



Adaptation of methanogenic microbial assemblages to antibiotics: The role of resistance genes and taxonomic composition[☆]

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ABSTRACT

Antibiotics are omnipresent contaminants in aquatic systems and can impact key ecosystem processes. Methanogenesis by anaerobic Archaea is such a process that has gained attention because antibiotics can increase their methane (CH₄) production dynamics – a 28-fold more potent greenhouse gas than carbon dioxide. Since such effects may depend on assemblage composition and antibiotic resistance, we investigated antibiotic effects on methanogenesis in sediments from a negligibly impacted site (reserve) and downstream of a wastewater treatment plant (WWTP). Prior to incubation, short-term pre-treatment with antibiotics aimed to stimulate adaptive responses. During incubation, antibiotics reduced methanogenesis speed in WWTP sediment (−7 %) but increased it in the reserve (10 %), with site-specific patterns linked to differences in prokaryotic assemblage composition and their gene expression. Methanomicrobia, a key methanogenic group, showed contrasting responses across sites, likely mediated by prokaryotic substrate dynamics, particularly within the acetate pathway. Pre-treatment effects on methanogenesis dynamics were minor (maximum Bayesian factor of 3.6), but subtle shifts in prokaryotic activity and composition were observed. Elevated antibiotic resistance gene expression in WWTP sediments reflected historical exposure but did not mitigate antibiotic impacts on methanogenesis. These findings show the vulnerability of methanogenic assemblages to antibiotics despite potential adaptations and emphasize the risks posed by pharmaceutical pollution to critical freshwater ecosystem functions.

1. Introduction

Pharmaceuticals are extensively utilized in human and veterinary medicine (Klein et al., 2018; Van Boeckel et al., 2014). Despite their long history of use, they are recognized as organic micropollutants of emerging concern in fresh- and groundwaters (Lapworth et al., 2012). Substances enter these ecosystems via various point and non-point sources including wastewater treatment plant (WWTP) effluents (Parra-Saldivar et al., 2021), manure application (Watanabe et al., 2010), or aquaculture (Cabello, 2006) and can spread via groundwater-surface water exchange (Buerge et al., 2009). Among pharmaceuticals, antibiotics are of particular concern due to their ubiquity in aquatic environments (Danner et al., 2019), where they

favour the development and horizontal transfer of antibiotic resistance genes (ARGs) (Novo et al., 2013) and induce direct toxicity towards microbes (Ding and He, 2010) that may propagate bottom-up (Bundschuh et al., 2017, 2009; Maul et al., 2006).

One freshwater process that has recently received growing attention – also in the context of antibiotics – is the production of methane (CH₄). Methane has 28 times the global warming potential of carbon dioxide (CO₂), contributes ~30 % to the observed global warming (IPCC, 2021) and is largely emitted from natural freshwater and wetland systems (Saunois et al., 2020). Although aerobic CH₄ production is reported (Mao et al., 2024), CH₄ is mainly synthesized by Archaea in anaerobic sediments in a multi-step cascade of hydrolysis, acidogenesis and acetogenesis involving diverse bacteria (Conrad, 2020). Antibiotics

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accumulate in freshwater sediments (Kerrigan et al., 2018; Zhou et al., 2011), exposing these bacteria and methanogenic Archaea directly. Despite a limited number of studies, current evidence suggests that antibiotics can increase CH₄ production by changing chemical conditions (Zhang et al., 2023) or directly affecting bacteria that produce methanogenic substrates (Bollinger et al., 2021). However, these studies often overlook pre-exposure of assemblages which stimulates the development and horizontal transfer of ARGs (Koonin et al., 2001; Ochman et al., 2000). In addition, toxicants can induce a shift towards more tolerant species (Tlili et al., 2016) and can alter the activity of assemblage members by inducing metabolic dormancy to withstand stressful conditions (Chebotar' et al., 2021). This in turn can shape the underlying functional capacity of communities and consequently also CH₄ production. These considerations question whether the effects observed on methanogenesis in presence of antibiotics are modified in direction and magnitude if communities are stress-adapted.

To address this, we compared a negligibly impacted assemblage (i.e., no pre-exposure to antibiotics) with one sampled downstream of a WWTP (stress-adapted). In the laboratory, both assemblages were either cultured with or without an antibiotic mixture for three weeks to provoke a short-term adaptation. Subsequently, these communities were incubated anaerobically in presence of increasing concentrations of antibiotics (three levels including toxicant-free control). We hypothesised that, due to pre-existing adaptation, effects of antibiotics on CH₄ production are less pronounced in the WWTP assemblage and that the adaptation invoked by the antibiotic pre-treatment is more effective due to horizontal transfer of ARGs (Koonin et al., 2001; Ochman et al., 2000). Mechanistic insights were gained via compound-specific stable isotope analysis (i.e., proxy for utilized substrates), 16S rRNA metabarcoding (i.e., active prokaryotic community) and gene expression profiling (i.e., active ARGs and metabolic processes).

2. Material & methods

2.1. Sampling sites and pre-treatment

Sediment was taken from the top ~20 cm layer of a site located in the Palatinate Forest-North Vosges Biosphere Reserve within the premises of the Eusserthal Ecosystem Research Station upstream from anthropogenic impacts (49°25' N; 7°96'E, called reserve henceforth) and a site ~500 m downstream of a WWTP discharge point close to Karlsruhe, Germany (49°4' N; 8°20' E, called WWTP henceforth). Unlike the reserve site, the latter site was assumed to have a diverse resistome due to constant exposure to antibiotics as shown in an earlier study (Brown et al., 2019). In 2 L beakers, 700 g of these sediments (n = 6) were pre-treated with 1 L unfiltered pond water (~90 µS cm⁻¹) from the reserve site dosed with 5000 µg L⁻¹ of a five-component mixture (amoxicillin, ciprofloxacin, erythromycin, sulfamethoxazole, and tetracycline each at 1000 µg L⁻¹) (Bollinger et al., 2021). The pre-treatment lasted for three weeks in total darkness at 20 ± 1 °C with weekly renewal of the water while keeping the sediment wet to ensure constant exposure to antibiotics. Samples of both old (after a week) and new water were stored at -80 °C for chemical analyses (see 2.3). In the following, we refer to this antibiotic treatment as “pre-treatment”, while the term “treatment” is used to describe antibiotic treatments at different concentration levels employed during the incubation study (see 2.2).

2.2. Incubation study

For the incubation study, pre-treated sediments were provided with fresh organic substrate in form of 20 g dry leaf powder (*Alnus glutinosa*) per kg wet sediment (Bollinger et al., 2021). Subsequently, 10.12 ± 0.46 g of this sediment were transferred to crimp top serum bottles (n = 20, V = 100 mL, ND20, VWR, Avantor Inc., six additional replicates for metabarcoding and gene profiling). Thereafter, 30 mL unfiltered pond water at pH 7 amended with sum concentrations of 0, 5, and 5000 µg L⁻¹

of the same five-component antibiotic mixture at equal mass fractions (designated as control, low, and high, respectively, Table S1) were added to the test system. This mixture represents five common antibiotic classes with relevant substances within these classes and consequently a wide range of toxic modes of action. The low concentration aligns closely with field-relevant levels (Danner et al., 2019), whereas the high concentration simulates more extremely polluted ecosystems (Fick et al., 2009). The headspace of the serum bottles was flushed with N₂ and hermetically sealed to warrant near-anaerobic conditions. Incubations proceeded in total darkness at 20 °C for 56 days. Water samples for antibiotic concentration analysis were collected at the start, middle, and end of the experiment and stored at -80 °C until further analysis.

2.3. Antibiotic analyses

Antibiotic concentration analysis followed Bollinger et al. (2024). Briefly, water samples were centrifuged and aliquots were transferred to amber vials, spiked with mass-labeled internal standards and methanol, and analyzed as direct injection using an ExionLC™ AD UHPLC coupled to a SCIEX Triple Quad™ 6500 tandem mass spectrometer. Quantification employed calibration standards with linearity of 0.9960–0.9994 (LOQ 0.4–5.0 µg L⁻¹, Table S2). Chemical recovery was on average 94–103 % for amoxicillin, ciprofloxacin, erythromycin, sulfamethoxazole, and 50 % for tetracycline, with the relative standard deviations of 4.2–9.3 % (n = 7, Table S2). Similar results were also obtained in the samples of MilliQ water spiked with the target antibiotics (n = 4, Table S2). Degradation was significant (51–84 % of initial concentration), with most of the target antibiotics below their respective LOQs by mid-to end-experiment (Table S3).

2.4. Greenhouse gases

The greenhouse gas measurements were carried out as described previously (Bollinger et al., 2024, 2021). Each week, 100 µL of headspace gas (V_i) from each incubation system was analyzed for CH₄ and CO₂ using a cavity-enhanced laser absorption spectrometer (UGGA, model 915-0011, Los Gatos Research Inc., USA) in a closed-loop setup. To determine the mole fraction in the headspace (x_h), we adjusted the equilibrium mole fraction (x_e, ppm) of the sample gas and loop gas, accounting for the loop volume (V_l, calibrated with certified CH₄ and CO₂ reference gases, Messer Industriegase, Germany) and the mole fraction of the background gas (x₀):

$$x_h = \left(\frac{V_l}{V_i} \right) \cdot (x_e - x_0) + x_e \quad (1)$$

The amount of dissolved CH₄ and CO₂ (n_w) was then estimated with Henry's law:

$$n_w = K_H \cdot x_h \cdot V_w \cdot f_1 \quad (2)$$

In this context, V_w represents the water volume, f₁ is a conversion factor (10⁻¹ Pa ppm⁻¹), and K_H is the Henry's law constant adjusted for the test temperature of 293.15 K (Lide, 2004; Weiss, 1974):

$$\ln(K_{H_{CH_4}}) = -115.6477 + 155.5756 \left(\frac{T_K}{100} \right)^{-1} + 65.2553 \cdot \ln \left(\frac{T_K}{100} \right) - 6.1698 \left(\frac{T_K}{100} \right) \quad (3.1)$$

$$\ln(K_{H_{CO_2}}) = -58.0931 + 90.5069 \left(\frac{T_K}{100} \right)^{-1} + 22.294 \cdot \ln \left(\frac{T_K}{100} \right) \quad (3.2)$$

Finally, CH₄ and CO₂ concentrations were standardized to the sediment dry-weight:

$$c(\text{CH}_4, \text{CO}_2) = \frac{n_w + (x_h \cdot V_h \cdot f_1) \cdot (R \cdot T)^{-1}}{m_s \cdot (1 + \Theta_g)^{-1}} \quad (4)$$

where V_h represents the headspace volume, R is the universal gas constant, m_s is the wet sediment weight, and Θ_g is the sediment's gravimetric water content.

2.5. Stable isotopes

Compound-specific isotope ratios were measured for five replicates per treatment on a weekly basis. Headspace gas was injected into a gas chromatograph (GC, Trace GC Ultra, Thermo Fisher Scientific, Germany) at 32 °C and separated on a capillary column (27.5 m length, 0.32 mm internal diameter, 10 µm film thickness, Agilent J&W PorapLOT Q, USA) connected to a ConFlo IV interface, which linked to a Delta V Advantage isotope ratio mass spectrometer (IRMS, Thermo Fisher Scientific, Germany). Injection volumes were adjusted to CH₄ and CO₂ concentrations in the samples to maintain consistent peak areas. Isotope ratios were reported in δ-notation relative to the Vienna Pee Dee Belemnite (VPDB) standard:

$$\delta^{13}\text{C} = \left(\frac{R_{\text{sample}}}{R_{\text{reference}}} - 1 \right) \cdot 1000 \text{ ‰} \quad (5)$$

These δ-values were then used to calculate the fractionation factor:

$$\alpha_c = \frac{\delta^{13}\text{C-CO}_2 + 1000}{\delta^{13}\text{C-CH}_4 + 1000} \quad (6)$$

CH₄ production primarily occurs through hydrogenotrophic methanogenesis (HM) from H₂ (indicated by higher α) and acetoclastic methanogenesis (AM) from acetate (indicated by lower α) (Whiticar, 1999).

2.6. RNA processing

As previously described (Bollinger et al., 2021), RNA was isolated from 2 g of sediment from each of the six replicate samples (see section 2.1) using the RNeasy PowerSoil Total RNA kit from Qiagen, adhering to the manufacturer's protocol. The RNA concentration was quantified with a Nanodrop2000 spectrophotometer (Thermo Fisher Scientific, Germany), and the integrity of the RNA was evaluated using the Agilent Bioanalyzer 2100 system.

2.7. Metabarcoding

To analyze the prokaryotic community in a comparable fashion, we followed our published protocol (Bollinger et al., 2021). Briefly, 2 µL of RNA extract from each replicate was converted to cDNA using the iScript synthesis kit (BioRad) with random primers. The hypervariable V4 region of the 16S rRNA gene was amplified using primers 515Fm (5'-GTGYCAGCMGCCGCGGTAA-3') and 806Rm (5'-GGACTACN VGG GTWCTAAT-3', Walters, 2015). For each replicate, 2 µL of cDNA ran through a program of initial activation at 98 °C for 30 s, followed by 26 cycles of 98 °C for 10 s, 63 °C for 30 s, and 72 °C for 30 s, and a 5-min final extension at 72 °C. To reduce PCR bias, three technical replicates were pooled afterward, and prepared for sequencing using the Next Ultra DNA Library Prep Kit for Illumina (NEB, USA). Library quality was checked with an Agilent Bioanalyzer 2100 system, and sequencing was performed on an Illumina MiSeq (2x300 nt, StarSeq, Mainz, Germany).

Reads were trimmed with cutadapt v1.18 (Martin, 2011) and then processed through the DADA2 pipeline (Callahan et al., 2016) with specific parameters: truncLen = c(192, 217), maxEE = 1, maxN = 0, and minOverlap = 20. Chimeras were removed using vsearch v2.13.7 (Rognes et al., 2016). Taxonomic classification was assigned to the resulting amplicon sequence variants (ASVs) using the SINTAX algorithm (Edgar, 2016) against the Greengenes database v13.5 (McDonald

et al., 2012). ASVs lacking taxonomic classification or with fewer than five occurrences across all samples were excluded (to eliminate potential artifacts, as per Bokulich et al. (2013)). The final ASV-to-sample matrix was used for statistical analyses (see section 2.9).

2.8. Metatranscriptome

Following earlier reports (Bollinger et al., 2024), approximately 2 µg of total RNA from each sample was used as the starting material for gene expression analysis. rRNA was selectively removed with the NEBNext rRNA Depletion Kit for Bacteria, following the manufacturer's instructions. The mRNA-enriched samples were then prepared for sequencing using the NEBNext Ultra II Directional RNA Library Kit. The quality-checked libraries were sequenced on an Illumina NextSeq 2000 (2x150 nt, StarSeq, Mainz, Germany).

The quality of the raw reads was assessed using FastQC (Andrews, 2010) and MultiQC (Ewels et al., 2016), then trimmed with Trimmomatic v0.39 (Bolger et al., 2014), using the following parameters: ILLUMINACLIP: NexteraPE-PE.fa:2:30:10, LEADING: 3, TRAILING: 1, SLIDINGWINDOW: 4:20 and MINLEN: 50. Residual rRNA were filtered using SortMeRNA v4 (Kopylova et al., 2012) with default settings. The mRNA reads were then assembled into contigs using Trinity v2.15.1 (Grabherr et al., 2011). Functional annotation used the SqueezeMeta v1.6.3 pipeline (Tamames and Puente-Sánchez, 2019), with assignments made to KEGG pathways. To analyze the ARG profile, open reading frame sequences were queried using BLAST against the CARD database (Alcock et al., 2023).

2.9. Statistics

CH₄ production over time was modelled as generalized logistic function (Bollinger et al., 2024; Grasset et al., 2021)

$$\text{CH}_4(t) = A + \frac{L - A}{(1 + v e^{-k(t-\tau)})^{\frac{1}{v}}} \quad (7)$$

where A and L are the initial and final concentrations of CH₄, k and τ are kinetic parameters and v modulates the symmetry of the curve. Following our previous rationale (Bollinger et al., 2024), τ (i.e., time until the inflection point is reached) is considered the most relevant endpoint of toxicity as it captures the first two successional phases of the incubation that involve the most relevant prokaryotic processes. Models were fit with the R package "brms" (version 2.20.4, chains: 4, iterations: 20000, thinning: 10, warmup: 16000) (Bürkner, 2017). Previously established priors (Bollinger et al., 2024) were used and sensitivity (Fig. S1) as well as validity (Fig. S2) were assessed likewise. For the latter, the standard variation (σ) was defined using a scaling factor (f_{scale}) determined with nonlinear least squares regression

$$\sigma = f_{\text{scale}} \cdot \frac{d\text{CH}_4}{dt} \quad (7a)$$

The models were 0.53 % inaccurate and 0.67 % imprecise.

Uncertainty in differences in τ between treatments, was assessed via contrasts of the posterior distribution as effect percentages. Posterior distributions were described using the maximum a posteriori estimate (MAP) and the 95 % highest-density credible interval (HDI). The Bayes factor (BF) was used to express the posterior odds of treatment effects on τ , indicating the likelihood of H_1 (e.g., treatment differences) versus H_0 (e.g., no treatment differences) given the data. For clarity and comparability, BF values were expressed as ≥ 1 , irrespective of effect direction.

Treatment effects on relative abundances from metabarcoding analyses were normalized to the control (ΔRA) for each treatment combination, allowing for consistent comparison across time points and treatments:

$$\Delta RA = \frac{\sum_{i=1}^n RA_{Ti} - RA_{Ci}}{\sum_{i=1}^n RA_{Ci}} \quad (10)$$

where RA_{Ti} and RA_{Ci} represent the relative abundance of ASVs in a treatment and control, respectively. Non-metric multidimensional scaling (NMDS) was performed on Hellinger-transformed relative abundances, based on Bray-Curtis dissimilarities, using the R package "vegan" (version 2.6–4) (Oksanen et al., 2022). Statistical significance was assessed using permutational multivariate analysis of variance (PERMANOVA) (Anderson, 2001), while species contributions to between-group dissimilarities were determined using SIMPER. Meta-transcriptional data was analyzed as transcripts per million (TPM) with the R package "SQMtools" (version 1.6.3) (Puentes-Sánchez et al., 2020).

All analyses were conducted in R (4.3.1) (R Core Team, 2023). Data and code are publicly available at Zenodo (<https://zenodo.org/doi/10.5281/zenodo.14679433>). Metabarcoding and metatranscriptome sequence data have been deposited in the NCBI BioProject database

(<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1207865>).

3. Results & discussion

3.1. Site-specific effects of antibiotics on methanogenesis without short-term adaptation

In absence of antibiotics, the CH_4 production was faster (~44 %) and higher (~74 %) at the WWTP site compared to the reserve (Fig. S3). While the latter might be driven by the availability of additional substrates in the sediment, the former might be a characteristic of groups predominant in this local assemblage. Notably, the WWTP sediment had a lower proportion of Firmicutes and Bacteroidetes but higher relative abundances of Proteobacteria and Acidobacteria (Fig. S4). However, these differences were less relevant at test start and thus might be evoked during the adaptation process to the anaerobic test system. These assemblage differences can also be influential in explaining the observed effects of antibiotics discussed in the following.

While the reserve sediment replicated antibiotic-enhanced

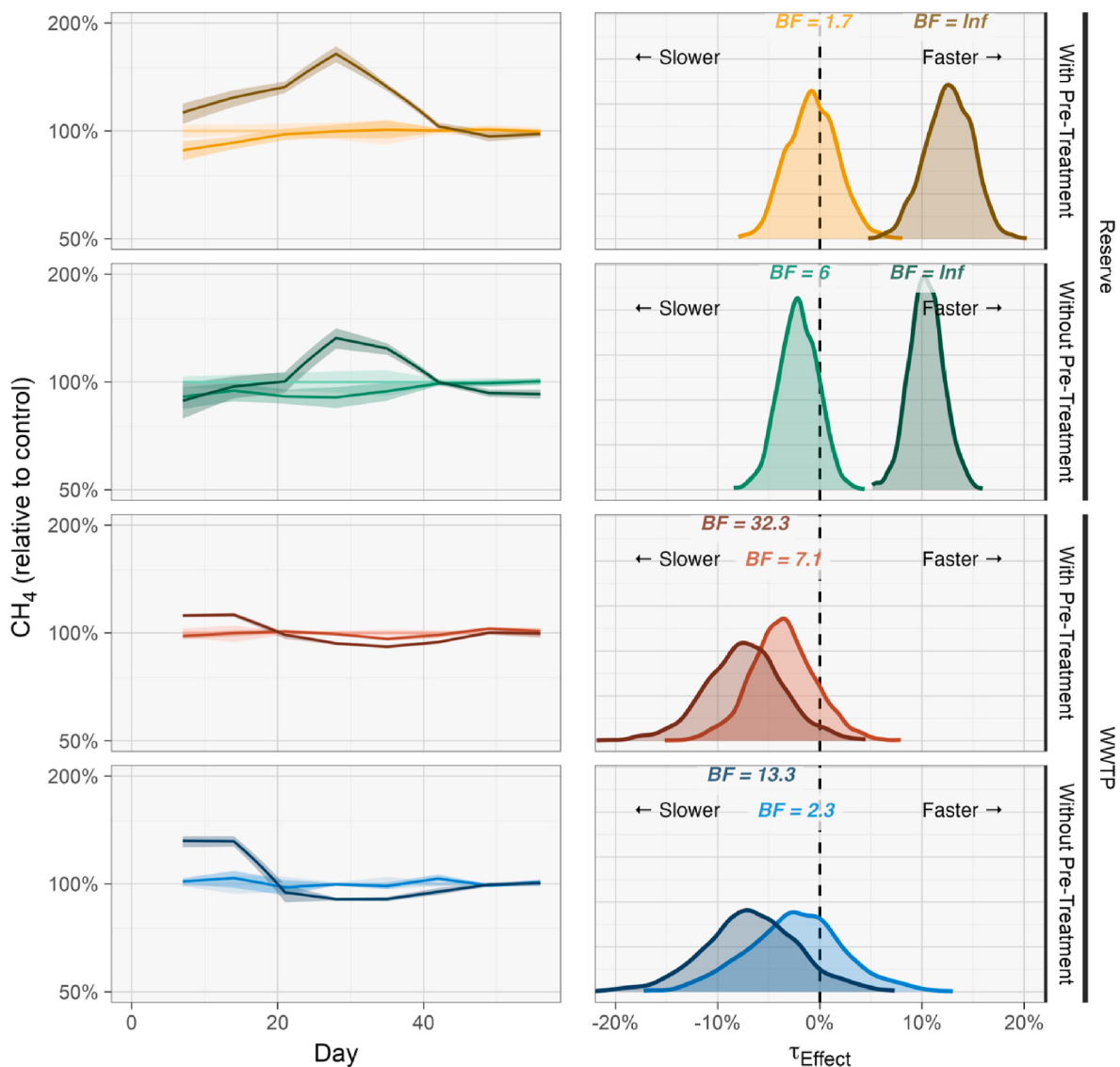


Fig. 1. CH_4 concentrations relative to the control (set to 100 %; mean $\pm$ 95 % confidence interval, left panels) and posterior density distributions of the kinetic parameter τ as effect size relative to the control (τ_{Effect} , right panels). Assemblage origin (reserve vs. WWTP) and pre-treatment are indicated by different rows and colors, while antibiotic concentration is represented by lightness (light: 0 $\mu g L^{-1}$, medium: 5 $\mu g L^{-1}$, dark: 5000 $\mu g L^{-1}$). The Bayes factor (BF) quantifies the odds ratio for the probabilities of τ_{Effect} being greater than or less than zero, with values always expressed as ≥ 1 . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

methanogenesis (Bollinger et al., 2024, 2021), the community from the WWTP site showed the opposite (Fig. 1). At the high antibiotic concentration, CH₄ production was 10.1 % (6.9–13.8) faster for the reserve but −7.1 % (−15.8 to 2.8) slower for the WWTP (Fig. 1). Moreover, effect dynamics (i.e., CH₄ concentration over time) were also inverted between sites. The reserve sediment initially showed reductive effects of antibiotics (−12 %) followed by positive effects during the exponential phase (33 %). Conversely, the WWTP sediment initially showed higher CH₄ production at the high concentration (32 %), which could indicate a higher initial antibiotic tolerance. Indeed, both expressed transcripts and number of ARGs were 1–2 orders of magnitude higher in the WWTP assemblage (Fig. 2) most likely as a consequence of their prior exposure towards antibiotics selecting for resistant species (Bengtsson-Palme and Larsson, 2016). In contrast, the positive effects on methanogenesis dynamics at the high concentration for the reserve sediment might indicate recovery because antibiotics were mostly undetectable in the middle of the experiment. The increase might therefore be explained by impaired substrate competitors (or competitive metabolic pathways) (McNichol et al., 2024) during the initial phase and a higher proportion of available substrate turned into CH₄. This would be in line with our previous hypothesis (Bollinger et al., 2024), that the presumed effect pathway of antibiotics on methanogenesis is mainly via bacteria-mediated substrate provision, instead of having a considerable direct toxic effect on methanogens. This is also underpinned by hydrolysis (mainly carried out by bacteria) being considered as rate-limiting step (Gonzalez et al., 2018). This fact complicates interpretation of the observed opposite effect patterns between sites given that hydrolysis is the first step in providing substrates for virtually every methanogenic pathway. Furthermore, linking structure to function in distant but interconnected metabolic systems is not trivial.

Nevertheless, both prokaryotic assemblages were significantly affected by antibiotics on structural level ($p < 0.0001$), while effects on the composition within the methanogenic Euryarchaeota were less pronounced (i.e., $p = 0.216$ for reserve and $p = 0.645$ for WWTP, Fig. 3). At the WWTP, contributions to dissimilarity were rather evenly distributed, for example, across classes of Proteobacteria (i.e., Alphaproteobacteria, Betaproteobacteria, and Gammaproteobacteria), while the classes Clostridia, Bacteroidia and Methanomicrobia contributed the most to assemblage dissimilarities at the reserve. Methanomicrobia, despite the lack of a significant assemblage shift within Euryarchaeota, were the class with the largest negative effects in the WWTP sediment

(−40 %, Fig. 3) and showed the second largest positive effects in the reserve (113 %, but only in the pre-treated assemblage). This pattern aligns with observations in methanogenesis (i.e., reduction for WWTP and increase for reserve, Fig. 1) and thus underpins the hypothesis of assemblage-mediated effects on methanogenesis with Methanomicrobia as the most important class. Understanding causes of this dissimilar response of Methanomicrobia between assemblages is thus a key objective for further research and might require physicochemical characterization, including concentrations of substrates. However, their ≤ 3.3 % dissimilarity contribution is still small compared to more abundant classes (e.g., Clostridia and Bacteroidia, Fig. 3), which may contribute indirectly to an altered methanogenesis by modifying available substrates.

Given uncertainties in inferring substrates from taxonomy, we focused instead on enzymatic activities through gene profiling. For both assemblages, methyl-coenzyme M reductase (MCR) was the most expressed enzyme of the CH₄ pathway which synthesizes but also decomposes CH₄ (Fig. 4). Midway through the experiment, the patterns of MCR effects closely mirrored those seen in Methanomicrobia. Both MCR activity and Methanomicrobia were reduced in the WWTP assemblage, while levels remained unchanged in the reserve assemblage (Figs. 3 and 4). A nearly identical pattern was observed for acetyl-CoA decarboxylase/synthase complex (ACDS), highlighting the importance of the acetoclastic pathway due to its importance for acetogenesis and acetate cleavage (Grahame et al., 2005). In support of this, the expression of acetate kinase and phosphate acetyltransferase showed disparate effect patterns between sites. While these enzymes were reduced at all time points in the WWTP assemblage, effects in both directions were present in the reserve. These enzymes catalyze key steps in the conversion of acetate into acetyl-CoA (Gorrell et al., 2005; Lipmann, 1944), which is subsequently processed into CH₄ and CO₂ during acetoclastic methanogenesis. A reduction in expressions of ACDS, acetate kinase and phosphate acetyltransferase, as observed in the WWTP assemblage, could indicate a lower acetoclastic methanogenesis, and may be a key insight in explaining the observed reductions in overall methane production. In line with literature (Conrad, 2020), acetate likely dominated as substrate for methanogenesis midway through the incubation, which is supported by lower fractionation factors of compound-specific $\delta^{13}\text{C}$ values of CH₄ and CO₂ (Fig. S5). Although differences to the control in $\delta^{13}\text{C}$ were present at the high concentration for both assemblages, these differences can also be driven by the different successional stage due to

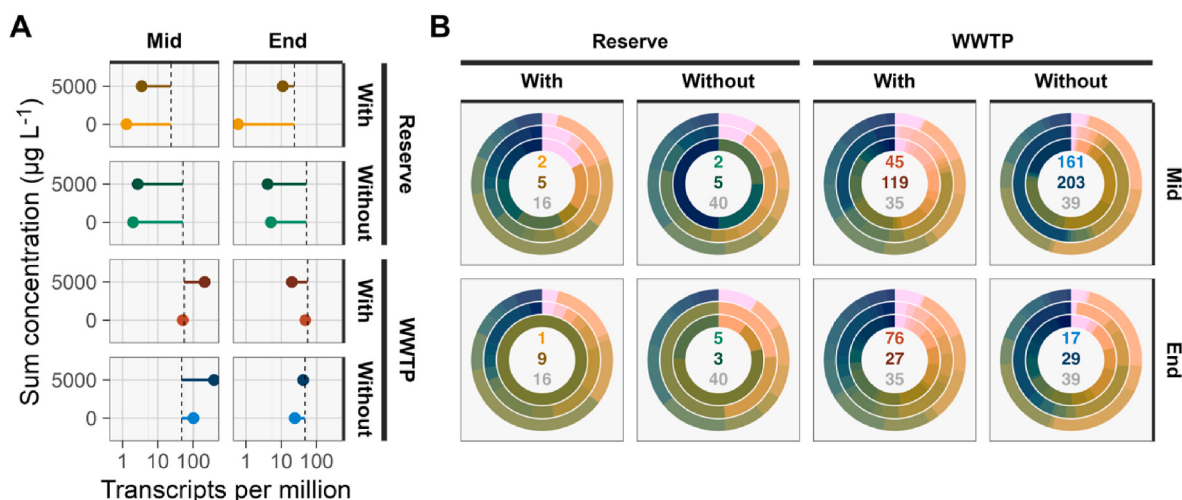


Fig. 2. Gene expression in transcripts per million (A) and ARG profile (B). In the left figure (A), vertical dashed lines represent ARG expression at the start of the experiment and lines connect to expression levels of the main experiment. In the right figure (B), fill color in the circles indicate different drug classes (for more details see Fig. S6) at 0 $\mu\text{g L}^{-1}$ (inner), 5000 $\mu\text{g L}^{-1}$ (middle), and at test start (outer). Text inside the circle displays the number of unique resistance genes at 0 $\mu\text{g L}^{-1}$ (top), 5000 $\mu\text{g L}^{-1}$ (middle), and at test start (bottom). Both figures are faceted by time point, assemblage (i.e., reserve vs. WWTP), and pre-treatment (i.e., with vs. without). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

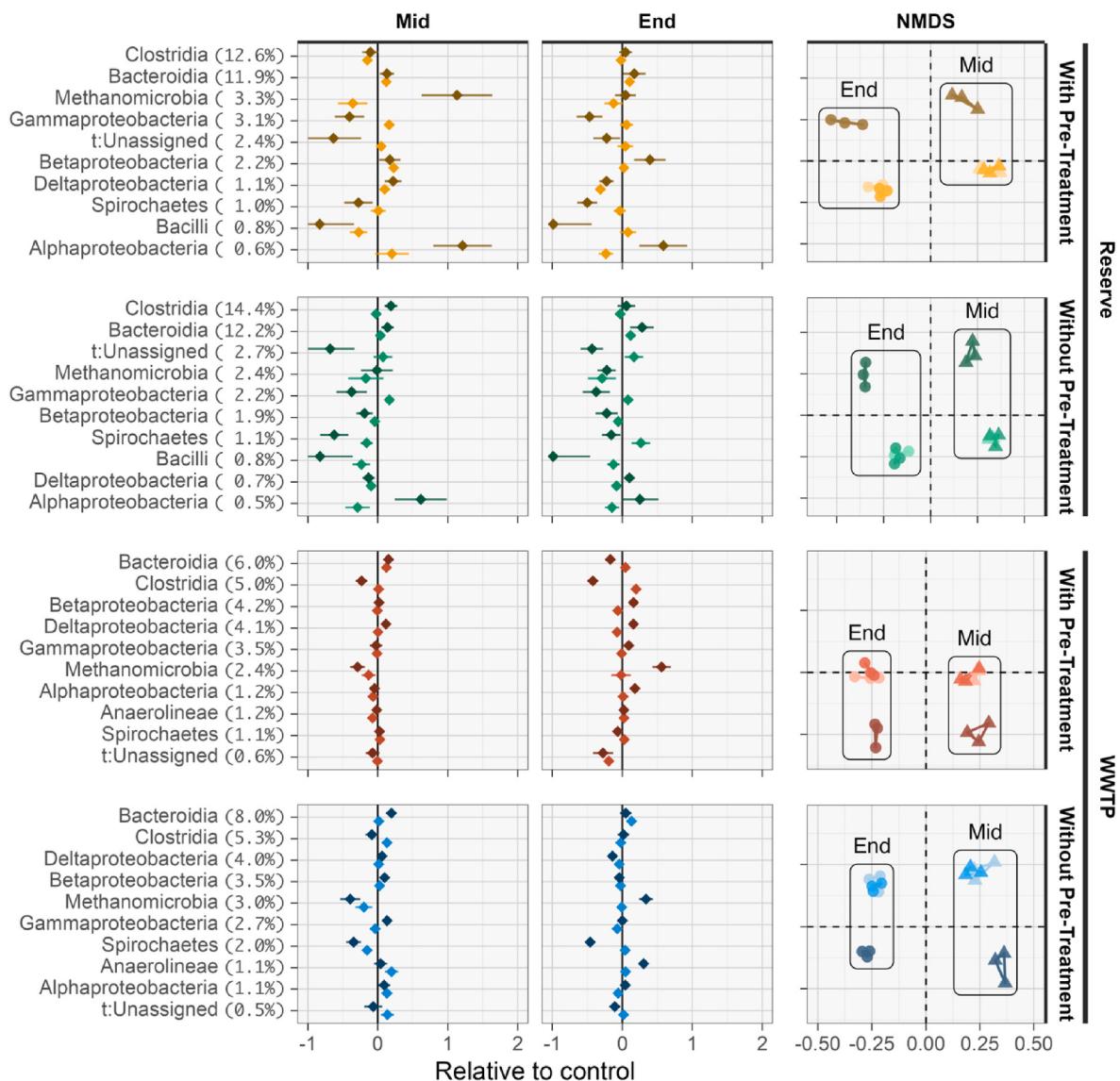


Fig. 3. Metabarcoding-based community analyses of all prokaryotes. The left and middle columns display average changes in relative abundance compared to the control, normalized to the average relative abundance in the control (expressed as relative change $\pm$ SE), for prokaryote classes at the mid (left column) and end (middle column) of the experiment. Negative values indicate reductions, while positive values represent increases in relative abundance. Taxonomic levels are ranked by their contribution to dissimilarity between the high antibiotic concentration and the control (averaged across both time points), with the contribution value shown in parentheses. The right column presents NMDS plots based on Hellinger-transformed relative abundances for prokaryotes. Assemblages from the same site (i.e., reserve vs. WWTP) but different pre-treatment and time point (triangle: mid, circle: end) share the same ordination space for better comparability (stress: 0.088 for reserve and stress = 0.055 for WWTP). Assemblage origin and pre-treatment are indicated by different rows and colors, while antibiotic concentrations are represented by lightness (light: $0 \mu\text{g L}^{-1}$; medium: $5 \mu\text{g L}^{-1}$; dark: $5000 \mu\text{g L}^{-1}$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

altered dynamics of CH_4 production (Bollinger et al., 2021). Taken together, this evidence reinforces our hypothesis that the acetate pathway is most important for understanding antibiotic effects (Bollinger et al., 2024) with gene profile differences helping explain site-specific methanogenesis responses.

3.2. Effects of short-term adaptation on antibiotic effects

For both assemblages, pre-treatment with antibiotics only showed minor changes in antibiotic effects during the main incubation experiment (Fig. 1). Effects on CH_4 production dynamics (i.e., time until inflection point, τ) were slightly increased in the reserve (up to 2.6 %) and slightly lower in the WWTP (down to -1.5 %, Fig. 1). Although this pattern could – in line with our hypothesis – suggest a higher adaptability of the WWTP assemblage, the effect sizes and statistical support

were negligible (BF up to 3.6), and the underlying processes are too versatile to support this interpretation. In combination with essentially unaltered expressions of ARGs between pre-treatments (Fig. 2), this suggests a limited capability for short-term adaptation. In contrast, high antibiotic treatment in the main incubation experiment nearly always elevated ARG expression. The absence of pre-treatment effects could therefore, be influenced by a lower number of generations (although higher ARGs could be expected within a handful of days) (Gullberg et al., 2011), a lower antibiotic concentration per gram of sediment, or the present prokaryotic assemblage. The latter is founded on assemblage filtering induced by the anaerobic conditions (but also substrate addition) which could have indirectly selected for species with greater resistance potential.

Although effects on methanogenesis dynamics and ARGs seem minor, the pre-treatment still had notable early-phase effects. Without

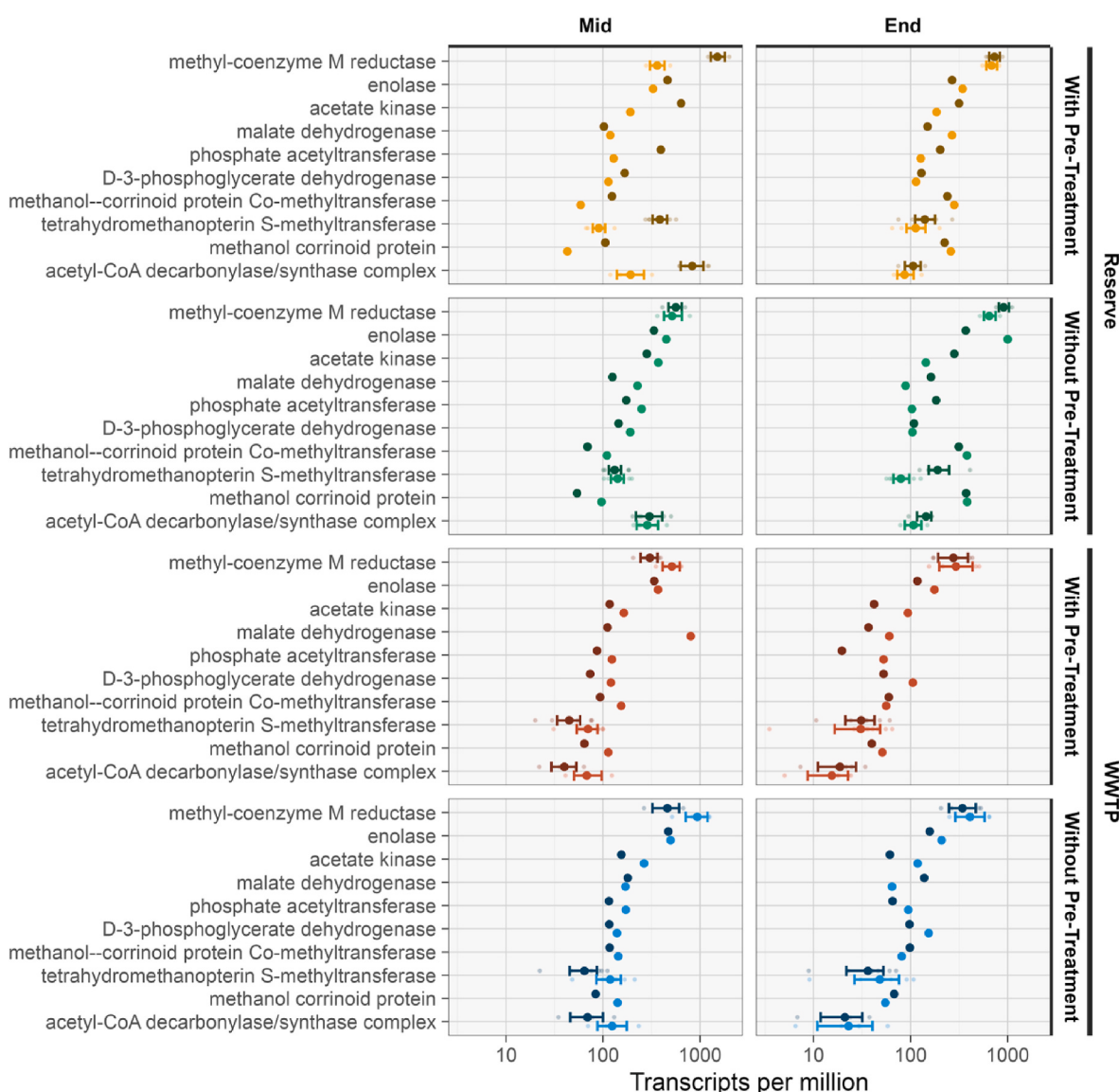


Fig. 4. The 10 most expressed transcripts from the KEGG methane metabolism pathway shown as transcripts per million. Transcriptions of subunits were assigned to the respective enzyme plotted as mean with bootstrapped 95 %-confidence intervals alongside raw values. Assemblage origin and pre-treatment are indicated by different rows and colors, while antibiotic concentrations are represented by lightness (light: 0 $\mu\text{g L}^{-1}$; dark: 5000 $\mu\text{g L}^{-1}$). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

pre-treatment, the reserve assemblage initially showed a decreased methanogenesis at the high concentration (as discussed above, Fig. 1). With antibiotic pre-treatment, however, this effect reversed (13 %). Since we previously interpreted this initial reduction as a sign of metabolic dormancy due to stress (Chebotar' et al., 2021), the positive effects when pre-treated with antibiotics could indicate an acclimatization on the metabolic level. Although effect directions stayed the same in the WWTP assemblage, effect sizes were reduced when pre-treated with antibiotics (i.e., 12 % instead of 32 % without pre-treatment).

The action of the pre-treatment is also reflected in prokaryotic assemblage differences ($p < 0.0001$) and their interactions with antibiotic effects ($p = 0.0168$ for reserve and $p = 0.0089$ for WWTP). In the reserve, Methanomicrobia almost doubled midway through the experiment at the high concentration when pre-treated with antibiotics. This is in sharp contrast to the unaltered relative abundance when not pre-treated. In addition to showing the effectiveness of the pre-treatment on the community level, this result also indicates a slight mismatch between structure (i.e., same relative abundance of methanogens) and function (i.e., increased methanogenesis). Further differences between

pre-treatments were not only visible in effect direction (e.g., Betaproteobacteria and Deltaproteobacteria), but also in effect size (e.g., Spirochaetes and Alphaproteobacteria) although many taxa showed no considerably altered effects (e.g., Bacteroidia and Gammaproteobacteria). WWTP sediment showed less clear patterns. Differences in effects on Methanomicrobia were lower in the WWTP (−29 and −40 % with and without antibiotic pre-treatment, respectively) compared to the reserve assemblage (−1 and 113 % with and without antibiotic pre-treatment, respectively) but matches the patterns observed in methanogenesis quite well (i.e., lower relative abundance and reduced methanogenesis, Figs. 1 and 3). Even though a community shift is likely the key to understanding changes in tolerance towards antibiotics (Thili et al., 2016), generalizing these mechanistically remains a prime challenge in such complex assemblages.

Also, the gene expression profiles indicate pre-treatment modulated antibiotic responses. Most notably, MCR increased at the high concentration mid-incubation in the pre-treated reserve assemblage, while no differences were present when not previously exposed to antibiotics (Fig. 4). This aligns with the above-mentioned slightly higher effects on

methanogenesis dynamics. ACDS, acetate kinase, malate dehydrogenase, and phosphate acetyltransferase also indicate disparate responses in the acetate pathway between pre-adapted and inert assemblages. Again, these differences were less apparent in the WWTP assemblage and could thus be a hint towards understanding divergent site responses.

In summary, significant impacts on assemblage composition and gene expression, did not translate to similarly substantial changes in antibiotic effects on CH_4 production dynamics after short-term adaptation. Along with effects observed in both assemblages (despite opposite directions, see 3.1), this suggests a notable susceptibility to antibiotics and potentially other chemical stressors, regardless of adaptation level. This is further supported by the expression of ARGs, hardly explaining any observed pattern in antibiotic effects on methanogenesis. Therefore, antibiotic exposure will nonetheless pose a risk even to adapted methanogenic assemblages and consequently for CH_4 production in future scenarios.

3.3. Effects of short-term adaptation on methanogenesis

The pre-treatment also had additional effects beyond changing antibiotic sensitivity. Comparing each antibiotic level of the pre-treated assemblages in the incubation experiment to its respective counterpart in the unexposed assemblage (e.g., control of sediment with pre-treatment vs. control of sediment without pre-treatment) revealed patterns deviating from the antibiotic action in the main experiment. For example, while the reserve and WWTP assemblage showed positive or negative effects on methanogenesis dynamics, respectively, the pre-treatment mildly accelerated methanogenesis in both assemblages (BF up to 4.7, Fig. 5). Apart from the high antibiotic treatment in the WWTP assemblage, CH_4 concentrations were highly increased in the initial stages of the incubation. Since these alterations were induced solely during the pre-treatment, it shows that previous antibiotic exposure can affect future methanogenesis but also that effect directions are context-dependent even in the same assemblage (i.e., given decreases in the main incubation for WWTP but increases when comparing pre-treatments directly). The absence of oxygen and addition of substrate can filter the community and therewith shape responses towards antibiotics. This could further complicate the generalization of effects, especially in natural environments where aerobic and anaerobic sediment layers interact via substrate transport. Moreover, the combination

of numerous stressors/factors could interact with antibiotic effects on methanogenesis. For example, the granularity of the sediment (Du et al., 2015), presence of oxidizing agents (that promote antibiotic degradation) (He et al., 2021) and temperature (Bollinger et al., 2024) can modulate the observed effects. However, the majority of findings stems from anaerobic digesters that are barely comparable to natural sediments (e.g., in terms of temperature and antibiotic exposure). Therefore, future efforts need to identify circumstances that reinforce increased methanogenesis in presence of antibiotics that extend the here taken approach with focus on prokaryotic assemblage composition.

CRediT authorship contribution statement

Eric Bollinger: Writing – original draft, Visualization, Validation, Software, Methodology, Investigation, Formal analysis, Conceptualization. **Johanna Mayer:** Writing – review & editing, Investigation. **Foon Yin Lai:** Writing – review & editing, Investigation. **Ralf Schulz:** Writing – review & editing, Resources, Funding acquisition. **Sabine Filker:** Writing – review & editing, Funding acquisition, Formal analysis, Conceptualization. **Mirco Bundschuh:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work the author(s) used ChatGPT in order to evaluate suggestions for the readability and language of the manuscript in rare individual cases. After using this tool/service, the author(s) reviewed and edited the content as needed and take(s) full responsibility for the content of the published article.

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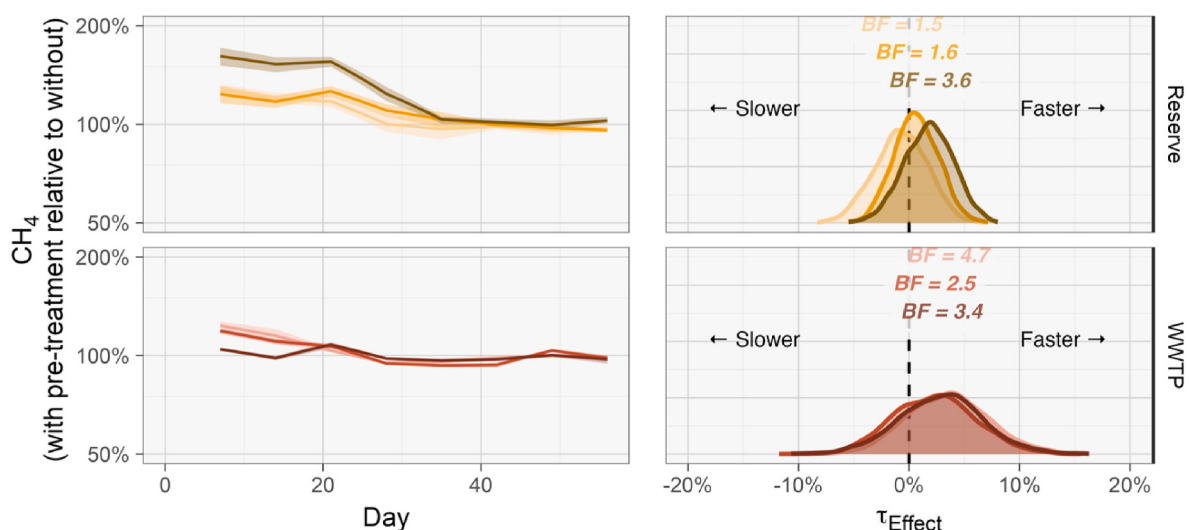


Fig. 5. CH_4 concentrations of different test concentrations from pre-treated sediments relative to sediments without pre-treatment (set to 100 %; mean $\pm$ 95 % confidence interval, left panels) and posterior density distributions of the kinetic parameter τ as effect size relative to sediments without pre-treatment (τ_{Effect} right panels). Assemblage origin is indicated by different rows and colors, while antibiotic concentration is represented by lightness (light: $0 \mu\text{g L}^{-1}$, medium: $5 \mu\text{g L}^{-1}$, dark: $5000 \mu\text{g L}^{-1}$). The Bayes factor (BF) quantifies the odds ratio for the probabilities of τ_{Effect} being greater than or less than zero, with values always expressed as ≥ 1 . (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Mirco Bundschuh & Sabine Filker reports financial support was provided by Carl Zeiss Foundation. Ralf Schulz reports financial support was provided by German Research Foundation. Foon Yin Lai reports financial support was provided by Swedish Research Council Formas. If there are other authors, they 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. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envpol.2025.126828>.

Data availability

Link to repository with DOI containing raw data and code is included in the manuscript

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