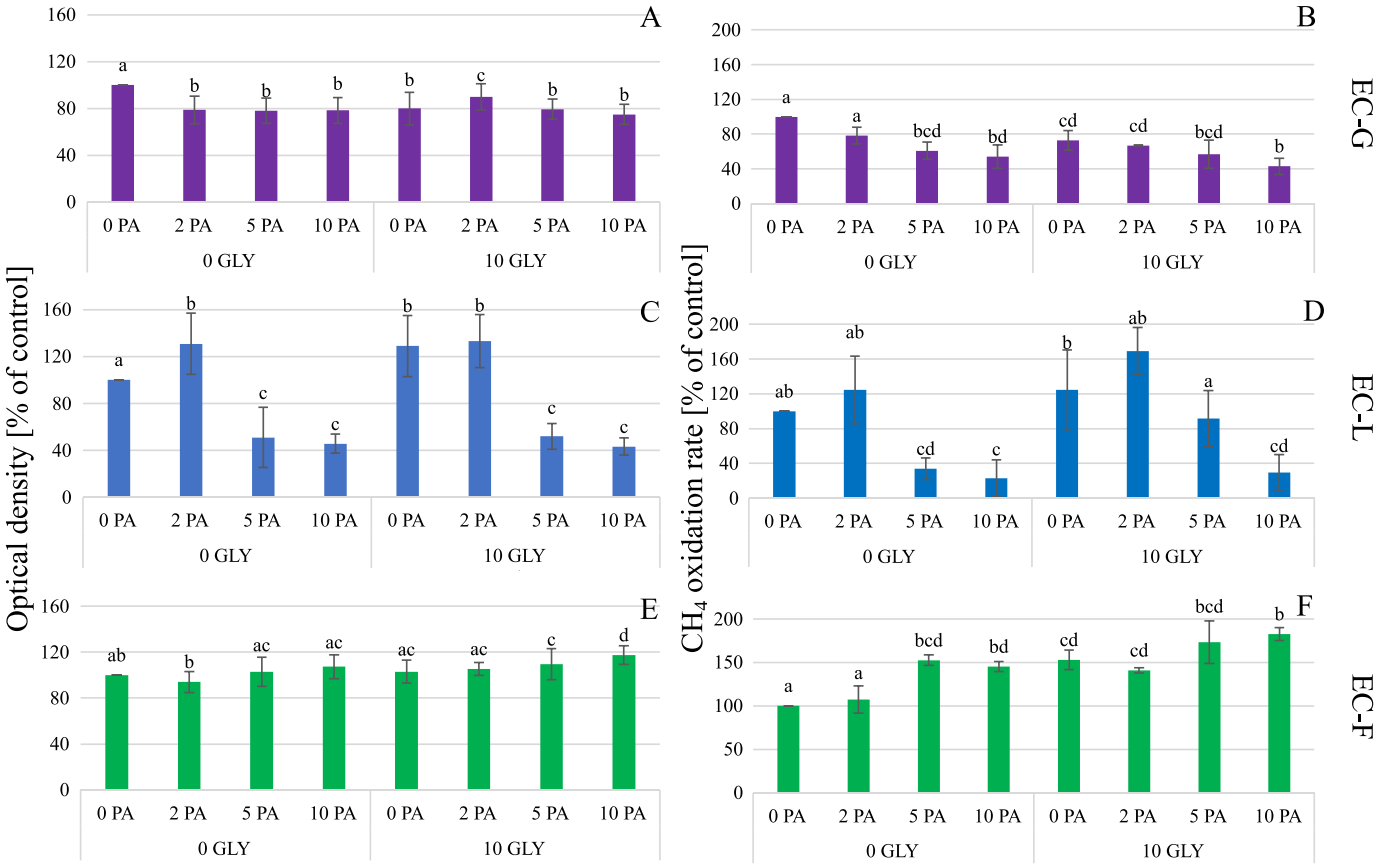

Fig. 5. Optical density (left, λ = 600 nm) and methane oxidation rate (right) of methanotrophic enriched cultures isolated from Gleysol (EC-G), Leptosol (EC-L) and Fluvisol (EC-F) after 48-hour exposure to glyphosate (0 and 10 μg mL^{-1}) and pelargonic acid (0, 2, 5 and 10 μg mL^{-1}). The figure shows parameter changes relative to the control treatment (0 μg mL^{-1} PA + 0 μg mL^{-1} GFP). The dashed red line separates treatments with 0 and 10 μg mL^{-1} GFP. Data are presented as mean ± SD for n = 3. Statistical analysis was performed on absolute values, separately for each enriched culture and parameter, and include Tukey HSD test at $p < 0.05$.

contrast, the ICM in Type II MB (e.g. *M. bryophila* and *Methylocystis*) are arranged parallel to the cytoplasmatic membrane (Belova et al., 2013) and were only slightly disorganised upon PA treatment (Fig. 3C, D). It seems that, in addition to the more PA-resistant composition of Type II MB cell membranes, their organization creates an additional physical barrier to PA penetration, allowing the cells to maintain their metabolic functions.

4.2. *Effect of GLY and PA GLY mixtures on methanotrophic bacteria*

Glyphosate may adversely affect microorganisms through a number of mechanisms. The most well-known are inhibition of the key enzyme in the shikimic acid pathway: 5-enolpyruvylshikimate^{-3}-phosphate synthase (EPSPS, AroA) and chelation of micronutrients (including Cu, Zn, and Ca ions) being key cofactors involved in the methane oxidation pathway. Recent findings suggest that glyphosate may also interfere with other enzyme proteins involved in key metabolic processes (ProA and AroH) in *E. coli* (Aldehoff et al., 2025). Despite this inhibitory potential, GLY exposure did not reduce but even significantly increased the cell numbers of *Methylocystis* in EC-G and EC-F (Fig. 7A,C).

With respect to EPSPS (AroA) inhibition, the literature indicates that the form of this enzyme present in methanotrophs is insensitive to glyphosate. The absence of any chelation-related effects may be explained by the low concentration of the herbicide in the culture medium. Regarding ProA and AroH, interference with the activity of these enzymes has so far been demonstrated only in *E. coli*. Further in-depth investigation of this proposed mechanism is therefore required before its potential toxicity to methanotrophic bacteria can be assessed. The results of the present study suggest that this mechanism was not a contributing factor in the methanotrophic bacteria examined here.

Notably, despite an increase in cell quantity, methanotrophic activity of *Methylocystis* in EC-G decreased (Fig. 5B). This discrepancy may be explained by the fact that *Methylocystis* is a facultative MB, capable of utilizing alternative carbon sources. It is possible that GLY, or its degradation products, provided an additional carbon source that was utilized alongside CH_4. The ability of *Methylocystis* to utilize multicarbon sources has already been demonstrated, including growth on acetate and ethanol (Belova et al., 2011; Im et al., 2011). Furthermore, *Methylocystis* has also been shown to grow on the degradation products of certain xenobiotics, such as anthracene (Magdy et al., 2022). Furthermore, the increased growth of *Methylocystis* under GLY exposure confirms that the herbicide does not inhibit its EPSPS. This is because bacterial EPSPS differs structurally among microorganisms. Those containing Type I EPSPS (not to be confused with Type I MB) are putatively sensitive to glyphosate, whereas microorganisms with Type II EPSPS (not to be confused with Type II MB) tend to be glyphosate-resistant (Leino et al., 2021; Rainio et al., 2021). Predictions of the classification of methanotrophic EPSPS proteins (https://ppuigbo.me/programs/EPSPSClass/), performed by Leino and co-workers (2021) suggest that the majority of both Type I and II MB — including the taxa important for the current study (*M. methanica* and representatives of *Methylocystis* and *Methylobacter)* — contain glyphosate-resistant EPSPS. These findings are consistent with the lack of inhibitory effects of GLY on the MB observed in our experiments (Fig. 5B,D,F).

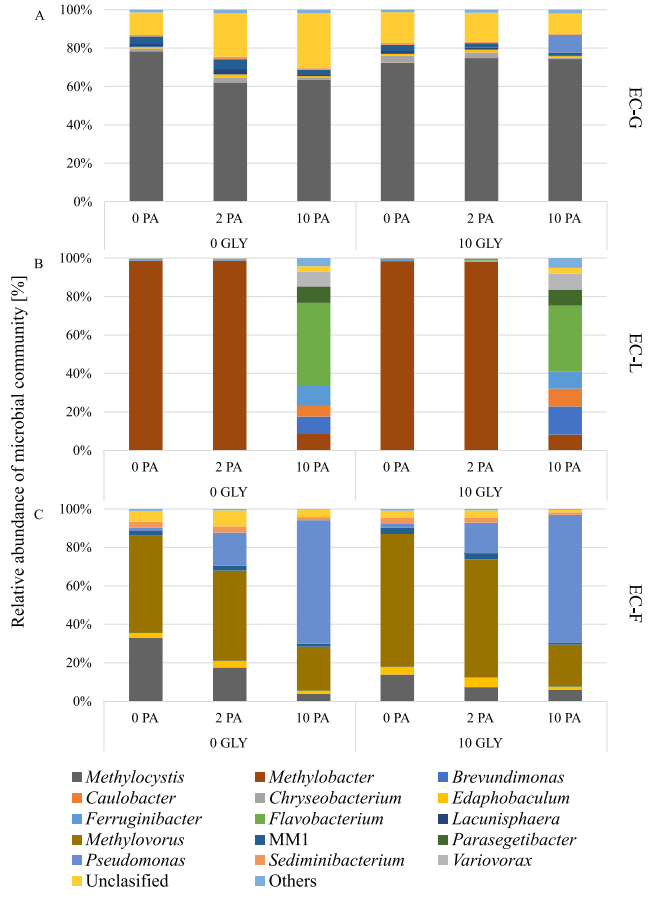

Fig. 6. Relative abundance of microbial community (Genus) in EC of the Gleysol (EC-G), Leptosol (EC-L) and Fluvisol (EC-F) after 48-hour exposure to GFP (0 and 10 μg mL^{-1}) and pelargonic acid (0, 2 and 10 μg mL^{-1}). The dashed red line separates treatments with 0 and 10 μg mL^{-1} GFP.

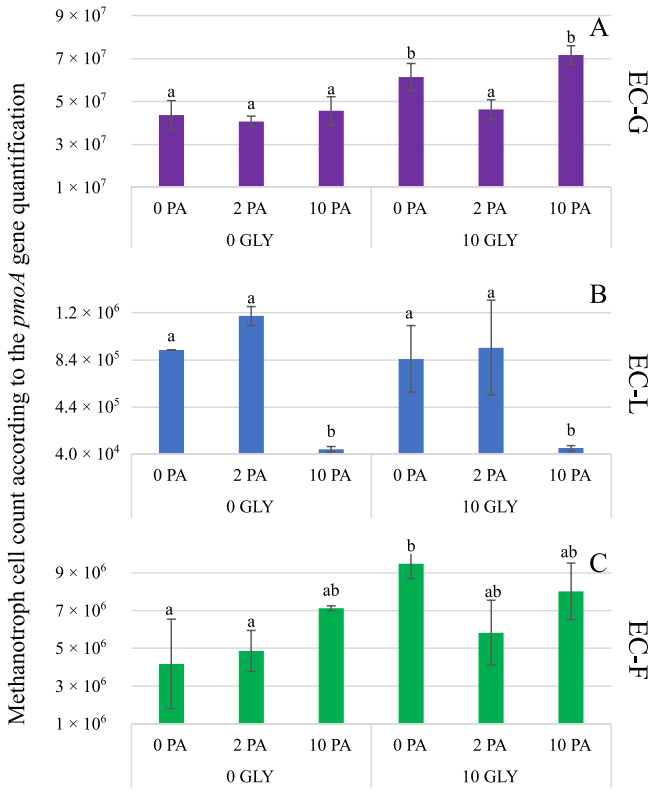

Fig. 7. Methanotroph cell counts based on dPCR amplification of the *pmoA* gene in EC-G, EC-L and EC-F after 48-hour exposure to PA (0, 2 and 10 μg mL^{-1}) and GFP (0 and 10 μg mL^{-1}) According to Chiri et al., methanotroph cells, on average, contain two copies of the *pmoA* gene. The dashed red line separates treatments with 0 and 10 μg mL^{-1} GFP. Data are presented as mean ± SD for n = 3. Statistical analysis: Tukey HSD test at $p < 0.05$ for each EC.

4.3. Implications for pesticide risk assessment

As mentioned in the introduction, PA is approved for use as safe for microorganisms (Álvarez et al., 2025). However, the risk assessment method applied, did not identify microorganisms as a primary target of PA, but rather as a factor contributing to its removal from the environment and thereby limiting its impact on other organisms. Our research shows that PA has differential effectiveness against various non-target microorganisms. In the case of MB, it selectively eliminates Type I MB cells. Upon exposure to PA, the variation in sensitivity among MB types may affect microbial community composition and their potential to oxidise CH_4. This is because Type I and II MB differ in terms of their substrate preferences and methane oxidation efficiencies. Type I MB are r-strategists, characterised by a rapid response to the presence of CH_4 and the ability to oxidise it with high efficiency. However, under conditions of limited CH_4 or oxygen availability, Type II MB become dominant. They are described as k-strategists and show greater resistance to environmental stressors (e.g. heat stress or oxygen limitation) (Knief, 2015). Nevertheless, they are characterised by a slower rate of CH_4 oxidation. Since the ecological niches of Type I and Type II are not identical, they often coexist in natural environments, complementing each other and providing a buffer for methane emissions — even fluctuating (Graham et al., 1993; Morris et al., 2002; Pfluger et al., 2011).

It should be noted that MB communities show high resilience to occasional disturbances. According to research by Lim et al. (2024), in the case of a single, non-permanent disturbance, the resulting decline in soil MB diversity and abundance is typically reversed within a few days. However, in the case of persistent or repeated disturbances, the collapse of the MB community can be long-lasting, and the restoration of its function may take several years.

Considering the above, the repeated use of PA throughout the year may could affect the diversity of MB communities — through the selective elimination of Type I MB — and may potentially limit the ability of soil ecosystems to oxidise methane. This is an issue that requires further research. Unfortunately, the impact of herbicides on microbial biodiversity, is not currently part of the pesticide safety evaluation system. The problem of insufficient risk assessment regarding the effects of pesticides on biodiversity was also highlighted by Solé et al. (2024). Although the authors mainly emphasised the need to expand the scope of pesticide ecotoxicity studies to include higher organisms and food web security, our findings suggest that their conclusions are equally relevant to microorganisms.

The current guidelines on pesticide ecotoxicity testing methodologies were formulated many years ago (Regulation (EC) No 1107/2009). Given the rapid growth in ecological knowledge and the growing development and availability of techniques for studying microbial communities in the environment (Clagnan et al., 2024; Ejaz et al., 2024), a revision of the existing methods used to assess the environmental safety of pesticides is necessary. This would enable the evaluation of the effects of agricultural practices on such an important group of microorganisms as MB.

5. Conclusion

In this study, investigating the effect of nonanoic (pelargonic) acid and glyphosate on methanotrophic bacteria, we found that the Type I MB (*M. methanica* and *Methylobacter* sp.) are more sensitive to nonanoic

acid than Type II MB (*M. bryophila* and *Methylocystis* sp.). This sensitivity may be related to the differences in composition and organization of the cellular membranes in both types. Nonanoic acid at a concentration of 10 μg cm^{-3} (corresponding to concentrations found after field application of the herbicide) significantly inhibited methanotrophic activity of Type I MB pure strains and enrichment cultures, indicating a potential risk of nonanoic acid-based herbicides to methane oxidation in soils. In contrast to nonanoic acid, an environmentally relevant dose of 10 μg cm^{-3} of glyphosate did not negatively affect the activity or growth of the methanotrophic bacteria.

The selective negative impact of nonanoic acid on Type I MB presented in this work, point to the necessity to revise pesticide safety evaluation systems by incorporating modern methods for studying microbial diversity. Due to the nature of this experiment (*in vitro* culture studies), the presented results may indicate the direction of the changes occurring in the soil methanotrophic community exposed to nonanoic acid and glyphosate. However, further experiments are needed to confirm whether these effects and mechanisms also occur in soil environment.

CRediT authorship contribution statement

Adam Furtak: Writing – review & editing, Writing – original draft, Visualization, Resources, Investigation, Formal analysis, Data curation, Conceptualization. **Anna Pytlak:** Writing – review & editing, Visualization, Supervision, Project administration, Funding acquisition, Conceptualization. **Andrzej Bieganowski:** Writing – review & editing, Validation. **Emil Zięba:** Resources. **Tomasz Skrzypek:** Resources. **Anna Szafranek-Nakonieczna:** Writing – review & editing, Resources. **Anna Bilokinna:** Resources.

Declaration of Competing Interest

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.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ecoenv.2026.120134.

Data availability

The data underlying this article are available in Zenodo (DOI: 10.5281/zenodo.17481486) and in the NCBI BioProject under accession number PRJNA1315137.

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