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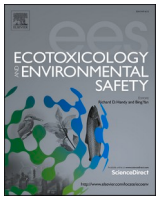

Antibiotics as a silent driver of climate change? A case study investigating methane production in freshwater sediments

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ABSTRACT

Methane (CH_4) is the second most important greenhouse gas after carbon dioxide (CO_2) and is inter alia produced in natural freshwater ecosystems. Given the rise in CH_4 emissions from natural sources, researchers are investigating environmental factors and climate change feedbacks to explain this increment. Despite being omnipresent in freshwaters, knowledge on the influence of chemical stressors of anthropogenic origin (e.g., antibiotics) on methanogenesis is lacking. To address this knowledge gap, we incubated freshwater sediment under anaerobic conditions with a mixture of five antibiotics at four levels (from 0 to 5000 μg/L) for 42 days. Weekly measurements of CH_4 and CO_2 in the headspace, as well as their compound-specific $\delta^{13}C$, showed that the CH_4 production rate was increased by up to 94% at 5000 μg/L and up to 29% at field-relevant concentrations (i.e., 50 μg/L). Metabarcoding of the archaeal and eubacterial 16S rRNA gene showed that effects of antibiotics on bacterial community level (i.e., species composition) may partially explain the observed differences in CH_4 production rates. Despite the complications of transferring experimental CH_4 production rates to realistic field conditions, the study indicated that chemical stressors contribute to the emissions of greenhouse gases by affecting the methanogenesis in freshwaters.

1. Introduction

Methane (CH_4) is the second most important greenhouse gas (GHG) with a 28-fold higher global warming potential (GWP) than carbon dioxide (CO_2) – a number which is considered an underestimation, when accounting for climate-carbon feedbacks (Myhre et al., 2013). A significant amount of CH_4 emissions originates from inland waters with an estimation of 14.5–31.1% (i.e., bottom-up estimates) and 2.7–6.5% (i.e., top-down estimates) of the global annual CH_4 emissions (Saunois et al., 2020). Following hydrolysis and fermentation of complex compounds from organic matter (OM) that accumulates in anoxic freshwater sediments (Cole et al., 2007; Regnier et al., 2013), specialized groups of archaea produce CH_4 from CO_2, H_2, methanol, methylamines, methylsulfides or acetate (Thauer et al., 2008). Acetate and H_2 are regarded as the most relevant substrates for CH_4 production, since acetoclastic (AM, i.e., methyl-type fermentation) and hydrogenotrophic (HM, i.e., CO_2-H_2 reduction) methanogenesis are the dominant metabolic pathways of methanogenic archaea (Gruen et al., 2018). Given the increasing atmospheric CH_4 levels since industrialization (Nisbet et al., 2014; Schaefer et al., 2016), researchers aim at understanding parameters driving CH_4 emissions from natural sources, including environmental factors (Borrel et al., 2011), bacterial community composition (Gutknecht et al., 2006; Laskar et al., 2018) and climate change (Dean et al., 2018). A factor that is, according to our knowledge, hardly assessed is the influence of potentially harmful substances of anthropogenic origin (e.g., antibiotics) through changes in the activity and composition of methanogenic prokaryotic communities.

The knowledge gap is particularly surprising as antibiotics are a frequently used measure to combat bacterial pathogens in human and veterinary medicine (Danner et al., 2019). Since antibiotics are only partially metabolized in the human or animal body, large amounts are released into wastewater treatment plants (WWTPs) where antibiotics are usually incompletely removed (Nnadozie et al., 2017). Tertiary WWTPs outmatch secondary WWTPs in terms of removal efficiency of antibiotics by additionally including physicochemical treatment steps (e.g., filtration, activated carbon and reverse osmosis, Burch et al., 2019;

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Gerba and Pepper, 2019). As a result of higher prevalence of tertiary WWTPs in industrialized countries, antibiotic concentrations in the low μg/L range can be found in European streams, whereas antibiotic levels in developing countries are generally one order of magnitude higher (Danner et al., 2019).

In the face of the consequential exposure towards antibiotics, studies have shown concentration-dependent effects on bacteria in single-species bioassays (see "Wikipharma" database, Molander et al., 2009). Moreover, toxicological studies on complex communities indicate potential changes in community structure (Laverman et al., 2015; Proia et al., 2013; Quinlan et al., 2011) as a result of selective pressure favoring strains that developed antibiotic resistance genes (ARG, Lupo et al., 2012; Nnadozie and Odume, 2019; Yang et al., 2018). Given that the methanogenic productivity is related to the microbial community structure (Ma et al., 2021), it seems plausible that the functioning of prokary communities is influenced by antibiotics as well. However, only few studies included methanogenesis as an endpoint of interest for effects of antibiotics and, if so, only considered technical systems such as sewage sludge bioreactors (Aydin et al., 2015; Lu et al., 2014; Reyes-Contreras and Vidal, 2015). To the best of our knowledge, no study has been testing antibiotic effects on CH_4 formation by freshwater prokaryotic communities, highlighting a relevant lack of information.

To address this, we conducted a chronic study (42 d) incubating freshwater sediments in an anoxic test system with four levels of antibiotics (including an antibiotic-free control). Weekly measurements of CH_4 and CO_2 concentrations and their compound-specific stable isotopic ratios of carbon were used to record the productivity of the bacterial community and the dominant pathway of methanogenesis (i.e., AM *vs.* HM), respectively. Since former studies showed a reduction in bacterial growth when exposed to antibiotics (Al-Ahmad et al., 1999; Kneifel and Leonhardt, 1992), a decreased productivity may be hypothesized. However, a potential community shift may also increase the microbial functioning under chemical stress if changes in the community structure are disconnected from their functional performance in favor of methanogenesis (cf. Feckler and Bundschuh, 2020; Shade et al., 2012). Ultimately, we incubated freshwater sediment at four levels of a five-component antibiotic mixture to address whether antibiotics can alter CH_4 production and its dominant pathway in freshwater sediments through changes in the prokaryotic community structure assessed by metabarcoding of archaeal and eubacterial 16S rRNA.

2. Materials and methods

2.1. Sediment sampling and processing

Sediment was taken from the upper 20 centimeters of a pond at the Eußerthal Ecosystem Research Station of the University of Koblenz-Landau (49°25′ N; 7°96′ E) in August 2020 and immediately processed for the experiment. The pond is fed by an upstream brook and therefore harbours a pristine prokaryotic community with a presumably low antibiotic resistance (Huerta et al., 2013). Serving as an additional carbon source in the experiment, leaves from black alder (*Alnus glutinosa* (L.) GAERTN.) trees were collected near Landau, Germany (49°12‘ N, 8°08‘ E) during fall 2020, dried at 60 °C for 48 h and pulverized in an electric mill. The pond sediment was spiked with 20 g of these leaves (dry weight) per kg sediment (wet weight; gravimetric water content: $\Theta_g = 2.67 \pm 0.05$ g g^{-1}). A homogeneous distribution of the leaf powder was realised by manually stirring the sediment with a scoop for 15 min. The added carbon corresponds to ~12% of the total organic carbon (Supplemental Material (SM) 1), which was determined via elemental analyses of leaves and spiked sediment (Flash HT elemental analyzer, Thermo Fisher Scientific, Germany). In order to remove inorganic carbon, the sediment was treated with 1 M HCl prior to the measurement (Fernandes and Krull, 2008).

2.2. Antibiotic treatment and analytics

The experiment included four levels (n = 20) of a mixture of the five antibiotics amoxicillin, ciprofloxacin, erythromycin, sulfamethoxazole and tetracycline (including an antibiotic-free control) prepared with analytical standards (Sigma-Aldrich, Germany). The mixture components were chosen to represent different classes of antibiotics with distinct modes of action (Table 1) and are reported in the μg/L range in freshwaters (Danner et al., 2019). Consequently, the low test concentration (i.e., 0.5 μg/L for sum of five antibiotics) is considered field-relevant for European and American and the medium concentration (i.e., 50 μg/L) for Asian and African freshwaters (Danner et al., 2019). The high concentration (i.e., 5000 μg/L) was chosen to test for concentration-dependency of effects but even higher values have been reported in the WWTP effluent and surface water of an extremely industrialized site (Fick et al., 2009; Larsson et al., 2007). To provide realistic levels of nutrients, stock solutions were prepared by serial dilution using unfiltered pond water from the same pond from which the sediment originated. A neutral pH of the test solutions was established by adding NaOH since anaerobic OM degradation is sensitive to changes in pH (Wang et al., 1993; Zhang et al., 2010). Three water samples per antibiotic level were sampled at day 0, 23 and 44 and stored frozen until chemical analysis.

Concentrations of antibiotics were measured through direct injection (i.e., without sample extraction). Briefly, water samples (2 mL) were centrifuged at 8000 rpm for 5 min at 7 °C. Then, an aliquot (400 μL) of the sample was transferred to an amber vial and fortified (20 μL) with the internal standard (IS) mixture of mass-labelled chemicals (1000 ng/mL). Together with the nine-point calibration curve (0.1–500 ng/mL; each fortified with mass-labelled standard solution at 50 ng/mL), antibiotic concentrations in the samples were analysed using a liquid chromatograph (ExionLC™ AD UHPLC system) coupled with tandem mass spectrometry (SCIEX Triple Quad™ 3500 system, LC-MS/MS). The injection volume was 10 μL. Chromatographic separation of the target antibiotics was performed on a Kinetex® biphenyl analytical column (100 × 2.1 mm, 2.6 μm, Phenomenex®) with mobile phases of Milli-Q water and methanol, each with 0.1% formic acid, running in a gradient programme (Table S1). Data acquisition was conducted using multiple reaction monitoring with two transitions for each analyte (Table S1). The calibration curve was analysed three times throughout sample analysis with linearity of 0.9985–0.9997 (Table S1). Concentrations of the target antibiotics were quantified with a compensation of IS amounts for potential instrumental variations (e.g. injection volume) and matrix effects. Two types of quality control samples were included, which were the Milli-Q water blank and the water samples spiked with native analytes as the positive matrix control. No contamination of the

Table 1
Substance name, class, mode of action and nominal concentration of the antibiotics.

Substance	Class	Mode of Action (Wishart, 2006)	Concentration (μg/L)
Amoxicillin	β-lactam	Inhibits bacterial cell-wall synthesis	0, 0.1, 10, 1000
Ciprofloxacin	Fluoroquinolone	Acts on bacterial topoisomerase II and topoisomerase IV	0, 0.1, 10, 1000
Erythromycin	Macrolide	Inhibits protein synthesis	0, 0.1, 10, 1000
Sulfamethoxazole	Sulfonamide	Inhibits bacterial dihydrofolic acid synthesis	0, 0.1, 10, 1000
Tetracycline	Tetracycline	Prevents binding of tRNA to the mRNA-ribosome complex, and thus interfering with protein synthesis	0, 0.1, 10, 1000
Mixture	All of the above	All of the above	0, 0.5, 50, 5000

target antibiotics was found in the Milli-Q water blank samples over the sample preparation and instrumental analysis. Matrix-spike samples showed satisfactory recovery of the chemicals, with an average (standard deviation; range; n = 4) of 80% (15%; 65–97%) for amoxicillin, 94% (8.5%; 80–104%) for ciprofloxacin, 108% (12.6%; 92–128%) for erythromycin, 117% (10.8%; 96–130%) for sulfamethoxazole, and 94% (19.6%; 70–127%) for tetracycline. The method quantification limit of the chemicals was at 0.2–4 μg/L (Table S1). Measurements indicate a successful test setup with a ready degradation of antibiotics. Data is presented in the supplemental material (Table S2).

2.3. Experimental setup

The experiment was conducted in airtight glass vessels (N = 80, Fig. 1). A total of 10.28 ± 0.40 mg wet weight of carbon spiked sediment and 30 mL of the test solution were transferred to each test vessel. The headspace of the test vessel was flushed with nitrogen for 30 s and hermetically sealed to restrain CH_4 oxidation. The test vessels were incubated in total darkness in a climate chamber at 20 ± 1 °C. The study was conducted over 42 days with weekly measurements of CH_4 and CO_2 concentrations in the headspace as well as their compound-specific isotope ratios of ^{13}C to ^{12}C.

2.4. Methane and carbon dioxide measurements

To quantify CH_4 and CO_2 in the headspace of each test vessel, 100 μL headspace gas was analyzed in a closed-loop with a cavity-enhanced laser absorption spectrometer (UGGA, model 915−0011, Los Gatos Research Inc., United States). After complete mixing of sample and loop gas, measurements of the equilibrium mole fraction x_e in the loop (ppm) were corrected for the volume of the closed loop V_l and the background mole fraction x_0

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

where x_h is the mole fraction in the headspace and V_i is the injected volume of the headspace. The volume of the loop was determined using a laboratory standard with certified levels of 5,000 ppm CH_4 and 20,000 ppm CO_2 (Messer Industriegase GmbH, Germany).

The amount of dissolved CH_4 and CO_2 (n_w) in the sample water was calculated using Henry's law

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

where K_H is Henry's constant for CO_2 and CH_4 ($3.3 \cdot 10^{-4}$ and $1.4 \cdot 10^{-5}$ mol Pa^{-1} m^{-3}, respectively), V_w is the volume of water and f_1 is a conversion factor assuming nearly normal atmospheric pressure in the headspace (i.e., 10^{-1} Pa ppm^{-1}). Data was finally expressed as μmol CH_4 or CO_2 normalized to the dry weight of the sediment ($c_{normalized}$) according to

$$c_{normalized} = \frac{n_w + (x_h \cdot V_h \cdot f_1)\cdot(R\cdot T)^{-1}}{m_s\cdot(1+\Theta_g)^{-1}} \quad (3)$$

where V_h is the volume of the headspace, R is the universal gas constant, T is the temperature of the system in Kelvin, m_s is the wet weight of the sediment and Θ_g is the gravimetric water content of the sediment.

2.5. Isotope ratio measurements

Although CH_4 can be metabolized from a variety of substrates, the high proportion of AM and HM makes methanogenesis from substrates other than acetate and H_2 in practice negligible (Gruen et al., 2018). Since enzymes involved in AM and HM discriminate substantially different against stable carbon isotopes (i.e., ^{12}C and ^{13}C), the dominant pathway of methanogenesis can be derived from the isotopic compositions of CH_4 ($\delta^{13}C$-CH_4) and CO_2 ($\delta^{13}C$-CO_2) by calculating a fractionation factor (α_c), where higher values represent a higher proportion of HM (Whiticar, 1999).

$$\alpha_c = \frac{\delta^{13}C\text{-}CO_2 + 1000}{\delta^{13}C\text{-}CH_4 + 1000} \quad (4)$$

For the determination of $\delta^{13}C$-CH_4 and $\delta^{13}C$-CO_2, headspace gas was

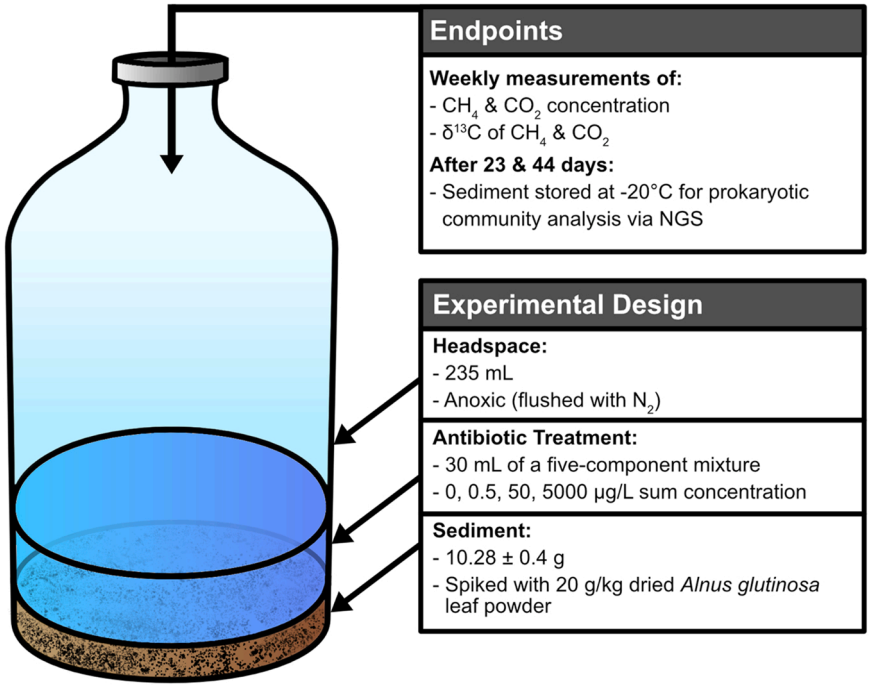

Fig. 1. Experimental scheme. The test was conducted in a closed anoxic system containing sediment spiked with *Alnus glutinosa* leaf powder and three levels of an antibiotic mixture plus an antibiotic-free control (n = 20). Concentrations of CH_4 and CO_2 in the headspace as well as their compound-specific $\delta^{13}C$ were measured weekly. At day 23 and 44 sediment samples were stored for prokaryotic community analyses using next generation sequencing.

injected into a gas chromatograph (GC, Trace GC Ultra, Thermo Fisher Scientific, Germany) at 32 °C, separated in a capillary column (length: 27.5 m, internal diameter: 0.32 mm, film thickness: 10 μm, Agilent J&W PoraPLOT Q, United States) coupled to a ConFlo IV and measured via a Delta V Advantage isotope ratio mass spectrometer (IRMS, Thermo Fisher Scientific, Germany). Injection volumes were based on prior concentration measurements to achieve a constant amount of substance in the system and therefore reduce any potential drift effects. Isotopic values are reported in the δ-notation relative to Vienna Pee Dee Belemnite (VPDB).

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

Compound-specific values of $\delta^{13}C$-CH_4 and $\delta^{13}C$-CO_2 were calibrated with an internal lab standard that was measured in duplicates after every tenth sample with 0.17‰ and 0.19‰ within-sequence precision (± 1 SD), respectively.

2.6. RNA extraction, reverse transcription, polymerase chain reaction (PCR) and sequencing

RNA was extracted from triplicate 2 g-subsamples taken at day 23 and 44 of all four antibiotic concentrations using Qiagens' RNeasy PowerSoil Total RNA kit according to the manufacturers' instructions. RNA concentrations were measured spectrophotometrically (Nanodrop2000, Thermo Fisher Scientific, Germany) and 2 μL RNA per sample were reverse transcribed into cDNA using random primers (iScript cDNA synthesis kit, Bio-Rad). Following the manufacturers' recommendation, 2 μL of cDNA were used as template for the amplification of the hypervariable V4 region of the 16S rRNA gene (ca. 300 bp fragment). The PCR mix included the primer pair 515Fm (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806Rm (5′- GGACTACNVGGGTWTCTAAT-3′, Walters et al., 2016) and NEBs Phusion High-Fidelity polymerase. The PCR protocol employed an initial activation step at 98 °C for 30 s, followed by 26 cycles consisting of 98 °C for 10 s, 63 °C for 30 s and 72 °C for 30 s, and a final extension for 5 min at 72 °C. To minimize PCR bias, three individual PCR reactions for each of the 24 samples were prepared. Afterwards, all replicate samples per time point (i.e., day 23, day 44) and antibiotic concentration (i.e., 0 μg/L, 0.5 μg/L, 50 μg/L, 5000 μg/L) were pooled and subjected to library preparation using the Next Ultra DNA Library Prep Kit for Illumina (NEB, United States). The quality of the final eight libraries was assessed with an Agilent Bioanalyzer 2100 system. Libraries were sequenced on an Illumina MiSeq platform, generating 2 × 250 bp paired-end reads by SeqIT, Kaiserslautern, Germany.

2.7. Sequence data processing, taxonomic assignment & statistical analyses

Initially, excessive primer overhangs were clipped from the raw reads using cutadapt v1.18 (Martin, 2011). Reads were then further processed using DADA2 (Callahan et al., 2016) as described for hypervariable taxonomic marker genes from metabarcoding studies (Forster et al., 2019) with the following criteria: filterAndTrim with truncLen= 230 and maxEE= 1. The truncation length criterion was determined by choosing the sequence position at which Phred assigned a quality score of ≥ 30 (Q3) for at least 51% of all reads in a dataset (=base call accuracy 99.9%, Ewing and Green, 1998). Reads were merged using 20 base pairs overlap with an allowed maximum mismatch of 2 and submitted to chimera identification and removal using vsearch v2.13.7 (Rognes et al., 2016). Taxonomy was assigned to resulting amplicon sequence variants (ASVs) using the SINTAX algorithm (Edgar, 2016) against the Greengenes database v13.5 (McDonald et al., 2012). After merging the ASV-contingency table with the taxonomic information, ASVs without any taxonomic assignment and ASVs which occurred only once and exclusively in one sample, and thus, may be artifactual sequences (Bokulich et al., 2013) were removed. The resulting ASV-to-sample matrix was then used for downstream statistical analyses. Prior to the analyses, the ASV-to-sample matrix was normalized to the lowest sequence number (n = 149.655). Rarefaction analyses were conducted to verify sufficient sequencing depth.

2.8. Statistics

Since CH_4 concentrations tend to span across orders of magnitude in incubation studies such as the present one (factor of ~60 in this study) heteroscedacity is present which may affect standard errors of the CH_4 production rates (Fig. S1 in SM). Therefore, the CH_4 production rates were log-transformed before performing statistical analyses. Since CO_2 concentrations span across a smaller range (factor of ~4 in this study), homoscedacity is given (Fig. S1 in SM) and therefore no data transformation was conducted.

To test for statistically significant differences in log-transformed CH_4 and raw CO_2 production rates among antibiotic treatments, an analysis of variance (ANOVA) of linear mixed effect models (LME) with time and antibiotic treatment as fixed effects was conducted. The replicate ID was included in the model as random effect to prevent pseudoreplication due to repeated measurements. A tukey-adjusted post-hoc test based on least-squared means was used for multiple comparisons among factor combinations (SAS Institute Inc., 2012). All calculations, statistics and data visualizations were conducted with R (3.5.3, R Core Team, 2019) using the packages "nlme", "emmeans" and "ggplot2". Raw data is made available via GitHub (https://github.com/EricBollingerResearch).

3. Results and discussion

3.1. CH_4 and CO_2 production over time

Across all antibiotic treatments, CH_4 concentration showed a linear increase in the headspace during the first three weeks, followed by an exponential increase from day 21–35, before production stagnated (Fig. 2a). The lower CH_4 production rate during the first three weeks compared to day 21–35 can be explained by remaining oxygen in the system, which inhibited the methanogenesis (Chae et al., 2010), or aerobic oxidation of methane (AOM) by methane-oxidizing bacteria (MOB, Bastviken et al., 2002). However, even under anaerobic conditions, several electron acceptors (e.g., SO_4^{2-}, Mn^{4+}, Fe^{3+}, NO_2, NO_3^-) were available in the test system oxidizing CH_4 to CO_2 in an interplay between mainly anaerobic methanotrophs (ANME) and sulphate-reducing bacteria (SRB, Cui et al., 2015). Consequently, both aerobic and anaerobic CH_4 oxidation contributed to high production rates of CO_2 and low production rates of CH_4 in the first weeks (Fig. 2c, d). Additionally, acetate was not limiting methanogenesis since typically more acetate is produced than can be consumed by methanogens at the beginning of anoxia (Chidthaisong et al., 1999). Thus, in the intial phase, CH_4 production was only limited by competition for H_2 (Conrad, 2002) and potentially off-set by oxidation. Consequently, the fractionation factor α_c was particularly high in this early stage of incubation (Fig. 3) suggesting a higher proportion of CH_4 in the headspace formed by HM.

In the subsequent phase of exponential increase of CH_4 during the experiment (day 21–35), isotopic compositions of CH_4 and CO_2 changed towards AM. The growing fraction of CH_4 formed by AM after the first three weeks is further supported by the increase in the molar ratio of CH_4 to CO_2 (Fig. 2e). Moreover, the increased proportion of the archaeal families *Methanosarcinaceae* and *Methanosaetaceae* (Fig. 4c), which inter alia comprise all acetoclastic methanogens (Whitman et al., 2006), further supports the elevation of acetogenic CH_4 also indicated by the isotopic ratio measurements. This is further confirmed by the relative decrease of the family *Methanoregulaceae*, which predominantly carry out HM (Oren, 2014). Consequently, the exponential increase of CH_4 is

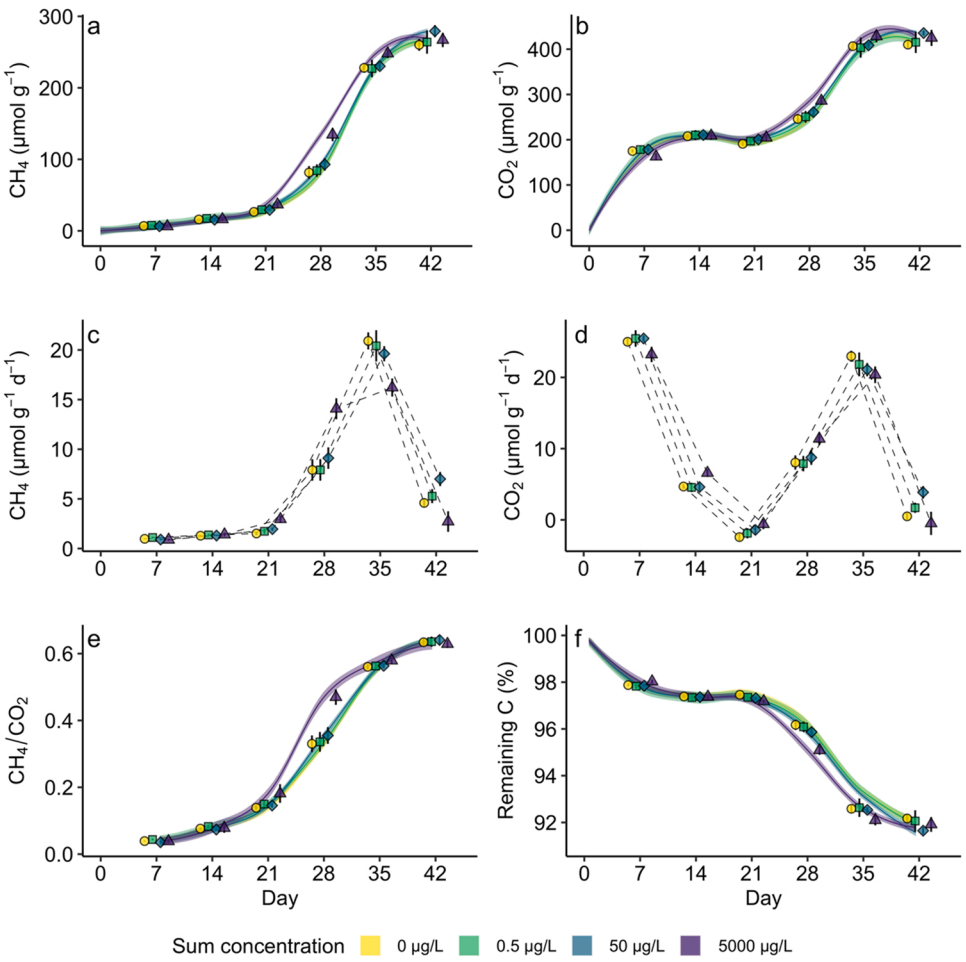

Fig. 2. Headspace concentrations (a, b) and production rates (c, d) of CH_4 (a, c) and CO_2 (b, d) as well as their molar ratio (e) and remaining carbon in the test system (f) over time. The control (yellow), low (green), medium (blue) and high (purple) concentrations were scattered symmetrically around the actual day of measurement to increase readability. Data is presented as mean with 99% confidence interval and either plotted with a LOESS regression (a, b, e, f) or dashed lines (c, d) to associate data of the same treatment. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

mainly triggered by an elevated utilization of acetate (Conrad, 2002). This can also be seen by the fractionation factor α_c shifting towards AM (i.e., lower values of α_c) during the period of increased productivity (Fig. 2b). Such an increment in both CH_4 and CO_2 productivity around day 30 was also observed in other incubation studies with macrophytes and phytoplankton as OM but not with terrestrial leaves (Grasset et al., 2018). Since the increase in the study by Grasset et al. (2018) was attributed to the complexity of the substrate, this might indicate a lower recalcitrance of *A. glutinosa* for metabolic pathways relevant for methanogenesis or a high enzymatic capability of the community tested in this study.

After 35 days the formation of both CH_4 and CO_2 stagnated (i.e., production rates close to zero), although only $8.1 \pm 0.46\%$ of the organic carbon in the test vessel has been processed at the termination of the experiment (Fig. 2f). Given that most readily available substrates are already utilized after the productivity increment past day 35 (Grasset et al., 2018), methanogenesis is mainly limited by the hydrolysis of more complex compounds (e.g., polysaccharides) and the resulting low and constant concentrations of acetate and H_2 (i.e., steady state, following the theoretical framework of Glissmann and Conrad, 2002).

3.2. Effects of antibiotics

As discussed above, in the initial 21 and after 35 days the factors determining CH_4 accumulation in the headspace were primarily of physico-chemical nature (i.e., accessibility of electron acceptors and polysaccharides, respectively). Since the exponential phase (~21–35 days) is the only period where methanogenesis is limited by microbial activity (van Hulzen et al., 1999; Vavilin et al., 2008), we argue that effects of antibiotics on methanogens can only be seen in this phase. Indeed, CH_4 production rates in the high antibiotic treatment (i.e. 5000 µg/L) were significantly ($p < 0.002$) higher than in the control after 21 and 28 days (94% and 78%, respectively) and significantly lower after 35 and 42 days (−22% and −41%, respectively). The same pattern occurred for the medium concentration (50 µg/L) at day 21, 28 and 35 (29%, 15% and −6%, respectively), despite the differences being only statistically significant at day 21 ($p < 0.001$). Contrary to what was observed in the high antibiotic treatment, the production rate of the medium antibiotic treatment was, with 52%, significantly higher than the control at day 42 ($p < 0.001$). Lower CH_4 production rates after 35 days should, however, not be interpreted as reductive effect of antibiotics on methanogenesis but rather as an earlier achievement of almost steady state conditions. Since all treatments plateaued at virtually the same headspace concentration of CH_4 and CO_2, it can be infered that antibiotics influenced the dynamic of methanogenesis in an incubation scenario but not the produced amount at steady state, which was rather determined by the quality of OM (Grasset et al., 2018). The temporal pattern (i.e., linear increase followed by exponential increase and steady state) of CH_4 concentration in the headspace was comparable for all antibiotic treatments. This suggests that the above stated effects on CH_4 production rates are likely a result of a faster achievement of degradation stages rather than a distinct mechanistic pathway of OM degradation. This is further supported by $\delta^{13}C\text{-}CH_4$ and $\delta^{13}C\text{-}CO_2$ of the control, where low and medium concentration are "following" the high concentration on a path from HM to AM over the course of the experiment (Fig. 3).

Overall, the effect of antibiotics on the dynamics of methanogenesis seems unequivocal. Although antibiotics could potentially be used as the

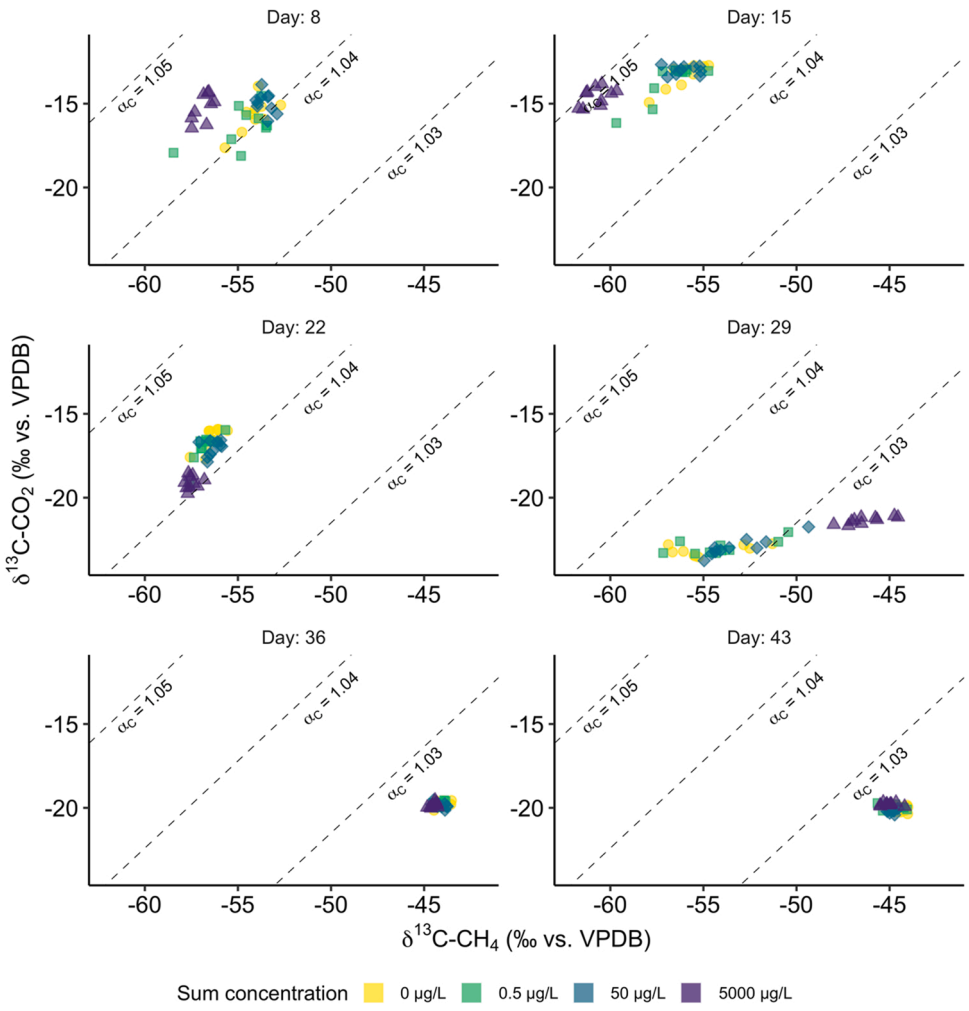

Fig. 3. δ^{13}C-CH_4 and δ^{13}C-CO_2 in the headspace over the course of the experiment. Data of the control (yellow circles), low (green squares), medium (blue diamonds) and high (purple triangles) concentrations are plotted as raw data. Plots include lines of constant fractionation factors (α_c, Eq. 4) for orientation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

energy source by bacteria (Dantas et al., 2008), the findings of this study are unlikely to be due to a nutritional effect, given that antibiotics added a maximum of 0.1% to carbon in the system. Therefore, toxic effects of antibiotics on microbes such as MOB (Tong and Xie, 2019) seem more relevant to explain the observed effects in functioning. Accordingly, the proportion of Gammaproteobacteria (i.e., the bacterial class that inter alia contains Type I MOB) and Alphaproteobacteria (i.e., the bacterial class that inter alia contains Type II MOB, Kojima et al., 2015; Mateos-Rivera et al., 2018) was affected at day 23 (Fig. 4). However, Proteobacteria comprise a variety of non-MOB (Bareither et al., 2013) and the only MOB family detected within the class of Alphaproteobacteria (i.e., *Methylocystaceae*) followed no obvious change in presence of antibiotics (data not shown). Furthermore, Gammaproteobacteria and Alphaproteobacteria are mostly aerobic MOB and despite a proposed contribution to anaerobic CH_4 oxidation (Mei et al., 2019) likely of minor importance for the tested scenario (but potentially relevant in natural habitats). Since ANME are present in many not exclusively methanotrophic bacterial classes (e.g., Deltaproteobacteria, Valentine, 2002) and archaeal groups related to methanogenic Methanosarcinales (Thauer and Shima, 2008), assessing the activity of specific enzymes involved in anaerobic CH_4 oxidation (e.g., ANME-1 methyl-coenzyme M reductase, Shima et al., 2012), could further focalize the mechanistic understanding in following studies.

Supporting the effect of antibiotics on the tested community, the proportion of the phyla Bacteroidetes and Firmicutes tended to increase with antibiotic treatment at day 23. This increment could undermine the observed increase in methanogenesis since a recent meta-regression revealed a positive relationship between those phyla and CH_4 production under anaerobic conditions (Ma et al., 2021). However, caution is advised since this correlation does not provide a mechanistic explanation and patterns observed in negatively correlated phyla, such as Actinobacteria and Proteobacteria (Ma et al., 2021), do not allow such deductions. Nevertheless, the present metabarcoding data imply an effect on bacterial groups involved in critical steps of the methanogenic pathway such as hydrolysis, acidogenesis and acetogenesis (e.g., Bacteroidetes, Proteobacteria and Actinobacteria, Amin et al., 2020).

The proportion of Euryarchaeota, the archaeal phylum of all known methanogenic species (Amin et al., 2020), was higher in antibiotic treatments especially on day 44, which suggests a displacement of other bacteria and probably a lower sensitivity towards antibiotics than eubacteria. Evidence from other research fields (e.g., clinical microbiology, Khelaifia and Drancourt, 2012) supports this hypothesis, while also exposing the effectiveness of antimicrobial agents targeting protein synthesis and the cell wall of archaea. Moreover, effects on methanogenic families within the phylum Euryarchaeota were observed. Mainly, *Methanosarcinaceae* seemed to supersede *Methanoregulaceae* at day 23, with almost no difference among antibiotic treatments at day 44 (except at the low concentration). While *Methanoregulaceae* are dominantly hydrogenotrophic (Oren, 2014), *Methansarcinaceae* tend to cover a broader spectrum of usable substrates (Juottonen, 2020). Due to the

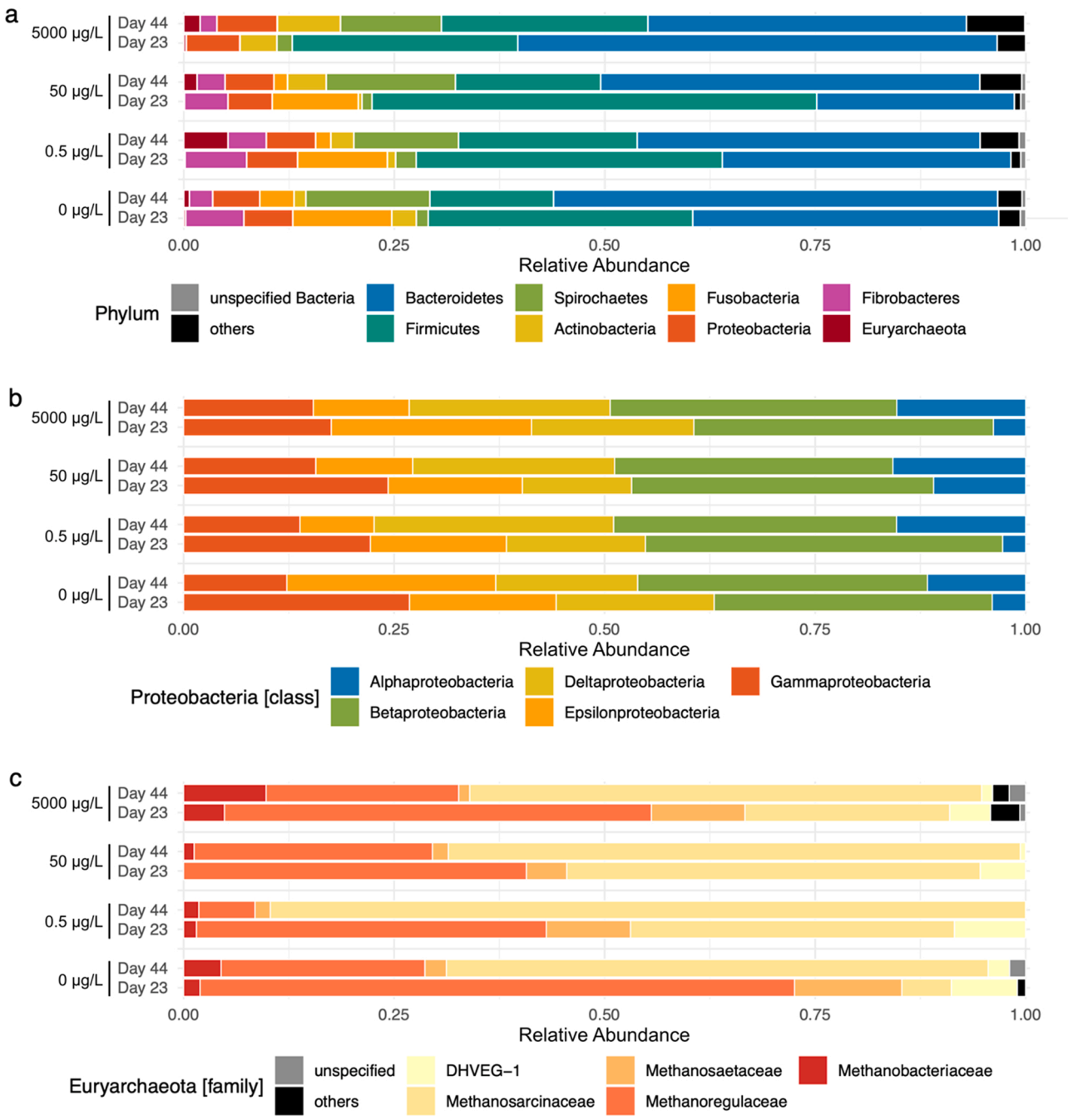

Fig. 4. Taxonomic composition of archaeal and bacterial communities in the sediments at days 23 and 44 exposed to increasing antibiotica concentrations. The bars show the relative proportion of genetic signatures to different taxonomic entities: (a) phylum-level taxonomy, (b) class-level taxonomy of the phylum Proteobacteria, and (c) family-level taxonomy of the phylum Euryarchaetoa.

different substrate requirements of these families, the observed effects could also be an indirect consequence of the direct effects on other bacterial groups (see above) and consequently different substrate availabilities between antibiotic treatments.

Despite the inherent intricacy of deducing microbial functioning from microbial community structure (i.e., complex network), the results of this study are, to the best of our knowledge, the first ones to demonstrate an influence of antibiotics on methanogenesis in a simulated freshwater system. Over and above that, methanogenesis was elevated although most studies investigating bioreactors reported reductions of CH_4 production as a consequence of antibiotic exposure (e.g. Aydin et al., 2015; Reyes-Contreras and Vidal, 2015; Rusanowska et al., 2020, but see: Lu et al., 2014). An additional mechanistic explanation of the increased productivity in this study might be provided by the fact that antibiotics can stimulate the release of extracellular polymeric substances in bacteria, which might represent an additional source of carbon utilized by methanogens (Lu et al., 2014).

3.3. *Perspectives on environmental relevance*

Translating the observed effects on CH_4 production to actual CH_4 emissions is intricate, subjecting the following extrapolations to the field to some uncertainty. In the field, CH_4 produced in anoxic layers of freshwater sediments may either be emitted rapidly (e.g., via ebullition) or by relatively slow diffusive transport towards and across the air-water interface (Bastviken et al., 2004). In the latter case, the produced CH_4

may largely be oxidized by MOB in oxic sediment layers or in the water column (Bastviken et al., 2002; Kankaala et al., 2006a; Sawakuchi et al., 2016). Consequently, CH_4-derived carbon can play a crucial role in freshwater food webs (Jones and Grey, 2011). This distinct pathway of carbon flux is especially, but not exclusively (Deines and Fink, 2011; Kankaala et al., 2006b), mediated by chironomids since they modify the oxic-anoxic interface in the upper sediment by bioturbation and burrow ventilation, which provides ideal habitats for MOB on which they feed (Deines et al., 2007). Therefore, potential effects of antibiotics on CH_4 production rates can theoretically lead to indirect effects within (Grey, 2016) and beyond (e.g., via the emergence of chironomids and other merolimnic insects, Richardson and Sato, 2015; Schulz et al., 2015) the borders of freshwater ecosystems. In consequence, the observed increase in CH_4 production can either fuel the biomass assimilation in and across the freshwater ecosystem or lead to higher CH_4 emissions. However, to validly estimate to which degree each process is happening is not straightforward and will likely depend on environmental conditions (e.g., temperature, presence of electron acceptors and OM quality, Borrel et al., 2011) as well as freshwater characteristics (e.g., surface area and water depth, Bastviken et al., 2004).

4. Conclusion

This study is, to the best of our knowledge, the first to show that antibiotics can influence methanogenesis in freshwater systems. Metabarcoding suggests an effect on eubacteria and archaea involved in processes relevant for methanogenesis. However, linking metabarcoding results to ecosystem functions is sophisticated and often requires trait-based approaches (Krause et al., 2014) for example by assessing functional genes relevant for the methanogenic process. Nevertheless, since the use of antibiotics is a rather new phenomenon at paleontological time scales, antibiotic pollution should be regarded as a factor potentially contributing to the recently observed increase in CH_4 emissions from natural sources.

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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.2021.113025.

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