Dissolved CH₄ coupled to Photosynthetic Picoeukaryotes in Oxic Waters and Cumulative Chlorophyll-a in Anoxia

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10 **Abstract.** CH₄ emissions from reservoirs are responsible for the majority of the atmospheric climatic forcing of these aquatic ecosystems, comparable to emissions from paddies or biomass burning. Primarily, CH₄ is produced during the anaerobic mineralization of organic carbon in the anoxic sediments by methanogenic archaea. However, the origin of the recurrent and ubiquitous CH₄ supersaturation in oxic waters (i.e., methane paradox) is still controversial. Here, we determined the dissolved CH₄ concentration in the water column of twelve reservoirs during the summer stratification and the winter

15 mixing. We obtained that the dissolved CH₄ concentration varied up to four orders of magnitude (0.02-213.64 μM), and all depths were consistently supersaturated (710-7082234 %) in both periods. Phytoplanktonic sources of carbon appear to determine the concentration of CH₄ in the reservoirs. In the anoxic waters, the depth-cumulative chlorophyll-a concentration, a proxy for the total phytoplanktonic biomass exported to sediments, determined the CH₄ concentration. In the oxic waters, the photosynthetic picoeukaryotes abundance significantly determined the dissolved CH₄ concentration both during the

20 stratification and the mixing. The mean depth of the reservoirs, as a surrogate of the CH₄ transport from sediment to the oxic waters, also contributed in shallow systems. Our findings suggest that photosynthetic picoeukaryotes can have a significant role in determining the CH₄ concentration in oxic waters and, in comparison to cyanobacteria, have been poorly explored as CH₄ sources.

1 Introduction

25 Lakes and reservoirs are significant sources of methane (CH₄) affecting climatic forcing in the atmosphere (Deemer et al., 2016). The contribution to the global budget of lakes is ca. 71.6 Tg CH₄ year⁻¹ (Bastviken et al., 2011), and of reservoirs ranges between 4 and 70 Tg CH₄ year⁻¹, representing up to 10 % of total CH₄ emissions (Deemer et al., 2016). Freshwaters only cover about 5-8 % of the Earth´s surface (Mitsch et al., 2012), but they emit much CH₄ than the ocean surface (Schubert and Wehrli, 2018). Traditionally, the net CH₄ production is attributed to archaeal methanogenesis, which produces methane

30 as an end product of organic matter degradation in anoxic conditions, and to methanotrophs, which consume it in oxic conditions (Schubert and Wehrli, 2018). In freshwater ecosystems, the anoxic sediments are a primary source of CH_4 (Segers, 1998), where methanogens are very sensitive to temperature and quantity and quality of the organic matter used as substrate (Marotta et al., 2014; Rasilo et al., 2015; Sepulveda-Jauregui et al., 2018; Thanh-Duc et al., 2010; West et al., 2012; Yvon-Durocher et al., 2014). They are also affected by the extent of anoxia in the sediments, as far as they are obligate

35 anaerobes and will not survive and produce CH_4 under aerobic conditions (Chistoserdova et al., 1998; Schubert and Wehrli, 2018). However, many observations from freshwaters and marine waters have detected CH_4 supersaturation in the oxic layers. A widespread phenomenon called the "methane paradox" (Bogard et al., 2014; Damm et al., 2010; Donis et al., 2017; Grossart et al., 2011; Kiene, 1991; Murase et al., 2003; Owens et al., 1991; Schmidt and Conrad, 1993; Schulz et al., 2001; Tang et al., 2014).

40 This persistent CH_4 supersaturation in oxic layers of marine and freshwater ecosystems requires extra inputs to compensate for the CH_4 losses by methanotrophy and the emissions toward the atmosphere. CH_4 inputs may become from anoxic sediments or from *in situ* sources in the oxic waters. The transport of CH_4 from the bottom and littoral sediments in shallow zones has been proposed to explain the supersaturation in the surface waters of some lakes (Bastviken et al., 2004; Encinas Fernández et al., 2016; Michmerhuizen et al., 1996; Murase et al., 2003; Peeters et al., 2019; Rudd and Hamilton, 1978). The

45 vertical transport may be relevant in small lakes, but in deep and thermally stratified systems, the vertical diffusion rates of dissolved gases across the thermocline are too low, and there is not apparent CH_4 upward movements from the hypolimnion (Peeters et al., 1996; Rudd and Hamilton, 1978). CH_4 diffusion from shallow sediments in littoral zones may be a significant source in the open surface of some lakes and reservoirs. However, lateral transport does not fully explain CH_4 supersaturation in the open ocean and other freshwater ecosystems, hence, other alternative *in situ* CH_4 sources likely occur

50 (Damm et al., 2010; DelSontro et al., 2018; Grossart et al., 2011; Owens et al., 1991; Schmidt and Conrad, 1993; Schulz et al., 2001; Scranton and Brewer, 1977; Tang et al., 2014; Tilbrook and Karl, 1995).

Previous works demonstrated the CH_4 production in oxic waters using stable isotope techniques in experiments and field samples (Bogard et al., 2014; DelSontro et al., 2018) and using molecular approaches (Grossart et al., 2011). There are different alternatives proposed as CH_4 sources in the literature. On the one hand, the occurrence of methanogenesis in micro-

55 anoxic zones in the guts of zooplankton, and within sinking particles (Angelis and Lee, 1994; Karl and Tilbrook, 1994). In both micro-niches, the CH_4 production was too low to sustain the total CH_4 supersaturation of the oxic waters (Schmale et al., 2018; Tang et al., 2014). On the other hand, there is a consistent link between dissolved CH_4 concentration and autotrophic organisms, primary production, and chlorophyll-a concentration (Bogard et al., 2014; Grossart et al., 2011; Owens et al., 1991; Schmidt and Conrad, 1993; Tang et al., 2014). Grossart et al., (2011) detected potential methanogenic

60 *Archaea* attached to photoautotrophs as *Chlorophyta* (*Eukarya*) and *Cyanobacteria* (*Bacteria*) in the epilimnion of an oligotrophic lake and confirmed the production of CH_4 in the presence of oxygen in laboratory incubations. If occurring, that symbiosis would require that the methanogens tolerate the oxygen exposure, contrary to general belief (Angel et al., 2011; Angle et al., 2017; Jarrell, 1985). New findings suggest that the link between phytoplankton and dissolved CH_4 may rely on

diverse metabolic pathways in *Bacteria* and *Eukarya*. These metabolic pathways contribute to the dissolved CH_4 in oxic

65 waters due to the degradation of methylated compounds. In the open ocean, bacteria decompose the abundant algal osmolyte dimethylsulfoniopropionate producing methane as a by-product (Damm et al., 2008, 2010, 2015; Zindler et al., 2013). Common methyl-containing substances as methionine produce methane in algae, saprotrophic fungi, and plants (Lenhart et al., 2012, 2015, 2016). Another reported pathway is the degradation of methyl-phosphonates (MPn) as an alternative source of phosphorus (P) in phosphate-starved bacterioplankton. The hydrolysis of these compounds, using the enzyme C–P lyase,

70 also releases methane as a by-product. This pathway appears in environments chronically P starved, as the ocean gyres, oligotrophic lakes, and microbial mats (Beversdorf et al., 2010; Carini et al., 2014; Gomez-Garcia et al., 2011; Karl et al., 2008; Repeta et al., 2016; Teikari et al., 2018; del Valle and Karl, 2014; Wang et al., 2017; Yao et al., 2016a). Recent studies using phytoplankton cultures and stable isotope techniques propose that the production of CH_4 may rely directly on the photoautotrophic carbon fixation of algae and *Cyanobacteria* (Bižić et al., 2020; Lenhart et al., 2016). All these alternative

75 sources of CH_4 in oxic waters, however, still have not been widely tested in reservoirs, despite the known high impact of these freshwater ecosystems in the global CH_4 emissions.

In this study, we measured the dissolved CH_4 concentration in the water column of twelve reservoirs covering a wide diversity (León-Palmero et al. in review) during the summer stratification and the winter mixing. We explored the potential sources of the dissolved CH_4 in the anoxic zone, where the classical methanogenesis occurs, and particularly in the oxic

80 zone. In the oxic zone, we considered the next potential CH_4 sources: 1) vertical and lateral transport; 2) *in situ* production by *Archaea*; 3) *in situ* production by methylphosphonates degradation; 4) *in situ* production by direct relationship with photoautotrophic carbon fixation using chlorophyll-a and the abundance of photosynthetic picoeukaryotes and cyanobacteria as surrogate.

2 Methods

85 **2.1 Study Reservoirs, Morphometry, and Vertical Profiles**

We sampled twice 12 reservoirs between July 2016 and August 2017 in southern Spain during the summer stratification and the winter mixing. The reservoirs were built between 1932 and 2003, and they differ in morphometry, chemical, trophic, and watershed characteristics (more details in León-Palmero et al., 2019, in review). We determined reservoir area, perimeter, and capacity using the next open databases: Infraestructura de Datos Espaciales de Andalucía (IDEAndalucia;

90 http://www.ideandalucia.es/portal/web/ideandalucia/), and the Ministerio para la Transición Ecológica (https://www.embalses.net/). The reservoir volume (m^3) divided by its surface area (m^2) will yield the mean depth (m). The equation is as follows (Eq. 1):

$$Mean\ depth\ (m) = \frac{Volume\ (m^3)}{Surface\ area\ (m^2)},$$ (1)

The shoreline development ratio (D_L) (Aronow, 1982) is a comparative index relating the shoreline length (i.e., the perimeter

95 of the reservoir) to the circumference of a circle that has the same area. The closer this ratio is to 1, the more circular the

lake. A large ratio (>>1) indicates the shoreline is more scalloped than a low ratio. The equation is as follows (Eq. 2):

$$D_L = \frac{Length\ of\ the\ shoreline\ (m)}{2\sqrt{\pi\ Area\ (m^2)}},\qquad(2)$$

The shallowness index (m^{-1}) was obtained by dividing the shoreline development index (D_L) by the mean depth (m), as

follows in eq. 3:

100 $$Shallowness\ index\ (m^{-1}) = \frac{D_L}{Mean\ depth\ (m)},\qquad(3)$$

We performed the vertical geochemical profiles of the reservoirs using a Seabird 19plus CTD profiler, coupled to Spherical

Underwater Quantum Sensor (LI-193R), and a fluorimeter Turner® SCUFA (model CYCLOPS–7). We obtained continuous

measurements of temperature, dissolved oxygen, conductivity, turbidity, density, PAR/Irradiance, fluorescence, specific

conductance, and salinity. We designed, based on the temperature and oxygen profile obtained, a discrete sampling with 6 to

105 9 depths along the water column. We took the water samples using a UWITEC sampling bottle. We also measured

barometric pressure using a multi-parameter probe (HANNA HI 9828) for the gas saturation calculations. We calculated the

saturation values (%) for dissolved oxygen as the ratio of the dissolved gas measured and the gas concentration expected in

equilibrium. We calculated the gas concentration in equilibrium, taking into account the differences in temperature, salinity,

and barometric pressure (Mortimer, 1956).

110 **2.2 Dissolved CH$_4$ in the water column**

We collected samples for dissolved CH$_4$ analysis in air-tight Winkler bottles by duplicate, preserved with a solution of HgCl$_2$

(final concentration 1mM) to inhibit biological activity and sealed with Apiezon® grease to prevent gas exchange. We stored

the samples in the dark at room temperature until analysis in the laboratory. We measured dissolved CH$_4$ using headspace

equilibration in a 50 ml air-tight glass syringe by duplicate or triplicate from each sample (Sierra et al., 2017). Then, we

115 analyzed the CH$_4$ concentration using a gas chromatograph (GC; Bruker® GC-450) equipped with Hydrogen Flame

Ionization Detector (FID). We daily calibrated the detectors using three standard gas mixtures with CH$_4$ concentrations of

1952, 10064, 103829 ppbv, made and certified by Air Liquide (France). We calculated the saturation values (%) as the ratio

between the concentration of the dissolved gas measured and the gas concentration expected in equilibrium considering the

temperature, salinity, and barometric pressure of each reservoir. We calculated the gas concentration in equilibrium using the

120 Bunsen functions for CH$_4$ (Wiesenburg and Guinasso, 1979; Yamamoto et al., 1976). We used the atmospheric gas

concentrations   provided   by   *The   Global   Greenhouse   Gas   Reference   Network*   website

(https://www.esrl.noaa.gov/gmd/ccgg/index.html), which is part of the National Oceanic and Atmospheric Administration

(NOAA) Earth System Research Laboratory in Boulder, Colorado. We calculated the 2016 global mean atmospheric

concentrations for CH$_4$ (Dlugokencky, 2019) from the 2016 global monthly mean. The differences among these values and

125   the local atmospheric concentrations are assumed to be small compared with the high dissolved concentrations obtained in the study reservoirs.

2.3 Chemical analysis in the water column

From the discrete sampling, we selected three or four relevant depths for C, N, and P analysis. We chose representative depths, covering the epilimnion, metalimnion (oxycline), and hypolimnion/bottom layers during the stratification period. We

130   determined total nutrients using unfiltered water, while we filtered the samples through 0.7 μm pore-size Whatman GF/F glass-fiber filters for the dissolved nutrients. We acidified the samples for dissolved organic carbon (DOC), total dissolved nitrogen (TDN), and total nitrogen (TN) samples with phosphoric acid (final pH<2). We measured DOC, TN, and TDN by high–temperature catalytic oxidation using a Shimadzu total organic carbon (TOC) analyzer (Model TOC-V CSH) coupled to nitrogen analyzer (TNM-1) (Álvarez-Salgado and Miller, 1998). We calibrated the instrument using a four-point standard

135   curve of dried potassium hydrogen phthalate for DOC, and dried potassium nitrate for TN and TDN. We analyzed two replicates and three to five injections per replicate for each sample. We purged the DOC samples with phosphoric acid for 20 min to eliminate all the dissolved inorganic carbon.

We measured the NO_3^- concentration using the ultraviolet spectrophotometric method, using a Perkin Elmer UV-Lambda 40 spectrophotometer at wavelengths of 220 nm and correcting for DOC absorbance at 275 nm (Baird et al., 2012). We

140   measured NH_4^+ and NO_2^- concentrations by Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Dissolved inorganic nitrogen (DIN) is the addition of the NO_3^-, NH_4^+, and NO_2^- concentrations. We measured total phosphorus (TP) concentration by triplicate using the molybdenum blue method (Murphy and Riley, 1962) after digestion with a mixture of potassium persulphate and boric acid at 120 °C for 30 min (Baird et al., 2012).

2.4 Phytoplankton, Chlorophyll-a and Primary Production in the water column

145   We determined chlorophyll-*a* concentration by filtering the particulate material of 500 to 2000 ml of water through pre-combusted Whatman GF/F glass-fiber filters. Then, we extracted the pigments from the filters with 95% methanol in the dark at 4 °C for 24 h (Baird et al., 2012). We measured chlorophyll-a (Chl-a) absorption using a Perkin Elmer UV-Lambda 40 spectrophotometer at the wavelength of 665 nm and for scattering correction at 750 nm. To obtain the integrated mean of chlorophyll-*a* (μg Chl-*a* L^{-1}), from the discrete points along the water column, we used the trapezoidal rule (León-Palmero et

150   al., 2019). To obtain the cumulative chlorophyll-a concentration in the whole water column (mg Chl-*a* m^{-2}), we summed the concentration of Chl-*a* from each stratum using the trapezoidal rule, as we did for the integrated chlorophyll-a before, but we omitted the division between the maximum depth.

We determined the abundances of cyanobacteria and photosynthetic picoeukaryotes using flow cytometry using unfiltered water. We collected and fixed the samples with a mixture of 1% paraformaldehyde and 0.05% glutaraldehyde for 30 min in

155   the dark at 4 °C. Then, we froze the samples in liquid nitrogen and stored them at −80 °C until analysis. We analyzed the samples in the FACScalibur flow cytometer equipped with the BD CellQuest Pro software for data analysis. We used

yellow-green 0.92 μm latex beads (Polysciences) as an internal standard to control the cytometer performance every day. We used different signals for groups determination: the side scatter (SSC), chlorophyll-a (red fluorescence, FL3), phycoerythrin (the orange fluorescence, FL2), and phycocyanin (the blue fluorescence, FL4); following the protocols and indications for
160   data previously published (Cellamare et al., 2010; Collier, 2000; Corzo et al., 1999; Gasol and Giorgio, 2000; Liu et al., 2014). In figure S13, we show a cytogram of the populations of cyanobacteria and photosynthetic picoeukaryotes.

We estimated gross primary production (GPP), net ecosystem production (NEP), and ecosystem respiration (R) by measuring temporal changes in dissolved oxygen concentration and temperature using a miniDOT (PME) submersible water logger during the stratification period. We recorded measurements every 10 minutes for 24-48 hours. We established the
165   start and ended time for photosynthesis as 30 minutes before sunrise and 30 minutes after dawn (Schlesinger and Bernhardt, 2013). We calculated the respiration rate during the night (the period between 60 minutes after dawn and 60 minutes before sunrise) (Staehr et al., 2010), and we assumed that the respiration rate overnight was similar to the respiration rate over the day. We used the equations proposed by Staehr et al. (2010) to calculate GPP, NEP, and R.

2.5 DNA analysis

170   We pre-filtered the water through 3.0 μm pore-size filters and extracted DNA following the procedure developed by Boström et al., (2004) for environmental samples. During the DNA extraction protocol, we combined a cell recovery step by centrifugation of 12 - 20 mL of the pre-filtered water, a cell lysis step with enzyme treatment (lysozyme and proteinase K), and finally, the DNA recovery step with a co-precipitant (yeast tRNA) to improve the precipitation of low-concentration DNA. DNA was quantified using a DNA quantitation kit (Sigma-Aldrich) based on the fluorescent dye bisBenzimide
175   (Hoechst 33258). Extracted DNA served as the template for PCR and quantitative PCR (qPCR) analysis to test the presence and abundance of the *mcrA* gene and the *phnJ* gene. For PCR analysis, we used the recombinant Taq DNA Polymerase (Thermo Fisher Scientific) using the Mastercycler X50 thermal cycler (Eppendorf). We ran the qPCR plates using SYBR Green as the reporter dye (PowerUp™ SYBR™ Green Master Mix, Thermo Fisher Scientific) in the Applied Biosystems 7500 Real-Time PCR System and the 7500 Software. In both cases, PCR and qPCR, we designed the standard reaction mix
180   recipes and the thermocycling conditions using the provider specifications and primer requirements. We chose specific primers from similar studies in freshwaters and pure cultures as positive controls. We targeted the alpha subunit of methyl-coenzyme reductase (*mcrA*) as a genetic marker to determine the existence and abundance of methanogenic *Archaea* in our samples. This gene appears to be an excellent marker since all known methanogens have the *methyl coenzyme-M reductase*, which is the enzyme responsible for the conversion of a methyl group to CH_4 (Grabarse et al., 2001). We used specific
185   primers from West et al. (2012) adapting their procedure. The forward primer was mcrAqF (5'-AYGGTATGGARCAGTACGA-3'), and the reverse primer was mcrAqF (5'- TGVAGRTCGTABCCGWAGAA -3'), and the annealing temperature was 54 ºC. We used a culture of *Methanosarcina acetivorans* (ATCC 35395) as a positive control. We also tested the presence of the *phnJ* gene, which encodes a subunit of the C-P lyase complex (Seweryn et al., 2015; White and Metcalf, 2007). This enzyme cleaves C-P bonds in phosphonate compounds releasing methane, and changes in

190   response to phosphate availability (Yao et al., 2016a). We ran the amplification with a pair of primers previously used by Fox et al., (2014); Karl (2008); and Yao et al., (2016). The forward primer was PhnJoc1 (5'-AARGTRATMGAYCARGG-3') and the reverse PhnJoc2 (5'-CATYTTYGGATTRTCRAA-3') adapting the PCR procedure from Yao et al., (2016). The annealing temperature was 52.5 ºC, and the positive controls were ran using a pure culture of *Rhodopseudomonas palustris* (ATCC 33872). We checked the result of the amplification by running 1.5 % (w/v) agarose gel electrophoresis. If we did not

195   detect amplification in the PCR or qPCR samples, we changed the standard procedure by increasing the DNA amount and the primers concentration to corroborate the negative results.

2. 6. Statistical tests

We conducted all the statistical analysis in R (R Core Team, 2014) using the packages car (Fox and Weisberg, 2011), nortest (Gross and Ligges, 2015), and mgcv (Wood, 2011). We performed the Shapiro-Wilk test of normality analysis and Levene's

200   test for homogeneity of variance across groups. We performed a one-way analysis of variance test (ANOVA) when the data were normally distributed. In case the data did not meet the assumptions of normality, we used the Kruskal-Wallis rank-sum test (K-W) or the Wilcoxon test. We analyzed the potential sources of dissolved CH_4 using simple regression analysis and generalized additive models (GAMs) (Wood, 2006). GAM is a generalized model with a linear predictor involving a sum of smooth functions of covariates (Hastie and Tibshirani, 1986, 1990). The model structure is shown in Eq. (4):

205     $y_i = f_1(x_{1i}) + f_2(x_{2i}) + \cdots + f_n(x_{ni}) + \epsilon_i \, ,$ (4)

Where the f_j are the smooth functions, and the ϵ_i are independent identically distributed $N(0, \sigma^2)$ random variables. We fit smoothing functions by penalized cubic regression splines. The cross-validation method (Generalized Cross Validation criterion, GCV) estimates the smoothness of the functions. We fitted the models to minimize the Akaike Information Criterion (AIC) and the GCV values. We provide details on these GAMs in Supplementary Table 5. We calculated the

210   percentage of variance explained by the model (adj R^2) and the quality of the fit (deviance explained). We also fixed the effect of each predictor to assess the contribution of the other predictor on the total deviance explained. Then, the sum of the deviance explained by two predictors can be different from the deviance explained by the model due to interactive effects.

3 Results and discussion

3. 1. Profiles description

215   We found pronounced differences in the concentration of dissolved CH_4 of the study reservoirs among depths and seasonal periods (Figs 1-3, Figs S1-9). The concentration of dissolved CH_4 varied up to four orders of magnitude from 0.06 to 213.64 μM during the summer stratification (n = 96), and it was less variable during the winter mixing (n = 84) ranging only from 0.02 to 0.69 μM. All depths were consistently supersaturated in CH_4, which ranged from 2224 % to 7082234 % during the stratification period, and from 710 % to 20006 % during the mixing period. The dissolved CH_4 concentration and the

220   saturation values were significantly higher during the stratification period than during the mixing period (V = 78, p-value <
0.001; V = 78, p-value < 0.001). These differences in the concentration of dissolved CH_4 are coherent with the differences
found in the CH_4 emissions from these reservoirs in the stratification and mixing periods (León-Palmero et al. in review).
The wide range in CH_4 concentrations found in this study covers from values found in boreal lakes (Donis et al., 2017;
Grossart et al., 2011; Murase et al., 2003; Tang et al., 2014), temperate lakes (West et al., 2016), to those found in tropical
225   reservoirs (Naqvi et al., 2018; Okuku et al., 2019). In surface waters, we found values from 0.06 to 8.18 µM, which is about
three times the minimum values and eighty times the maximum values found in the surface waters of Lake Kivu (Africa) by
Roland et al., (2017). We found similar values to the concentrations reported in subtropical and tropical reservoirs (Musenze
et al. 2014, and references therein).

The dissolved CH_4 profiles were uniform during the winter mixing in all the reservoirs (Figs. 1b-3b, Figs S1b-9b), whereas
230   during the summer stratification, we found considerable differences in the concentration of dissolved CH_4 in the water
column (Figs. 1a -3a, figs S1a-9a). Based on the differences found during the stratification in the dissolved CH_4 profiles, we
sorted the reservoirs in three types. The first type included six reservoirs, in which the dissolved CH_4 profile increased from
the oxycline to the anoxic bottom, just above the sediments, where reached its maximum. When the oxycline and the
thermocline were spatially coupled, the dissolved CH_4 concentration increased exponentially from the thermocline along the
235   anoxic hypolimnion to the sediments. The reservoirs Béznar, San Clemente, and Iznájar showed this profile (Fig. 1a and figs.
S1a and 2a). The existence of a sizeable almost anoxic hypolimnion led to a massive accumulation of CH_4 in this layer. The
differences in the CH_4 concentration between the surface and bottom waters were up to three orders of magnitude, as we
found in Béznar (from the 0.25 to 56.17 µM; Fig. 1a), San Clemente (from the 0.23 to 45.15 µM; Fig S1a), and Iznájar (from
the 0.82 µM to 213.64 µM; Fig. S2a). When the oxycline and the thermocline were not spatially coupled, the dissolved CH_4
240   concentration increased just above the sediments where the anoxic-oxic interface was closed to the bottom. The reservoirs
Cubillas, La Bolera, and Francisco Abellán showed this profile type (Figs. S3a, S4a, and S5a). This accumulation of CH_4 in
the hypolimnion and above sediments might be related to the high rates of methanogenesis in the sediments and its
subsequent diffusion to the water column. Dissolved CH_4 concentration declines at the oxycline level, where the highest
rates of CH_4 oxidation are usually measured (Oswald et al., 2015, 2016). The CH_4 profiles in this group were similar to the
245   ones found in tropical eutrophic and temperate reservoirs (Naqvi et al., 2018; West et al., 2016). The second profile type
presents a small peak of metalimnetic CH_4, concomitant with peaks of dissolved oxygen, chlorophyll-a, photosynthetic
picoeukaryotes, and cyanobacteria (Fig. 2a). In the Negratín reservoir, we found the maximum concentration of CH_4 in the
oxic hypolimnion. Unlike several previous works (Blees et al., 2015; Grossart et al., 2011; Murase et al., 2003), we did not
find a metalimnetic CH_4 maximum. Donis et al. (2017) augmented that the observed metalimnetic CH_4 maximum
250   represented only an accumulation physically driven. The third profile type included five reservoirs, in which the dissolved
CH_4 profile presented a CH_4 accumulation more significant in the epilimnion than in the hypolimnion. The reservoirs
Jándula, Bermejales, Rules, El Portillo, and Colomera showed this profile type (Fig. 3a, Figs. S6a–9a). These reservoirs had

a mean CH$_4$ concentration in the water column lower than the reservoirs from the first type. Similar profiles have been reported in boreal lakes (Murase et al., 2003; Tang et al., 2014).

255 **3. 2. CH$_4$ sources in the water column**

We found two well-differentiated groups of CH$_4$ data based on the dissolved oxygen concentration (D.O.) (Fig. S10). The first dataset included the samples with a D.O. lower than 7.5 μM (n = 18, hereafter anoxic samples). These samples belong to the hypolimnion and bottom of the study reservoirs during the stratification period. The second dataset included the samples with D.O. higher than 7.5 μM (n = 160, hereafter oxic samples). All the samples from the mixing period (n = 82)
260 and most of the samples from the stratification period (n = 78) belong to this second dataset. We found significant differences (W = 2632, p-value < 0.001) between the concentration of CH$_4$ in the anoxic samples (median = 15.79 μM, min = 0.35 μM, max = 213.64 μM) and in the oxic samples (median = 0.15 μM, min = 0.02 μM, max = 8.17 μM).

3. 2. 1. CH$_4$ sources in anoxic waters

Archaeal methanogens are obligate anaerobes that decompose the organic matter and produce CH$_4$ in anoxic environments,
265 as freshwater sediments. We analyzed the presence of the methanogenic *Archaea* in the water column of the anoxic samples, by targeting the typical genetic marker of this group: the alpha subunit of methyl-coenzyme reductase, determined by the gene *mcrA*. We did not detect the amplification of the *mcrA* gene in the PCR or the qPCR analysis. Therefore, we assumed that the methanogenic *Archaea* are not present as free-living microorganisms in a significant number in the water column of the anoxic samples. We did not find a significant relationship between the water temperature and the dissolved CH$_4$
270 concentration in the anoxic samples from the water column (n=17, p-value = 0.66), even though methanogenesis is a microbial process very susceptible to temperature (Marotta et al., 2014; Sepulveda-Jauregui et al., 2018; Yvon-Durocher et al., 2014). These two results (the no detection of the *mcrA* gene and the absent of relationship of dissolved CH$_4$ with water temperature) suggest that CH$_4$ production is not happening in the water column of the study reservoirs. Segers (1998) reported that in freshwaters the organic matter decomposition in the anoxic sediments produces dissolved CH$_4$. We presumed
275 that methanogenic *Archaea* must be present in the sediments, where they produce CH$_4$ that diffuses up to the water column developing vast accumulations of CH$_4$ in the hypolimnion.

Methanogenesis in the sediments may be affected by organic matter quantity and quality (West et al., 2012). In the study reservoirs, the dissolved organic carbon concentration did not show a significant relationship with the dissolved CH$_4$ concentration (n=12, p-value = 0.10). We examined the importance of the autochthonous organic matter produced by
280 primary producers using the total cumulative chlorophyll-a (Chl-a, mg m^{-2}). The cumulative Chl-a is a surrogate for the vertical exportation of the phytoplankton biomass and their by-products for the whole water column. We obtained that the CH$_4$ concentrations in anoxic samples depended on the cumulative Chl-a following a power function (CH$_4$ = 3.0 10^{-4} Cumulative Chl-a $^{2.28}$; n=17, adj R^2=0.40, p-value <0.01) (Fig. 4). The autochthonous organic matter was a better predictor

for the concentration of CH_4 in anoxic waters than the dissolved organic matter likely because methanogenesis is mainly
affected by the origin of the organic matter. Previous experimental studies have demonstrated that the addition of algal
biomass on sediment cores increase the CH_4 production more than the addition of terrestrial organic matter (Schwarz et al.,
2008; West et al., 2012, 2015). The stimulation of the methanogenesis rates appears to be related to the lipid content in
phytoplankton biomass (West et al. 2015). West et al. (2016) found a significant relationship between the chlorophyll-*a*
concentration in the epilimnion and the potential methanogenesis rates from sediment incubations. In this study, we
corroborate the importance of the autochthonous-derived organic matter on the CH_4 concentrations, considering the
phytoplankton biomass of the whole water column.

3. 2. 2. CH_4 sources in oxic waters

In this study, we observed CH_4 supersaturation in all the samples of the oxic waters ranging from 827 % to 363131 %, and
the dissolved CH_4 concentration ranged from 0.02 μM to 8.18 μM. To explain the origin of this CH_4 supersaturation we
tested different hypotheses: (1) the lateral and vertical CH_4 transport from littoral and deep layers, (2) the in-situ CH_4
production by methanogenic *Archaea,* or methyl-phosphonate degradation under extreme P-limitation, and 3) the CH_4
production by other processes linked to phytoplankton.

Vertical and lateral CH_4-transport from anoxic environments

Several previous works pointed out that CH_4 supersaturation in oxic waters can be explained by the vertical transport from
the bottom sediments, and the lateral inputs from the littoral zones that are in contact with shallow sediments where
methanogenesis occurs (Bastviken et al., 2004; Encinas Fernández et al., 2016; Michmerhuizen et al., 1996). To test the
importance of the lateral and vertical transport explaining the concentration of CH_4 in oxic waters, we used two
morphometric parameters: the mean depth (m) as a proxy for the vertical transport and the shallowness index as a proxy for
the lateral transport. We also studied the influence of wind speed on surface waters. The dissolved CH_4 concentration was an
exponential decay function of the reservoir mean depth (Fig. 5a) both during the stratification period ($CH_4 = 4.0 \ 10^{-2} \ e^{(50.0/ \text{mean depth})}$, adj $R^2 = 0.95$) and during the mixing period ($CH_4 = 3.7 \ 10^{-2} \ e^{(22.9/ \text{mean depth})}$, adj $R^2 = 0.54$) (Fig. 5a). We observed
that at mean depths shallower than 16 meters, the dissolved CH_4 concentration increased exponentially (Fig. 5). Several
studies have proposed that the vertical transport of CH_4 from bottom sediments explains the supersaturation in surface waters
(Rudd & Hamilton 1978, Michmerhuizen et al. 1996, Murase et al. 2003, Bastviken et al. 2004). However, the vertical
diffusion rates of dissolved gases across the thermocline are too low in deep and thermally stratified systems and no
movements of methane upwards from the hypolimnion have been detected (Rudd and Hamilton, 1978). The shallowness
index increases in elongated and dendritic lakes with more impact of the littoral zone and decreases in near-circular lakes,
with low shoreline length per area. In this study, we did not find a significant relationship between the shallowness index and
the dissolved CH_4 concentration (Fig. 5b). Surface winds can cause wave mixing, promoting the CH_4 transport from littoral
zones. We studied the relationship between the wind speed and the CH_4 concentration in surface waters, but we did not find

a significant relationship during the stratification period (p-value = 0.43) or the mixing period (p-value = 0.40). In the study reservoirs, wind speeds were relatively low ranging from 0 to 4.11 m s^{-1}. The diffusion from the shallow water zones may be an important process in some areas closed to the littoral zone, but not in the open waters. Then, these lateral inputs may not be enough to explain extreme CH$_4$ supersaturations (DelSontro et al., 2018).

320 **CH$_4$-production by methanogenic *Archaea* or methyl-phosphonate degradation**

The ubiquitous CH$_4$ supersaturation found in oxic waters may not be fully explained, in many cases, by the vertical and lateral transport and it seems that there is an in situ production of CH$_4$ (Bogard et al., 2014; DelSontro et al., 2018; Grossart et al., 2011). However, the ultimate mechanisms involved in this production are not clear enough. We studied the presence of the methanogenic *Archaea* in the oxic samples by targeting the gene *mcrA* by PCR and qPCR, but we were unable to detect

325 this gene (Fig. S11). This result indicates that methanogenic *Archaea* are not present in a significant number in the water column of the oxic samples in the study reservoirs. The classical methanogens (i.e., *Archaea* with the *mcrA* gene) are obligate anaerobes without the capacity to survive and produce CH$_4$ under aerobic conditions (Chistoserdova et al., 1998). Previous studies showed that methanogens may tolerate oxygen exposure in soils (Angel et al., 2011; Angle et al., 2017) and detected potential methanogenic *Archaea* attached to photoautotrophs in lake oxic waters (Grossart et al., 2011).

330 We also considered the possibility of methylphosphonates (MPn) degradation as an in situ CH$_4$ source. This metabolic pathway appears in the bacterioplankton under chronic starvation for phosphorus (Karl et al., 2008). Diverse pieces of evidence have shown that marine bacterioplankton can degrade the MPn and produce CH$_4$ through the C–P lyase activity in typically phosphorus starved environments, as the ocean gyres (Beversdorf et al., 2010; Carini et al., 2014; Repeta et al., 2016; Teikari et al., 2018; del Valle and Karl, 2014). Freshwater bacteria can also degrade the MPn and produce CH$_4$, as it

335 has been demonstrated in Lake Mantano (Yao et al., 2016b, 2016a). Lake Mantano is an ultra-oligotrophic lake with a severe P deficiency (below 0.050 µmol P L^{-1}) due to the permanent stratification, iron content, and extremely low nutrient inputs (Crowe et al., 2008; Sabo et al., 2008). The ratio of dissolved inorganic nitrogen (DIN) to total phosphorus (TP) (µmol N: µmol P) is widely used to evaluate P-limitation (Morris and Lewis, 1988). DIN:TP ratios greater than 4 are indicative of phosphorus limitation (Axler et al., 1994). In the study reservoirs, the TP concentration ranged from 0.13 to 1.85 µmol P L^{-1}

340 during the stratification period, and from 0.10 to 2.17 µmol P L^{-1} during the mixing period. The DIN:TP ratio ranged from 15 to 985 during the stratification period, and from 28 to 690 during the mixing period. The more extreme P-limitation conditions, the higher the CH$_4$ production by methylphosphonates (MPn) degradation is. However, we did not find a significant relationship between the DIN:TP ratio and the CH$_4$ concentration (Fig. 6). We also analyzed the presence and abundance of the gene *phnJ*, which encodes the enzyme complex C–P lyase that hydrolyzes the MPn and changes in

345 response to phosphate availability. We did not detect the *phnJ* gene in the PCR or the qPCR analysis in any of the study samples (Fig. S12). These results indicate that the MPn degradation was not a quantitatively relevant source of CH$_4$ in the oxic waters of the study reservoirs. Our results are in concordance with Grossart et al. (2011), who did not detect CH$_4$

production by adding inorganic phosphate or methylphosphonates to lake samples in laboratory experiments. Although we used different methodologies, both studies may indicate that MPn degradation is only an important source of CH_4 in ultra-

350 oligotrophic systems, as Lake Mantano or ocean gyres.

CH_4-production coupled to photosynthetic organisms

Previous studies have consistently reported CH_4 production in oxic waters associated to phytoplankton in the field, in situ incubations, and in floating mesocosms (Bogard et al., 2014; Grossart et al., 2011; Owens et al., 1991; Schmidt and Conrad, 1993; Tang et al., 2014). In the study reservoirs, we analyzed the relationship between phytoplankton and the dissolved CH_4

355 concentration using the gross primary production (GPP, g O_2 m^{-3} d^{-1}), the concentration of chlorophyll-a (Chl-a, µg L^{-1}), and the abundance of photosynthetic picoeukaryotes (PPEs, cell mL^{-1}) and cyanobacteria (CYA, cell mL^{-1}). The PPEs are marine and freshwater microorganisms of ca. 3.0 µm or less in size. In the freshwaters, the PPEs include species from different phyla, as non-colonial *Chlorophyta* (green algae), and *Haptophyta*. We show the vertical profiles of the Chl-a concentration and the abundance of PPEs and CYA profiles of each reservoir in figs. 1, 2, 3, and figs. S1-9. The abundance of

360 cyanobacteria ranged from 1513 to 204201 cells mL^{-1} and was more than one order of magnitude higher than the abundance of PPEs that ranged from 32 to 7450 cells mL^{-1}. Using optical microscopy, we determined that the main groups of photosynthetic picoeukaryotes in the study reservoirs. PPEs were non-colonial green algae from the order *Chlorococcales* (class *Chlorophyceae,* phylum *Chlorophyta),* and the genus *Chrysochromulina* spp., (class *Coccolithophyceae,* phylum *Haptophyta*).

365 We did not find a significant relationship between the gross primary production (GPP) and the dissolved CH_4 concentration (p-value = 0.077, n = 12, Table 1). The Chl a concentration showed a significant relationship with the GPP (p-value < 0.01, n = 12, adj R^2 = 0.55), but the abundance of cyanobacteria or the abundance of the photosynthetic picoeukaryotes did not show a significant relationship with the GPP (p-value = 0.911, n = 12; p-value = 0.203, n = 12, respectively).

We found significant potential relationships between the Chl-a concentration, the abundances of the photosynthetic

370 picoeukaryotes, and the abundance of cyanobacteria with the concentration of dissolved CH_4 during the stratification period (Fig. 7a, 7b, and 7c respectively, and Table 1). During the mixing period, the only predictor of the dissolved CH_4 concentration was the abundance of photosynthetic picoeukaryotes (Fig. 7b). The variance explained, and the slope of the relationship (i.e. the exponent in the power relationship) between the dissolved CH_4 and the abundance of photosynthetic picoeukaryotes was higher during the stratification than during the mixing (Table 1). By comparing the stratification slopes,

375 the effect per cell of PPEs on CH_4 concentration was slightly higher than the impact of cyanobacteria (Table 1). These results agree with previous studies that showed a closed link between the CH_4 concentration and the autotrophic organisms, primary production, or chlorophyll-a concentration (Bogard et al., 2014; Grossart et al., 2011; Schmidt and Conrad, 1993; Tang et al., 2014).

In this study, we show that the PPEs abundance was a better predictor of the CH_4 concentration than the abundance of

380 cyanobacteria. In the study reservoirs, the PPEs group included members from green algae and *Haptophyta*, which are

regular components of the marine plankton. Therefore, these results may be relevant also for marine waters. Cyanobacteria have received more attention as potential producers of CH_4 in oxic conditions than photosynthetic picoeukaryotes (Berg et al., 2014; Bižić et al., 2020; Teikari et al., 2018). Grossart et al. (2011) also detected CH_4 production in laboratory cultures of cyanobacteria and green algae. Overall, these results indicate a clear association between the CH_4 production and the

385 photoautotrophs from both *Eukarya* and *Bacteria* domains. The pathways involved in the CH_4 production may be related to the central photosynthetic metabolism or the release of methylated by-products different from methylphosphonates during the photosynthesis. Lenhart et al. (2016) demonstrated the CH_4 production using ^{13}C-labeled bicarbonate in the eukaryotic microalgae *Emiliania huxleyi*. More recently, Bižić et al., (2020) also detected CH_4 production from ^{13}C-labeled bicarbonate in marine, freshwater, and terrestrial cyanobacteria cultures. In both studies, the autotrophic organisms uptake bicarbonate in

390 the reductive pentose phosphate cycle (Calvin-Benson cycle) (Berg, 2011). Carbon fixation is a crucial step of photosynthesis in cyanobacteria, algae, and plants (Berg, 2011; Burns and Beardall, 1987). Therefore, CH_4 production may be a common pathway in the central metabolism of photosynthesis of all the cyanobacteria and algae in freshwaters and marine environments.

On the other hand, the production of CH_4 can also be related to the production of methylated compounds during

395 photosynthesis. Lenhart et al. (2016) also detected the CH_4 production in *E. huxleyi* cultures from the sulfur-bound methyl group of the methionine. Common substances as methionine can act as a methyl-group donor during the CH_4 production in plants and fungi (Lenhart et al. 2012, 2015). Besides, algae use part of the methionine for the synthesis of dimethylsulfoniopropionate (DMSP), an abundant osmolyte, the precursor of dimethyl sulfide (DMS), and dimethylsulphoxide (DMSO). These methylated substances produce methane during their degradation (Damm et al., 2008,

400 2010, 2015; Zindler et al., 2013). These substances, together with other organosulphur compounds, can also produce CH_4 abiotically (Althoff et al., 2014). The production of DMSP, DMS, and other methylated substances as isoprene, has been extensively studied in marine phytoplankton, showing that taxa as photosynthetic picoeukaryotes and the cyanobacteria are relevant sources (Shaw et al., 2003; Yoch, 2002). Recent studies have reported that freshwater algae and cyanobacteria also produced DMS and isoprene (Steinke et al., 2018). Further studies are needed to quantify the potential role of the methylated

405 by-products as CH_4 sources in freshwaters.

Modeling the CH_4 production in oxic waters

The explanation of the CH_4 oversaturation in oxic waters may relay on the interaction of several processes as the transport from anoxic environments and the biological activity (DelSontro et al., 2018). In this study, we found that vertical transport (mean depth as surrogate), water temperature, and the abundance of photosynthetic picoeukaryotes and cyanobacteria had a

410 significant effect on the dissolved CH_4 concentration. We combined these explanatory variables with significant effects using generalized additive models (GAMs). The GAM model for the stratification period (n=78) had a fit deviance of 82.7% and an explained variance (adj R^2) of 81.4 % (Table S1). The explanatory variables, in decreasing order, were: the photosynthetic picoeukaryotes abundance ($\log_{10}$ PPEs), the reservoir mean depth, the cyanobacteria abundance ($\log_{10}$ CYA),

and the water temperature (Fig. 8a). The function obtained was: $Log_{10} CH_4 = -4.05 + 3.4 \; 10^{-1} Log_{10} PPEs + e^{(6.7/ mean depth)} + 1.7 \; 10^{-1} Log_{10} CYA + 2.7 \; 10^{-2}$ Temperature. The abundance of PPEs was the variable explaining most of the variance of dissolved CH_4 concentration ($Log_{10} CH_4$) during the stratification period, with an effect higher than the cyanobacteria abundance. Figure 8b-e shows the partial responses of each explanatory variable. The GAM model for the mixing period (n=82) only included two explanatory variables: the reservoir mean depth and the abundance of the photosynthetic picoeukaryotes. The reservoir mean depth was the variable explaining most of the variance of the dissolved CH_4 concentration ($Log_{10} CH_4$) during the mixing period, closely followed by the abundance of PPEs (Fig. 9a). The function for the model was: $Log_{10} CH_4 = -2.07 + 1.5 \; e^{(-0.04 \; mean \; depth)} + 1.8 \; 10^{-1} Log_{10} PPEs$, with a fit deviance of 53.9 % and an explained variance (adj R^2) of 52.1 % (Table S1). In Figure 9b and 9c, we show the partial response plots for both variables. These results show that the abundance of photosynthetic picoeukaryotes can be key for explaining the dissolved CH_4 concentration in oxic waters, even though they have received less attention than cyanobacteria in previous studies (Berg et al., 2014; Bižić et al., 2020; Teikari et al., 2018). Finally, we have also included a simple model to explain the dissolved CH_4 concentration ($Log_{10} CH_4$) in both periods (n=160) using common explanatory variables like the water temperature (ºC) and chlorophyll-a concentration (Chl-a, μg L^{-1}). The function for the model was: $Log_{10} CH_4 = -1.22 + 3.2 \; 10^{-2} \; e^{(0.13 \; Temperature)} + 2.3 \; 10^{-1} Log_{10} Chl-a$. The GAM model had a fit deviance of 49.7 % and an explained variance (adj R^2) of 48.8 % (Table S1). Overall, during the stratification period, the in situ CH_4 production was coupled to the abundance of photosynthetic picoeukaryotes in oxic waters (Fig. 8a). This CH_4 source by photosynthetic picoeukaryotes can be crucial in large, deep lakes and reservoirs, and the open ocean since the impact of the CH_4 transport from sediments (i.e., mean depth) decreases with increasing depths. In deeper reservoirs, the thermal stratification during the summer produced that the vertical diffusion rates of CH_4 from sediments is limited. Rudd and Hamilton, (1978) did not detect any movement of CH_4 upwards from the hypolimnion during the stratification. Bogard et al., (2014) also suggested that CH_4 produced in the oxic water column is a significant component of CH_4 fluxes, especially in deep systems. This CH_4 produced in the oxic layer can reach up to 90% of total CH_4 emissions during the stratification period (Donis et al., 2017).

In contrast, during the winter mixing, the mean depth had a more considerable influence than the in situ production by photosynthetic picoeukaryotes (Fig. 9a). The photosynthetic picoeukaryotes identified in the study reservoirs are considered indicators of eutrophic conditions and are bloom-forming genera (i.e., *Chlorococcales* and *Chrysochromulina* spp.) (Reynolds, 1984; Willén, 1987; Edvardsen and Paasche, 1998). Global future estimations suggest a rise in eutrophication and algal bloom over the next century due to climate change and the growing human population (Beaulieu et al., 2019). In that situation, photosynthetic picoeukaryotes as *Chlorococcales* and *Chrysochromulina* spp., and cyanobacteria, would lead to an increment in CH_4 production and emissions. Further studies are needed to better understand the role of the photosynthetic picoeukaryotes in the production of CH_4 in oxic waters, and to quantify their influence in the methane oversaturation and CH_4 fluxes from inland and oceanic waters.

4 Conclusions

The dissolved CH$_4$ concentration in the study reservoirs showed a huge variability (i.e. up to four orders of magnitude), and presented a seasonal pattern. Surface waters were always supersaturated in CH$_4$. The concentration of CH$_4$ was closely

450   linked to the phytoplankton dynamics. In the anoxic waters, the depth-cumulative chlorophyll-a concentration, a proxy for the total phytoplanktonic biomass exported to sediments, determined the CH$_4$ concentration. In the oxic waters, we considered different potential CH$_4$ sources, including the vertical and lateral transportation of CH$_4$ from anoxic zones and *in situ* production by different approaches. The mean depth of the reservoirs, as a surrogate of the CH$_4$ transport from sediment to the oxic waters, contributed in shallow systems. We did not detect methanogenic *Archaea* or methylphosphonates

455   degradation target genes (i.e. *mcrA* and *phnJ* genes, respectively*)*, which suggests that these pathways are not responsible for the in situ production of CH$_4$ in the oxic waters in the study reservoirs. We found that dissolved CH$_4$ was coupled to the abundance of photosynthetic picoeukaryotes (PPEs) during both periods, and to chlorophyll-a concentration and the abundance of and cyanobacteria during the stratification period. These PPEs were non-colonial green algae from the order *Chlorococcales* (class *Chlorophyceae,* phylum *Chlorophyta),* and the genus *Chrysochromulina* spp., (class

460   *Coccolithophyceae,* phylum *Haptophyta*). Finally, we combined the explanatory variables with significant effects and tested their relative contribution on the CH$_4$ concentration using generalized additive models (GAMs). The abundance of PPEs was the variable explaining most of the variance of dissolved CH$_4$ concentration during the stratification period, with an effect higher than the cyanobacteria abundance. During the mixing period the the reservoir mean depth and the abundance of the PPEs were the main drivers for CH$_4$ concentration. In a simpler model, we can also predict the dissolved CH$_4$ concentration

465   in both periods using water temperature and chlorophyll-a concentration. Our findings show that the abundance of PPEs can be key for explaining the dissolved CH$_4$ concentration in oxic waters, and, in comparison to cyanobacteria, have been poorly explored as CH$_4$ sources. These coupling of the abundance of the PPEs and the dissolved CH$_4$ concentration is novel, and further studies should determine the ultimate mechanism involved in methane production.

Data availability

470   Additional figures and tables can be found in the supplementary information. The dataset associated with this manuscript is available at Pangaea (XXXXXXXX).

Author contribution

E.L.-P., R.M.-B. and I.R. contributed equally to this work. R.M.-B. and I.R. designed the study and obtained the funds. E.L.-P., R.M.-B., and I.R. contributed to data acquisition during the reservoir samplings. E.L.-P. processed most of the chemical

475   and biological samples. A.C. performed flow cytometry and molecular analysis. A.S. collaborated with the dissolved CH$_4$

analysis using gas chromatography. E.L.-P., R.M.-B. and I.R. analyzed the data and discussed the results. E.L.-P. wrote the first draft manuscript, which was complemented by significant contributions of R.M.-B. and I.R.

Competing interests

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be
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Figures

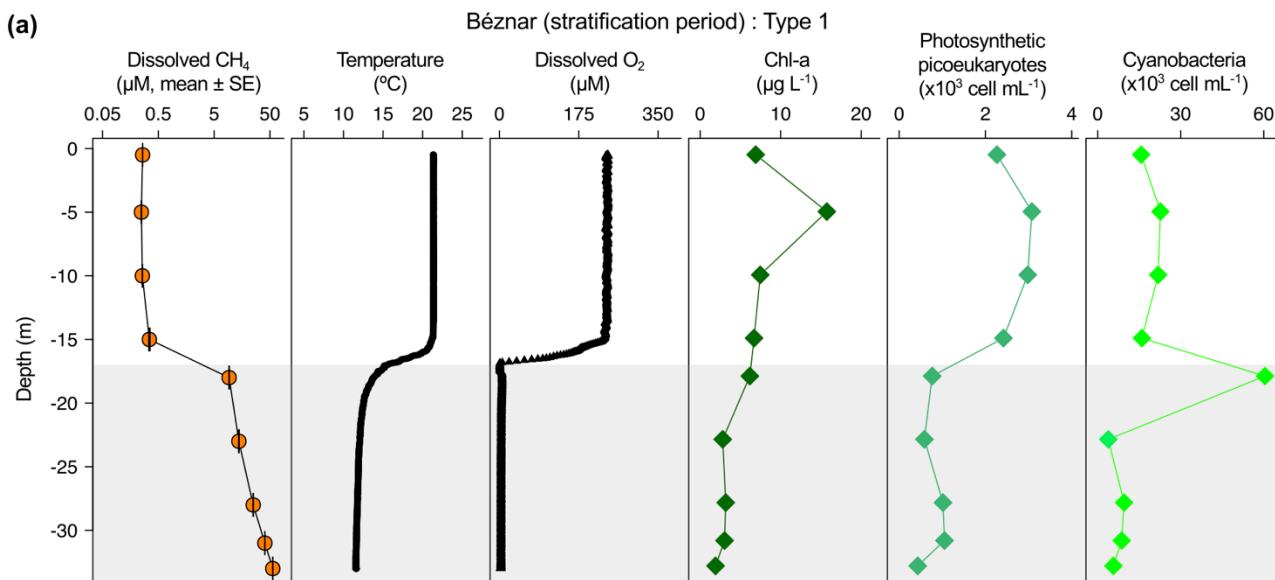
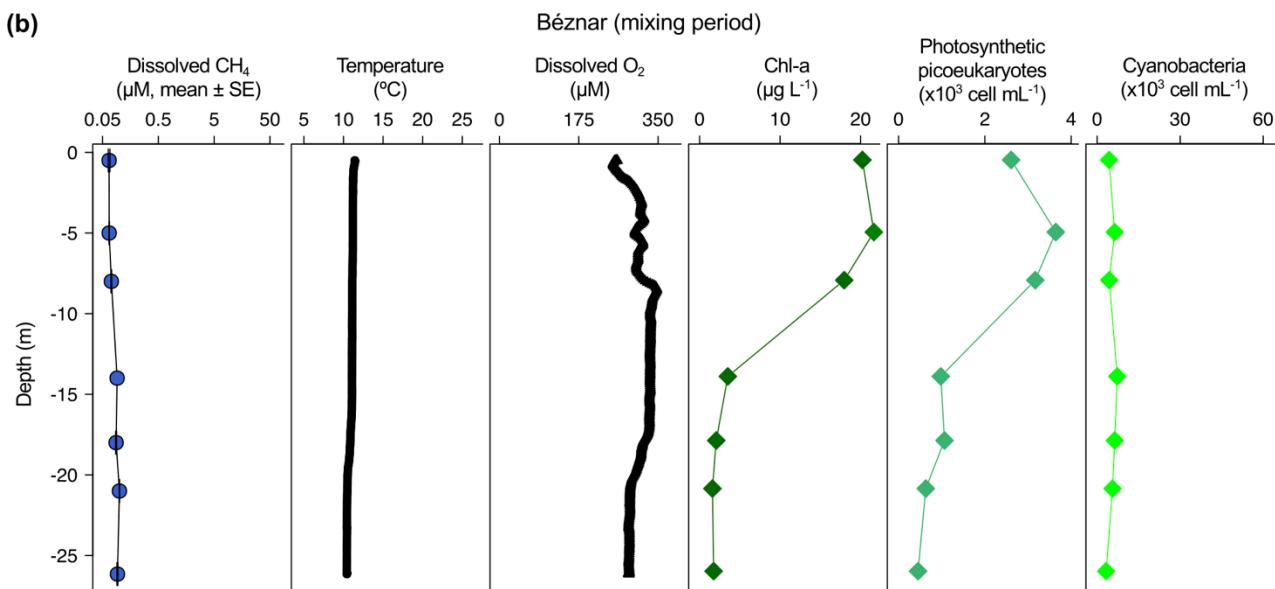



Figure 1: **Vertical profiles of physicochemical and biological variables in Béznar reservoir.** Dissolved methane concentration (CH$_4$, µM), temperature (°C), dissolved oxygen concentration (DO, µM), chlorophyll-a concentration (Chl-a, µg L^{-1}), abundance of photosynthetic picoeukaryotes (cell mL^{-1}) and abundance of cyanobacteria (cell mL^{-1}) during the stratification period (**a**) and the mixing period (**b**). The grey area represents the anoxic zone (DO < 7.5 µM).

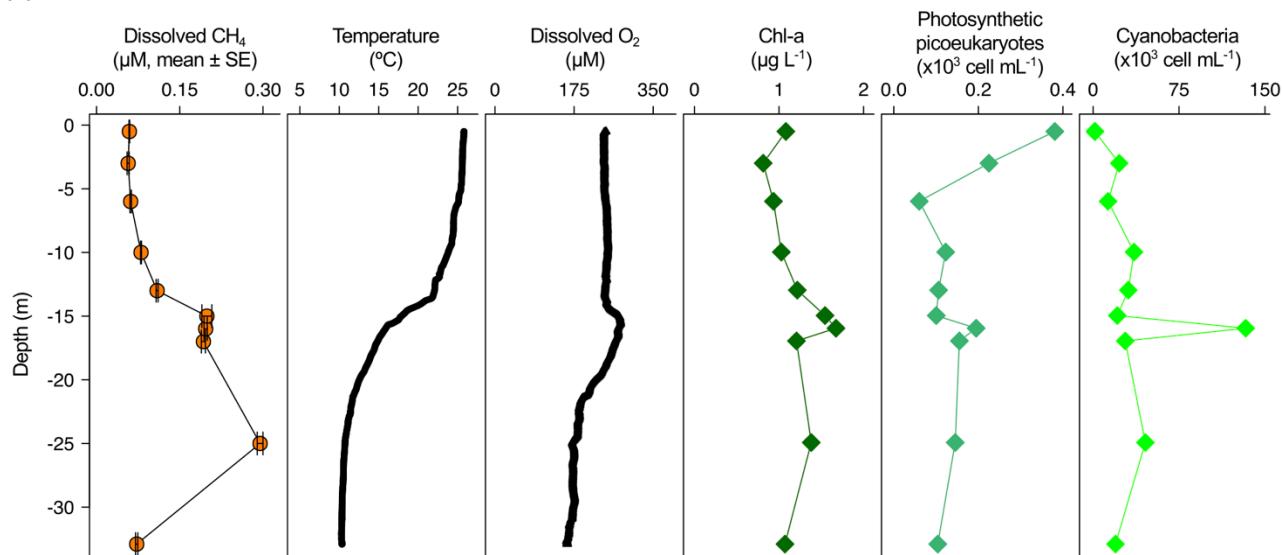
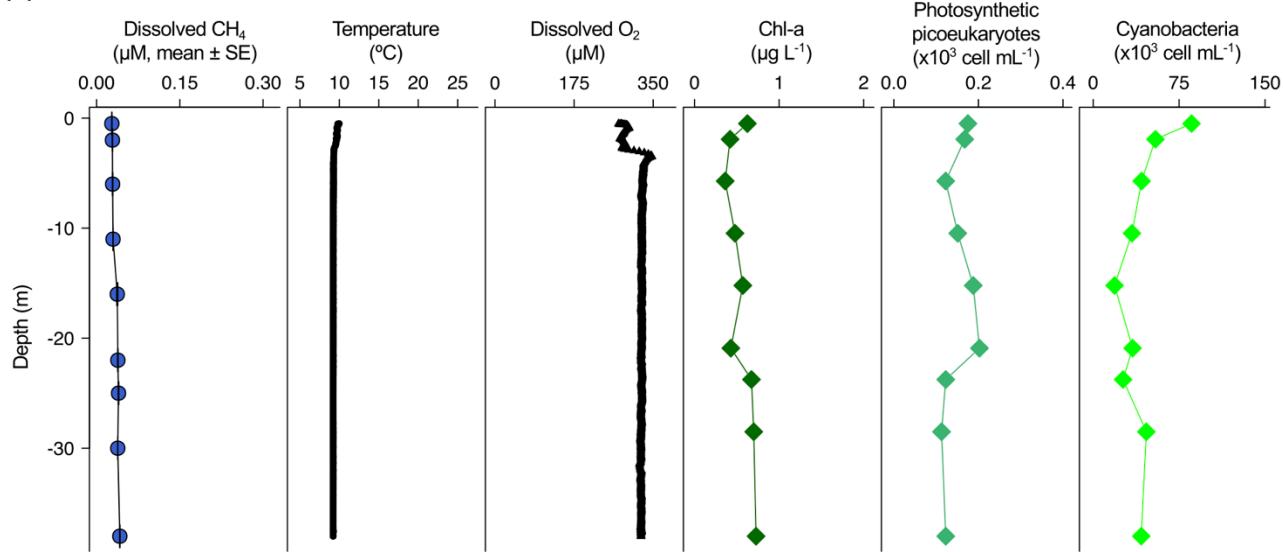



Figure 2: Vertical profiles of physicochemical and biological variables in Negratín reservoir. Dissolved methane concentration (CH_4, µM), temperature (ºC), dissolved oxygen concentration (DO, µM), chlorophyll-a concentration (Chl-a, µg L^{-1}), abundance of photosynthetic picoeukaryotes (cell mL^{-1}) and abundance of cyanobacteria (cell mL^{-1}) during the stratification period (**a**) and the mixing period (**b**). The grey area represents the anoxic zone (DO < 7.5 µM).

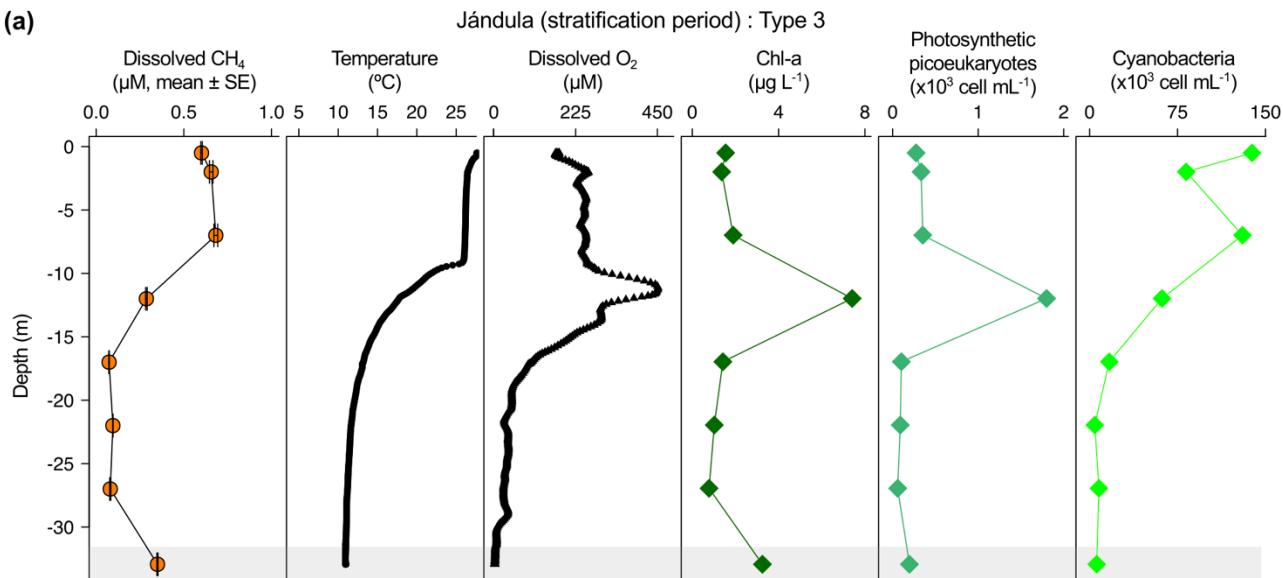
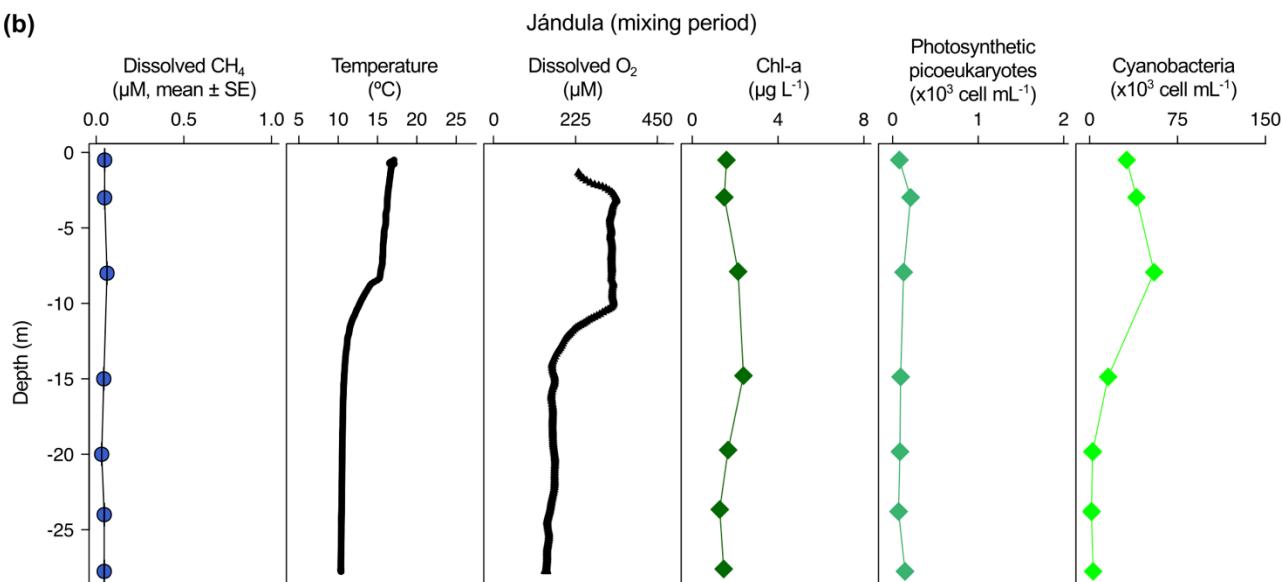
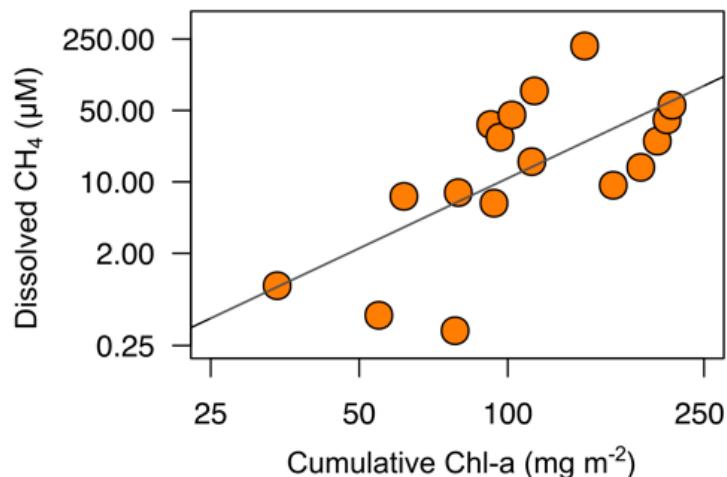
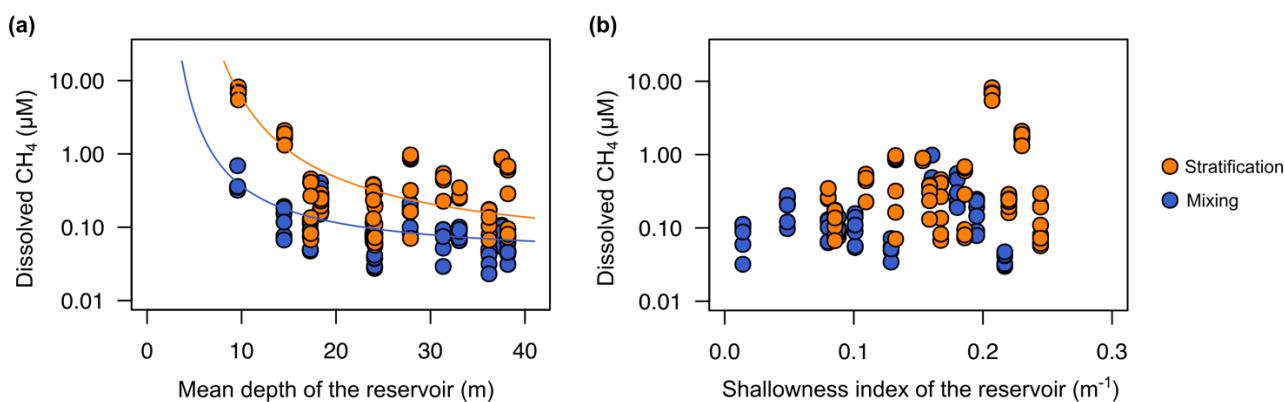



Figure 3: Vertical profiles of physicochemical and biological variables in Jándula reservoir. Dissolved methane concentration (CH$_4$, μM), temperature (°C), dissolved oxygen concentration (DO, μM), chlorophyll-a concentration (Chl-a, μg L^{-1}), abundance of photosynthetic picoeukaryotes (cell mL^{-1}) and abundance of cyanobacteria (cell mL^{-1}) during the stratification period (**a**) and the mixing period (**b**). The grey area represents the anoxic zone (DO < 7.5 μM).

760 **Figure 4:** Power relationship between the depth-cumulative chlorophyll-a concentration and the concentration of dissolved CH$_4$ in the anoxic waters during the stratification period. Note that both axes are in logarithmic scale.

765 **Figure 5: Reservoir morphometry and the dissolved CH$_4$ concentration in the oxic zone.** (a) Exponential decay relationships of the dissolved CH$_4$ concentration and the mean depth (m) during the stratification period and the mixing period. (b) Scatterplot of dissolved CH$_4$ concentration and the reservoir shallowness index.

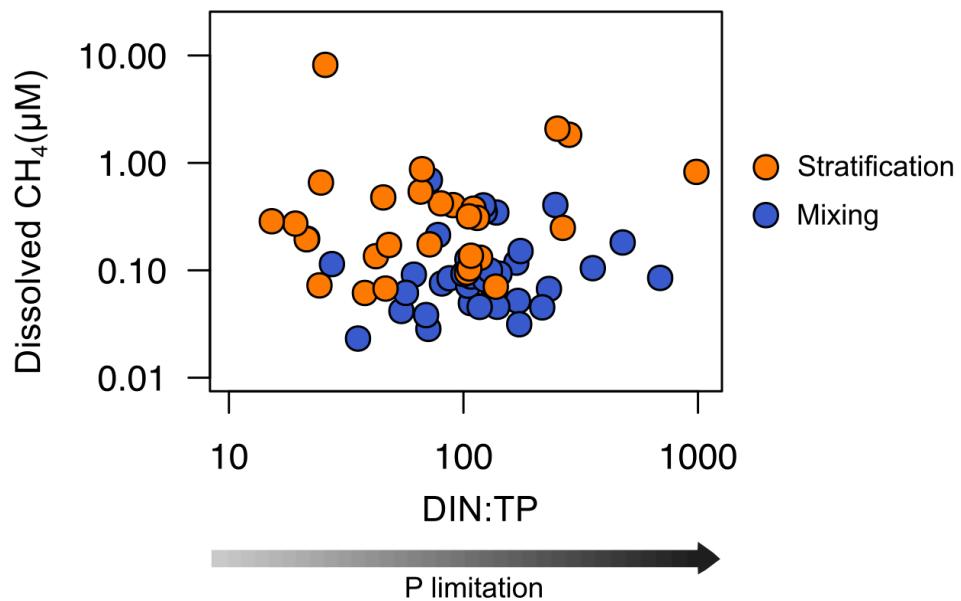



Figure 6: Phosphorus limitation and the dissolved CH₄ concentration in the oxic waters. Scatterplot of dissolved CH$_4$ concentration
770 and the ration between dissolved inorganic nitrogen (DIN) and the total phosphorus (TP) (μmol N : μmol P). Note the logarithmic scale in
both axes.

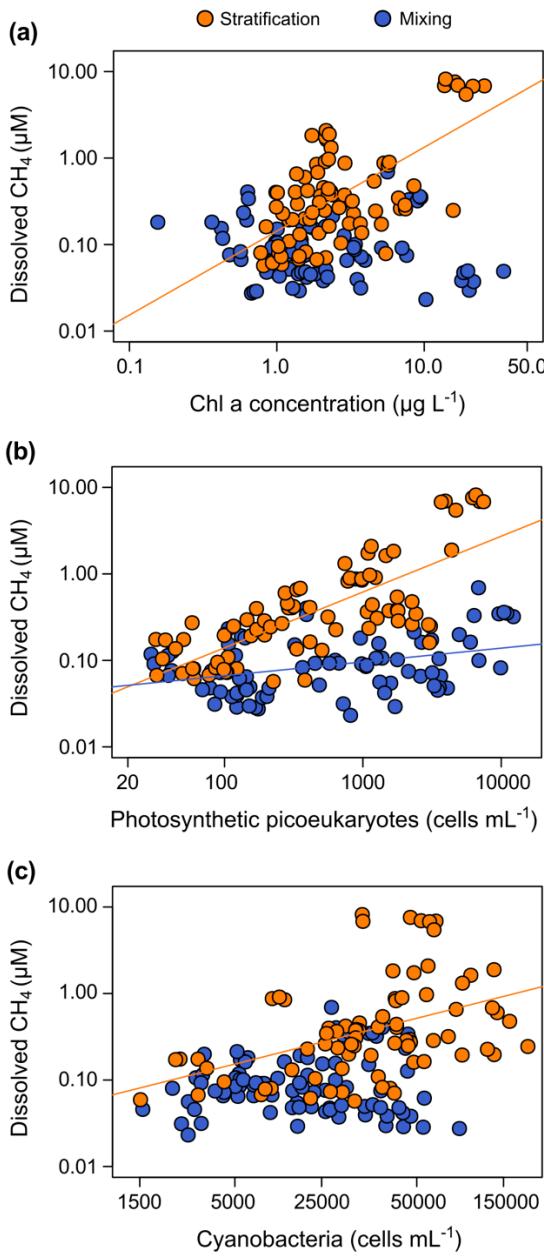



Figure 7: Phytoplanktonic variable coupled with the dissolved CH$_4$ concentration in the oxic waters. (a) The relationship between dissolved CH$_4$ concentration and the chlorophyll-a concentration. A power function (CH$_4$ = 0.14 Chl-a$^{0.97}$; n = 78, adj R^2=0.40, p-value <0.001) explained the dissolved CH$_4$ concentration during the stratification period. **(b)** The relationships between dissolved CH$_4$ concentration and the abundance of photosynthetic picoeukaryotes (PPEs) during the stratification period (CH$_4$ = 7.2·10^{-3} PPEs $^{0.65}$; n = 78, adj R^2=0.55, p-value <0.001) and the mixing period (CH$_4$ = 3.2·10^{-2} PPEs $^{0.16}$; n = 82, adj R^2=0.12, p-value <0.001). **(c)** The relationship between dissolved CH$_4$ concentration and the cyanobacteria abundance (CYA, cell mL^{-1}). A power function explained the dissolved CH$_4$ during the stratification period (CH$_4$ = 1.7·10^{-3} CYA$^{0.53}$; n = 78, adj R^2=0.17, p-value <0.001).

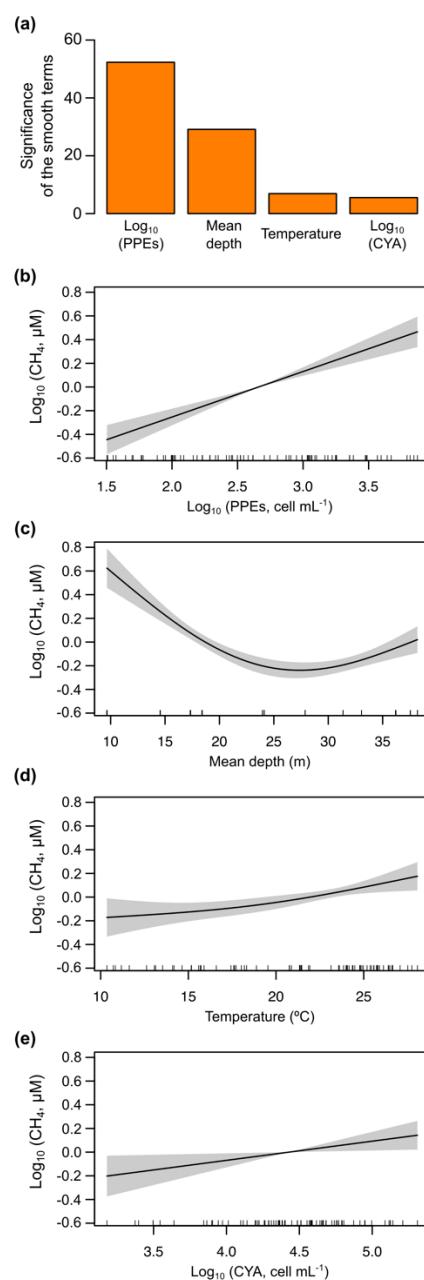



Figure 8. **Results of the Generalized Additive Model (GAM) fitted for the concentration of dissolved CH$_4$ in the oxic waters during the stratification period. (a)** Bar plot showing the significance of the smooth terms from the fitted GAM model. **(b-e)** Partial response plots from the fitted GAM model showing the additive effects of the covariates on the dissolved CH$_4$ concentration: the photosynthetic picoeukaryotes abundance (log$_{10}$ PPEs) **(b)**, the mean depth **(c)**, the cyanobacteria abundance (log$_{10}$ CYA) **(d)**, and water temperature **(e)**. In partial response plots, the lines are the smoothing functions and the shaded areas represent 95% point-wise confidence intervals. Rugs on x-axis indicate the distribution of the data. More details are provided in Table S1.

785

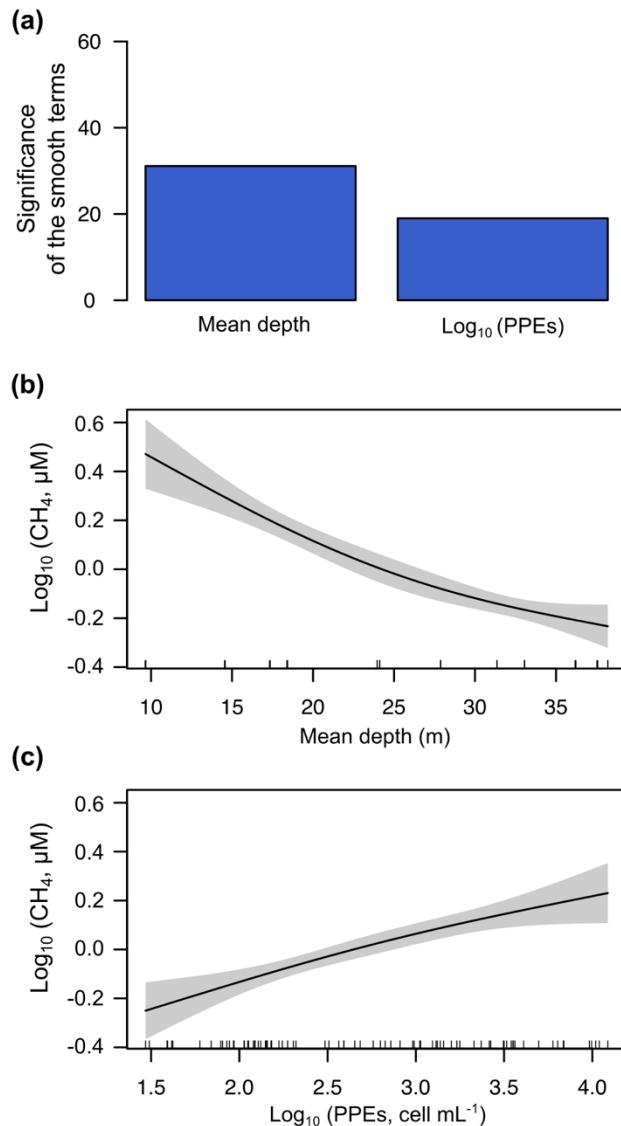



Figure 9. **Results of the Generalized Additive Model (GAM) fitted for the concentrations of CH₄ in the oxic waters during the mixing period. (a)** Bar plot showing the significance of the smooth terms from the fitted GAM model. **(b** and **c)** Partial response plots from the fitted GAM model showing the additive effects of the covariates on the dissolved CH₄ concentration: the mean depth **(b)** and the abundance of photosynthetic picoeukaryotes (log₁₀ PPEs) **(c)**. In partial response plots, the lines are the smoothing functions and the shaded areas represent 95% point-wise confidence intervals. Rugs on x-axis indicate the distribution of the data. More details are provided in Supplementary Table 1.

Tables

Table 1. Equations for the relationships between the phytoplankton and the dissolved CH_4 concentration in the oxic waters.

	Stratification + Mixing	Stratification period	Mixing period
Chl-a concentration ($\mu g\ L^{-1}$)	$CH_4\ (\mu M) =$ $0.12\ Chl\text{-}a^{0.44}$ p-value < 0.001 Adj R^2: 0.11	$CH_4\ (\mu M) =$ $0.14\ Chl\text{-}a^{0.97}$ p-value < 0.001 Adj R^2: 0.40	Not significant p-value = 0.469
Gross primary production (GPP, $g\ O_2\ m^{-3}\ d^{-1}$)		Not significant p-value = 0.077	Not measured
Photosynthetic picoeukaryotes abundance (PPEs, cell mL^{-1})	$CH_4\ (\mu M) =$ $2.0 \cdot 10^{-2}\ PPEs^{0.35}$ p-value < 0.001 Adj R^2: 0.19	$CH_4\ (\mu M) =$ $7.2 \cdot 10^{-3}\ PPEs^{0.65}$ p-value < 0.001 Adj R^2: 0.57	$CH_4\ (\mu M) =$ $3.2 \cdot 10^{-2}\ PPEs^{0.16}$ p-value < 0.001 Adj R^2: 0.12
Cyanobacteria abundance (Cyanobacteria, cell mL^{-1})	$CH_4\ \mu M =$ $9.9 \cdot 10^{-4}\ CYA^{0.53}$ p-value < 0.001 Adj R^2: 0.19	$CH_4\ \mu M =$ $1.7 \cdot 10^{-3}\ CYA^{0.53}$ p-value < 0.001 Adj R^2: 0.17	Not significant p-value = 0.666

800