Reduced methane oxidizing activity by sediment methanotrophs in shallow coastal zones with high methane emissions

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Abstract

Background Coastal zones are transitional areas between land and sea where large amounts of organic and inorganic carbon compounds are recycled by microbes. Especially shallow zones near land have been shown to be the main source for oceanic methane (CH4) emissions. Water depth has been predicted as the best explanatory variable, which is related to CH4 ebullition, but exactly how sediment methanotrophic bacteria mediates these emissions along water depth is unknown. Here, we investigated the activity of methanotrophs in the sediment of shallow coastal zones with high CH4 emissions within a depth gradient from 10−45 m. Field sampling consisted of collecting sediment slices from eight stations along a coastal gradient (0−4 km from land) in the coastal Baltic Sea. We combined real-time measurements of surface water CH4 concentrations, acoustic detection of CH4 seeps in the bottom water, and sediment DNA plus RNA sequencing.

Results The relative abundance and CH4 oxidizing activity (pMMO; particulate methane monooxygenase) of the dominant methanotroph Methylococcales was significantly higher in deeper costal offshore areas (36−45 m water depth) compared to adjacent shallow zones (10−28 m). This was in accordance with the shallow zones having CH4 concentrations in the surface water, as well as more CH4 seeps from the sediment. Furthermore, our findings indicate that the low prevalence of Methylococcales and their activity was restrained to the euphotic zone (indicated by PAR data, photosynthesis proteins, and 18S rRNA data of benthic diatoms). This was also indicated by a positive relationship between water depth and the relative abundance of Methylococcales and pMMO.

Conclusions We detected a low relative abundance of methanotrophs and CH4 oxidizing activity in shallow coastal areas, this can partly explain the difference in CH4 emissions between shallow and deep coastal areas (and the relationship between CH4 emission and water depth). Potentially a reduced activity of methanotrophs also facilities the build-up of CH4 bubbles in the sediment. CH4 emissions from the study area has previously been calculated to be comparable to that of subarctic lakes, and it is suggested that shallow coastal waters, similarly to inland waters, are hotspots for CH4 emissions.

Background

Coastal zones are transitional areas between land and sea where microbes in the water and sediment cycle large amounts of organic and inorganic carbon compounds [1]. Such zones have recently been shown to be the main source for oceanic methane (CH_4) emissions [2]. CH_4 is a potent greenhouse gas that has increased ~2.5 times in the atmosphere since the industrial revolution [3], and is today at ~1.85 ppm [4], and contributes to approximately 20% of tropospheric radiative forcing [5]. Furthermore, the annual atmospheric CH_4 concentration measured during 2014−2017 was record high since 1980 [4]. The majority of CH_4 emissions are derived from human activities (~60%) such as livestock [6], rice paddies [7, 8], hydropower dams [9], and waste management [10]. However, natural aquatic systems such as inland waters are reported to contribute a significant portion to CH_4 emissions (30 % or more) [10−12]. In marine ecosystems, coastal zones have the highest contribution to global CH_4 emissions [2, 13], with shallow

inshore waters closer to land being estimated to have an annual CH_4 emission 370 times higher compared to that in the open ocean [12, 14, 15]. Globally, shallow water depths in coastal zones are linked to higher CH_4 emissions [2], but environmental predictors have been unable to explain this relationship [2]. It is therefore possible that biological mechanisms are partly able to explain the discrepancy between coastal shallow and deeper areas. However, this has not been fully investigated and would help to increase the understanding of the controls of CH_4 cycling in coastal areas.

The cycling of CH_4 in natural aquatic ecosystems is driven by microbial consumption and oxidative processes [16]. In brief, the majority of CH_4 is produced in anoxic zones in sediments as a result of the reduction of e.g. CO_2, acetate, or methanol by anaerobic methanogenic archaea [17]. Large parts of the produced CH_4 diffuses upwards in the sediment and is oxidized to CO_2 by anaerobic methanotrophic archaea (ANME) [18], anaerobic methanotrophic bacteria [19], and eventually by aerobic methanotrophic bacteria in the oxic sediment surface or the water column [20]. These aerobic methanotrophic bacteria thrive on produced CH_4, and have traditionally been divided into two types: Type I belonging to the Gammaproteobacteria family Methylococcales; and Type II belonging to the Alphaproteobacteria family Methylocystaceae and Beijerinckiaceae [21]. Both types use the enzyme methane monooxygenase (MMO) to oxidize CH_4, and are able to utilize either the particulate form (pMMO, i.e. bound to the intracellular membrane) and/or the soluble form (sMMO, i.e. enzyme complex in the cytoplasma) [21]. The importance of methanotrophic bacteria to limit CH_4 emission has previously been shown, e.g. Bornemann M, Bussmann I, Tichy L, Deutzmann J, Schink B and Pester M [22] used pMMO primers (subunit A, *pmoA*) and clone-libraries to identify methanotrophs (taxonomic order Methylococcales) in the pelagic area of Lake Constance, and found that these bacteria contributed substantially to CH_4 removal in the bottom water directly above the sediment surface. Bacterial members belonging to e.g. the class Methylococcales are ubiquitous [23], and metagenome plus metatranscriptome analysis have shown that they dominate aerobic CH_4 oxidation in wetland soil [23], and are important in removing CH_4 escaping from benthic CH_4 seeps [24]. Methanotrophs are therefore essential key players in regulating CH_4 emission to the atmosphere from aquatic environments. Although methanotrophs play a key role in CH_4 cycling and emission to the atmosphere, it is still not fully understood what environmental factors control these populations in aquatic sediments.

Major environmental factors controlling methanogenesis in the coastal zone include temperature and labile organic matter load that influence bacterial activity and oxygen availability in the sediment [25, 26]. CH_4 produced by methanogens are oxidized in the sediment or water column by methanotrophs. Main factors shown to control methanotrophy include CH_4 availability and oxygen availability [27], and differences in adaptation among methanotrophic bacteria have been shown as a response to varying pH, salinity, and oxygen concentration [28]. Laboratory studies have also shown that methanotrophs and their activity are stimulated when other heterotrophic bacteria are present [29, 30]. Ammonium (NH_4^+) and CH_4 can be oxidized by both ammonia oxidizing and methanotrophic bacteria, although methanotrophs oxidize CH_4 more efficiently and vice versa [31]. High concentrations of NH_4^+ have, thus, been reported to

have an inhibitory effect on methanotrophic activity [32, 33]. Additionally, controlled experimental studies have investigated the role of light availability in mediating methanotrophic activity, but showed contrasting results with both inhibition [34, 35] and stimulation being reported [36]. Despite this, there is a knowledge gap on the underlying reasons as to why higher CH_4 emission to the surface waters occur in shallow coastal areas. It has been suggested that shallow areas have well-mixed waters where CH_4 can reach the surface waters easily, and bubbles from CH_4 seeps in the seafloor can quickly escape to the atmosphere [12]. However, what role CH_4 oxidation has in regulating such emissions in these shallow coastal areas and what environmental factors determine CH4 oxidizer activity is unknown. Such knowledge is critical to our understanding of the contribution of coastal ecosystems to global CH_4 budgets.

The aim of the study was to investigate and elucidate why CH_4 emission is higher in shallow inshore coastal zones compared to adjacent deeper offshore areas. We tested the following hypotheses: a) the relative abundance of methanotrophs is higher in shallow inshore sediments where previous studies have found high concentrations of CH_4 in the water column; b) relative abundance and activity of methanotrophs are positively related to concentrations of surface water CH_4 and number of CH_4 seabed seeps; and c) bottom water oxygen and pore water NH_4^+ concentrations regulate methanotrophic activity in the sampled sediments.

Results

In this study, a multidisciplinary team of scientists aboard the research vessel (R/V) Electra participated on a cruise conducted in the Western Gulf of Finland during June 2017 and September 2018. Field sampling consisted of collecting sediment slices (top 0–2 cm) from eight stations along a coastal gradient (0–4 km from land, 10–45 m water depth) in the Storfjärden bay, Tvärminne, Finland (n = 3 per station; Fig. 1 and Table 1). The stations were divided into four offshore sites (stations 5, 7, 10, 13; 36–45 m deep) and four inshore sites (stations 11, 12, 15, 16; 10–28 m deep). Sediment was extracted for metagenomic DNA and total RNA sequencing to identify CH_4-related microbial populations and active metabolism. During the 2018 sampling campaign this data was coupled to i) real-time measurements of CH_4 in the 0.5–1.0 m water surface (first presented in Humborg C, Geibel MC, Sun X, McCrackin M, Mörth C-M, Stranne C, Jakobsson M, Gustafsson B, Sokolov A, Norkko A and Norkko J [37]), and ii) acoustic data of the seafloor and bottom water to identify CH_4 seeps. Furthermore, CTD casts in the study area were used to collect water column profiles of light (PAR) and oxygen concentrations.

Water column parameters. During the sampling campaigns salinity ranged 6.5–7.0 ppt, with higher salinity in the bottom water. In the surface water salinity did not differ between the inshore and offshore stations during sampling and neither could salinity explain the increase in water surface CH_4 concentrations across the study area (see published geochemistry data in Humborg C, Geibel MC, Sun X, McCrackin M, Mörth C-M, Stranne C, Jakobsson M, Gustafsson B, Sokolov A, Norkko A and Norkko J [37]). Temperature ranged 3.4–8.9°C (2017 early June) and 6.02–15.82°C (2018 late September), with

higher temperatures in the surface water. CTD profiles of the water column from twelve locations inside the study area showed that photosynthetically active radiation (PAR) light reached a water depth of 28 m at sites < 30 m water depth, and the inshore stations would therefore have been illuminated (Additional File 1: Fig. S1). The bottom water was oxic with oxygen concentrations between 7.6–8.6 ml/l in the study area (Additional File 1: Fig. S1).

CH_4 concentrations in the surface water. CH_4 concentrations in the surface water were higher in the inshore shallow stations close to land (23.4–40.6 nM, n = 4 stations) compared to the offshore areas (16.2–23.4 nM; n = 3 stations, Fig. 1 and Table 2).

Alpha and beta diversity. In the 0–2 cm sediment surface prokaryotic alpha diversity ranged between 7.0–7.9 (Shannon's H, 16S rRNA 2018 data) and only station 13 was slightly lower compared to stations 7 and 11 (7.2 ± 0.3 compared 7.5 ± 0.3 and 7.5 ± 0.1, respectively; One-Way ANOVA, $F_{6,14}$ = 2.75, Post hoc Tukey tests, P = 0.049; one standard deviation shown; Additional File 1: Fig. S2). NMDs of Bray-Curtis beta diversity showed that the offshore stations 7, 10, and 13 clustered differently compared to inshore stations 11, 12, and 16, with station 15 being a "boundary" station located between these two clusters (PERMANOVA 9999 permutations, F = 8.2, P < 0.001 for the whole model; Additional File 1: Fig. S2). A full list of the prokaryotic classifications and sequence counts is available in Additional File 2: Data S1.

Methanotrophic bacteria in inshore and offshore sediments. Gammaproteobacteria had the highest relative abundance of the prokaryotic community in the 0–2 cm sediment surface when comparing phyla and Proteobacteria classes between stations (Fig. 2A). In the RNA-seq 16 rRNA data the relative abundance of Gammaproteobacteria ranged between 8–29 % (Fig. 2A), while in the metagenome 16S rRNA gene the relative abundance was between 14 and 24 % for all stations (Fig. 2A). The relative abundance of the Type I CH_4 oxidizing bacteria Methylococcales (dominated by the family Methylomonaceae, with majority of sequences (up to 64%) aligning to *Methyloprofundus;* Additional File 2: Data S1) was significantly higher in stations located offshore when compared to the more shallow inshore stations (DNA and RNA data, Kruskal-Wallis tests, df = 1, H = 17.3, P = 0.000032 (2017 DNA data), df = 1, H = 14.78, P = 0.000120 (2018 DNA), df = 1, H = 14,73, P = 0.000124 (2018 RNA data); Fig. 2 B). In the DNA and RNA data *Methylococcales* had a relative abundance up to 1.7% and 11.6% of the whole microbial community, respectively (Additional File 2: Data S1), indicating that methanotrophic bacteria had a key role in metabolic processes in the sediment. CH_4 concentrations measured in the 0.5–1.0 m water surface showed a negative relationship with the relative abundance of Methylococcales in the sediment, with lower CH_4 concentrations in the offshore stations (Fig. 3A) where Methylococcales activity was higher. Furthermore, CH_4 concentration in the water column correlated negatively with the relative abundance of Methylococcales for both the 2018 DNA (rho = −0.769, P = 0.000046, n = 21); and 2018 RNA data (rho = −0.766, P = 0.000052, n = 21). That Methylococcales was associated with offshore sites further away from the coast was also indicated by positive correlations with water depth (DNA data, rho = 0.800, P = 0.000013; RNA data, rho = 0.907, P = 0.000000015). In addition to a higher relative abundance of Methylococcales, RNA transcripts attributed to the protein family AMO/pMMO also correlated

negatively with measured concentrations of CH_4 (RNA data, rho = −0.760, P = 0.000064, n = 21; See Additional File 2: Data S2 for all protein calssifications; Fig. 3). RNA transcripts attributed to AMO/pMMO were also significantly higher in the offshore stations (FDR < 0.05, test between all stations individually; Fig. 3B and Table 1), while functional genes in the metagenome attributed to AMO/pMMO were available at all stations with little difference in CPM values (counts per million sequences) (1429−1652 CPM; Table 2), showing that the potential to oxidize CH_4 was available at all sites. The soluble form of MMO was not detected in the DNA or RNA dataset (Additional File 2: Data S2 and Data S3). AMO/pMMO sequences were classified against the UniProtKB database to separate AMO and pMMO sequences. The results showed a large difference in pMMO CPM values between the offshore stations (8742 ± 2342 CPM) compared to the more shallow inshore stations (58 ± 175 CPM, Kruskal-Wallis test, df = 1, H = 14.7, P = 0.000124; Fig. 4). These pMMO sequences were affiliated with the reference species *Methylococcus capsulatus* in the UniProtKB database.

Because light has been indicated to inhibit CH_4 oxidation we also analysed the amount of RNA transcripts attributed to proteins in the Gene Ontology (GO) category Photosynthesis (Table 2 and Additional File 2: Data S2). Photosynthesis proteins in the sediment surface had a negative correlation with both the relative abundance of Methylococcales (DNA data, rho = −0.655, P = 0.001; RNA data, rho = 0.787, P = 0.00002, both n = 21), and AMO/pMMO enzymes (rho = −0.760, P = 0.00006, n = 21). Photosynthesis proteins were also negatively correlated with water depth (rho = 0.676, P = 0.0008, n = 21; Additional File 1: Fig. S3). Moreover, 18S rRNA data of diatoms with a higher relative abundance of benthic genera such as *Amphora* and *Nitzschia* in the inshore stations provides further indication that these stations were euphotic (Additional File 1: Fig. S4). This in accordance with the PAR data that indicated the inshore areas to be illuminated while offshore bottom zones were in darkness.

Our results clearly show that Methylococcales were the major methanotroph active in our sediments, while other methanotrophic bacteria were absent or present in low relative abundances in the dataset. The Type II methanotrophic family Methylocystaceae (belonging to Alphaproteobacteria) was not present in the dataset, while the Type II family Beijerinckiaceae had less than 0.06 % relative abundance in each sample (Additional File 2: Data S1). Similarly, the Verrucomicrobia family Methylacidiphilaceae had less than 0.06% relative abundance in each sample (Additional File 2: Data S1). Finally, the NC10 phylum known to contain anaerobic methanotrophic bacteria had less than 0.02% relative abundance in each sample (Additional File 2: Data S1).

Pore water ammonium concentrations. NH_4^+ analyses showed that the pore water concentration of NH_4^+ was higher in the offshore stations (308 ± 59 μM) compared to the inshore stations (196 ± 49 μM; One-Way ANOVA, $F_{6,14}$ = 33.1, P = 0.00000017, with Tukey post hoc test between stations, P < 0.01; Table 2). However, in the DNA and RNA dataset ammonia oxidizing bacteria/archaea and anaerobic ammonium oxidation (anammox) Planctomycetes together contributed to less than 2.5% of the whole microbial community (Additional File 1: Fig. S5). Furthermore, AMO sequences showed no differences in CPM values between the offshore and inshore stations (196 ± 90 CPM, Kruskal-Wallis test, H = 0.4, P = 0.83;

Fig. 4), suggesting that pore water NH_4^+ concentrations did not explain the difference in methanotrophic activity between inshore and offshore areas.

Methanotrophic and methanogenic archaea in the sediment. Archaea had a low relative abundance in the 0−2 cm sediment (1−5 %; Fig. 2), and methanotrophic archaea (ANME) had a less than 0.06 % relative abundance (Additional File 2: Data S1). Methanogenic archaea represented < 15 % of all archaea and did not show a difference in relative abundance among inshore and offshore stations (based on RNA-seq extracted 16S rRNA data, One-Way ANOVA post hoc Tukey tests for each station; Additional File 1: Fig. S6). However, the 16S rRNA relative abundance of methanogenic archaea was indicated to be associated with sites close to the coast by correlating positively with the measured CH_4 concentrations in the water surface (2018 RNA data, Spearman's rank correlations, rho = 0.672, *P* = 0.0008, *n* = 21), and negatively with water depth (rho = −0.707, *P* = 0.0003). RNA transcripts and genes attributed to proteins affiliated with the GO category methanogenesis were found at all stations with low CPM values (< 800 CPM; Table 1). This finding suggests that higher CH_4 concentrations in the surface water in the study area was unrelated to methanogenic activity in the 0−2 cm sediment surface (e.g. if surface sediment had been oxygen deficient). A full list of proteins, including all methanogenesis and photosynthesis proteins, can be found in Additional File 2: Data S2 (RNA) and Additional File 2: Data S3 (DNA).

Methane escape from the sediment. Acoustic data of the seafloor and bottom water was collected in the study area during 2018, and CH_4 seeps were defined as either trains of bubbles or bubble plumes (Fig. 5). The results showed that the prevalence of CH_4 seeps in sediment surface was greater in shallow areas compared to deeper areas (Fig. 6A), further suggesting that CH_4 availability did not explain the lower relative abundance of methanotrophs in the inshore stations. Moreover, the amount of CH_4 seeps km^{-1} was negatively correlated with water depth (Pearson correlation, r = −0.83, *P* < 0.000001, *n* = 52; Fig. 6B).

Discussion

Shallow coastal zones are known to have high CH_4 concentrations in the water column compared to deeper waters [12, 14, 15]. This was also indicated by our acoustic data of CH_4 seeps and the real-time measurements of CH_4 in the surface water [37]. Here we build on these results and hypothesized that: 1) methanotrophic activity (i.e. CH_4 cycling) is higher in the shallow inshore stations, and 2) the relative proportion of methanotrophs are positively related to the CH_4 concentrations and number of CH_4 seabed seeps. However, our results presented here instead suggest that these higher CH_4 concentrations in shallow coastal zones are partly explained by low methanotrophic activity. This was indicated by the significantly lower relative abundance of 16S rRNA sequences (DNA and RNA data) classified as methanotrophic bacteria, and lower RNA transcripts attributed to CH_4 oxidation (pMMO) in the inshore areas. The metagenome data also showed the pMMO gene to be present at all stations (inshore and offshore), further indicating a decreased activity on CH_4 oxidation in the inshore areas. A majority of the Methylococcales sequences were classified to the genus *Methyloprofundus,* an obligate aerobic

methanotroph only able to utilize the pMMO pathway [38], which was in accordance to our RNA transcript data. Compared to a previous study measuring CH_4 from a 1 km to 59 km land-to-sea gradient (Osudar R, Matoušů A, Alawi M, Wagner D and Bussmann I [15]) here we also sampled sediment and used fine-scale gradients from the coast (0−4 km). Our results therefore further imply that shallow coastal zones are hotspots for CH_4 emission due to low methanotrophic activity. This is important because microbial aerobic CH_4 oxidation has been estimated to account for 50−90% removal of CH_4 before it escapes the sediment surface [27]. Additionally our findings suggest that a reduced activity of methanotrophs facilitate build-up of CH_4 bubbles in the sediment, which helps to further drive high CH_4 emissions from shallow coastal zones.

In the study area, water depth strongly correlated with the increase in relative abundance and activity of methanotrophs, with lower amounts of seabed CH_4 seeps and CH_4 concentrations in the surface water. Abiotic factors that have been shown to affect methanotrophs and their activity along water depth are e.g. salinity [39], oxygen and CH_4 availability [27], ammonium concentrations [32, 35],and light [35, 36]. At the time of sampling there was just minor changes in salinity in the studied stations (range 6.5−7.0), compared to previous studies having a factor from freshwater-to-marine salinity[12, 14, 15]. Furthermore, a separate study conducted at the same time in the study area found no significant link between CH_4 concentrations in the water and salinity (full details in Humborg C, Geibel MC, Sun X, McCrackin M, Mörth C-M, Stranne C, Jakobsson M, Gustafsson B, Sokolov A, Norkko A and Norkko J [37]) indicating that salinity does not explain the patterns of relative abundance and activity of methanotrophs in our study. The bottom water was oxygenated at all studied stations and was therefore unlikely to be a limiting factor for methanotrophs in the study area. In addition, the real-time measurements and acoustic data of CH_4 also showed more CH_4 availability in the inshore areas (compared to offshore), and it is therefore unlikely CH_4 was a limiting factor inhibiting growth and activity of methanotrophs. The 0−2 cm sediment surface was sliced and the data showed a very low relative abundance of methanogens and their activity at all studied stations. Considering that methanogenesis occurs in anoxic sediment [17] these findings indicate that the sediment surface was oxygenated by the bottom water in the study area.

We measured higher pore water NH_4^+ concentrations in the offshore stations, and most of the measured NH_4^+ likely derived from organic matter mineralization in the suboxic and anoxic layers [40] in and below the 0−2 cm sediment slices. It seems unlikely that methanotrophs might have actively oxidized NH_4^+. For example, the amount of RNA transcripts attributed to pMMO was on average 47 times higher than AMO in the offshore areas. Furthermore, the real-time CH_4 measurements and acoustic data showed lower CH_4 concentrations in the surface water and less seepages from the offshore sites. If methanotrophs were thriving on NH_4^+ more RNA transcripts for AMO than pMMO would be expected considering ammonia oxidizing bacteria oxidize NH_4^+ more effectively [31]. Considering that ammonia oxidizing bacteria/archaea [41] and anammox Planctomycetes [42] together contributed less than 2.5% of the whole microbial community in our dataset (compared to 11.6% for *Methylococcales* in the RNA data), it is more likely that the availability of CH_4 was driving growth of methanotrophs in the offshore stations.

Moreover, in a laboratory experiment NH_4^+ concentrations below 36 mM have been observed to not influence methanotrophic activity[33] (our highest measurement was 0.4 mM NH_4^+). In addition, the NH_4^+ data does not explain why methanotrophs had very low relative abundance in the inshore sediments where NH_4^+ was also available. We hypothesized that NH_4^+ would influence methanotrophy in the studied system along the coastal gradient. Our results instead show that pore water NH_4^+ did not drive inhibition of methanotrophs or methanotrophic activity in the studied system.

Considering that the geochemistry data (CH_4 water concentrations and CH_4 seabed seeps) and biology data (relative abundance of methanotrophic bacteria and RNA transcripts attributed to pMMO) both showed a positive relationship with water depth and that the main environmental factor changing along this gradient was light intensity, we suggest that illumination might influence sediment microbial communities. That the inshore stations were euphotic was indicated by 1) the CTD profiles showed that PAR light reached 28 m in the study area; 2) photosynthesis mRNA data (Fig. 7) that showed a decrease with water depth; and 3) benthic diatoms such as *Amphora* and *Nitzschia* [43] in the inshore stations based on 18S rRNA data (Additional File 1: Fig. S4). Previous studies conducted in a reservoir and pelagic lake water have shown light to inhibit methanotropy and increase CH_4 water concentrations in northern South America and central Japan [34, 35]. These studies included using bacterial cultures that would remove influencing factors on methanotrophy such as photosynthesis (i.e. producing oxygen) [27]. Moreover, Garcia SL, Szekely AJ, Bergvall C, Schattenhofer M and Peura S [44] investigated the microbial community in a boreal lake with and without snow on the ice cover, and found that the relative abundance of methanotrophic bacteria decreased and CH_4 water concentrations increased when the snow cover was removed and illumination increased in the water column. Additionally, the activity of NH_4^+ oxidizing bacteria are known to be inhibited by light availability [45], and this can further explain why light might have an effect on methanotrophs as the enzymes ammonia monooxygenase (AMO) and MMO are highly similar and evolutionary related [46]. However, light has also been observed to stimulate methanotrophic activity in wetland sediments (Florida, USA) [47], and polar lake water (north-west Russia) while investigating bacterial cultures [36]. These contrasting results in the literature could indicate that illumination affects various methanotrophic species differently or indirectly through other ecosystem processes. Further work is needed to investigate if light has a negative effect on methane oxidation in shallow coastal ecosystems.

Conclusions

On a global scale CH_4 emissions from coastal zones are higher at shallower water depths [2], and we also detected this relationship between water depth and CH_4 seeps and CH_4 concentrations in the studied costal ecosystem. We detected a lower relative abundance of methanotrophic bacteria and CH_4 oxidizing activity in the sediment of shallow coastal areas when compared to adjacent deeper offshore areas. This could partly explain the difference in CH_4 emissions in coastal zones, and reduced activity of methanotrophs could also facilitate build-up of CH_4 bubbles in the sediment. Here we further increase

knowledge with *in situ* molecular and geochemical data collected in a marine coastal system. We also suggest that light might have a negative effect, directly or indirectly, on the relative abundance and pMMO activity benthic methanotrophy in shallow coastal zones. This is significant because natural aquatic environments are estimated to contribute to at least 30% of the global CH_4 emissions [10]. For example, CH_4 emissions from inland waters are known to significantly contribute to the atmospheric budget (estimated to 77 Tg C yr^{-1}) [48]. Humborg C, Geibel MC, Sun X, McCrackin M, Mörth C-M, Stranne C, Jakobsson M, Gustafsson B, Sokolov A, Norkko A and Norkko J [37] calculated a daily sediment flux-water column CH_4 reservoir of 2.5 mmol m^{-2} (or 30 mg C m^{-2}) in the coastal waters of Tvärminne during September 2018 (same sampling campaign as reported in this study). This is within the range of CH_4 emissions reported from subarctic lakes [49], and it is suggested that shallow coastal waters, similarly to inland waters, are hotspots for CH_4 emission. Moreover, limited methanotrophic activity could also explain why shallow coastal waters in rapidly changing ecosystems like the East Siberian artic shelf have higher CH_4 emissions compared to the deeper offshore water [50, 51]. Significant CH_4 emissions from the artic subsea might therefore only occur in the shallowest parts due to a limited activity of methanotrophs. Our results imply that methanotrophs, rather than solely methanogens, play a key role in shallow coastal zones regulating CH_4 emissions. Globally, low methanotrophic activity in the sediment could partly explain the substantial amount of CH_4 emissions from shallow inland water bodies and reservoirs [52]. Furthermore, the results suggest that low CH_4 oxidizing activity by methanotrophs might explain why shallow parts of the costal rim has higher CH_4 emissions. This is an overlooked mechanism that can potentially contribute to explain the dynamics of greenhouse emissions from marine ecosystems.

Methods

Sediment collection and water column profiles. Sediment slices (top 0−2 cm) were collected along coastal gradients (0−4 km, 10−45 m water depth) on board R/V Electra in Storfjärden bay close to the Tvärminne Zoological Station (TZS), Tvärminne, Finland (Fig. 1 and Table 1). Samples were collected during 2017 June 4−5 and 2018 September 20−23. All samples were collected using a GEMAX twin gravity corer in combination with acrylic tubes (height: 80 cm, inner diameter: 80 mm). From each core the top 0−2 cm sediment surface layer was sliced into either plastic bags (freezer bags, 2017 sampling) or autoclaved 215 ml polypropylene containers (Noax Lab; 2018 sampling). During June 2017 sediment was collected for DNA extraction from eight stations (due to logistical reasons RNA was not collected), while during September 2018 sediment was collected from seven stations for both DNA and RNA extraction (*n* = 3 per station for both years). The stations were divided into four offshore sites (stations 5, 7, 10, 13; 36−45 m deep) and four inshore sites (stations 11, 12, 15, 16; 10−28 m deep). For the 2018 sampling sediment slices from each station was aseptically homogenised inside the containers and 2 ml sediment transferred into 2 ml cryogenic tubes (VWR), flash frozen in liquid nitrogen, and stored at −80°C at TZS. All collected sediment for DNA was stored at −20°C on the boat until reaching the coast of Sweden (Stora Uttervik, lat. 58.848372, long. 17.541019). The sediment samples on the boat were then transferred into a cooling box filled with ice bars and transported to Stockholm University (~1 hour). The

flash frozen 2 ml sediment for RNA were transported from TZS to Stockholm University on dry ice, and stored again at −80°C until RNA extraction. The DNA data was used to investigate methanotrophic microorganisms in the sediment, while RNA was used to identify active methanotrophs and the activity of the enzyme methane monooxygenase.

CTD profiles of PAR light and oxygen concentrations (SEA-Bird SBE 911 plus) were collected in the study area from 12 locations during the 2018 sampling campaign between September 19–23. This information was used to infer how light and oxygen availability might have affected methanotrophic bacteria.

Real-time measurement of methane in the surface water. During the 2018 sampling campaign CH_4 concentrations at a depth of 0.5–1.0 m of the water column were measured *in situ* using a Water Equilibration Gas Analyser System (WEGAS), and a full method description and results are presented in Humborg C, Geibel MC, Sun X, McCrackin M, Mörth C-M, Stranne C, Jakobsson M, Gustafsson B, Sokolov A, Norkko A and Norkko J [37]. In brief, circulation pumps equipped to a seawater inlet transfer seawater into an equilibrator with showerhead. The gas is transferred through a gas handling system, and is analysed for CH_4 concentrations by a cavity ring-down spectrometer gas analyser (Picarro G2131-i). This system also tracked temperature and salinity as long as Electra was cruising.

Acoustic data of methane seeps from the sediment. Acoustic data were collected during the 2018 sampling September 19–22. The acoustic data were collected with a Simrad EK80 wide band transceiver, transmitting through a hull mounted Simrad ES70–7C split beam transducer with a center frequency of 70 kHz. Position and attitude information were provided to the echo sounder as an integrated solution by a Seapath 330+ GPS/GLONASS navigation and motion reference system. The dataset was match filtered with an ideal replica signal using a MATLAB software package provided by the system manufacturer (Lars Anderson, personal communication, 2014). Seeps were defined as either trains of bubbles or bubble plumes (many bubbles overlapping in vertical structures) and were identified through visual inspection of the processed acoustic data. The number of seeps per km was derived by applying a running average with a window size of 0.2 km along the cruise track. For calculations of seeps per km as a function of depth, the total ship track (about 65 km) as well as the number of observed seeps (in total 1975 observations) were divided into 1 m seafloor depth bins ranging from 5 to 60 m. The number of seeps in each depth bin was then divided by the track length within each depth bin. Note that the tendency of decreasing number of seeps per km with increasing depth becomes significantly stronger if accounting for the footprint of the echo sounder beam Additional File 1: Fig. S7. This is because the beam footprint increases with depth. While this should provide a more accurate picture in theory, there might be issues with overlapping seeps (multiple seeps being counted as one), and the actual seep distribution might be somewhere in between.

Ammonium analyses. Sediment frozen at −20°C sampled during 2018 was thawed and 20 ml of sediment was transferred into 50 ml centrifuge tubes (Sarstedt). Pore water was extracted by centrifugation at 2200 × g at 9°C followed by filtration of 10 ml supernatant through a 0.45 μm polyethersulfone membrane filter (Filtropur S 0.45, Sarstedt). The pore water was then stored at −20°C

until NH_4^+ analyses. Pore water samples were analyzed colorimetrically (Multiskan GO spectrophotometer, Thermo Scientific) for ammonium concentrations and the modified salicylate-hypochlorite method of Bower CE and Holm-Hansen T [53] was used.

Nucleic acids extraction and sequencing. Ten g and two g of sediment were used to extract DNA and RNA using the DNeasy PowerMax Soil kit (QIAGEN) and RNeasy PowerSoil kit (QIAGEN) kits, respectively. DNase treatment was conducted on extracted RNA by using the TURBO DNA-free kit (Invitrogen). This was followed by ribosomal depletion with the RiboMinus Transcriptome Isolation Kit (ThermoFisher Scientific). Library preparation of DNA and RNA samples were prepared with the ThruPLEX DNA-seq (Rubicon Genomics) and TruSeq RNA Library Prep v2 (Illumina) kits, respectively. The Illumina NovaSeq6000 platform was used to sequence DNA and RNA, with one S2 and S4 lane used for the DNA and RNA samples (a paired-end 2 × 150 bp setup), respectively. All samples were sequenced at the Science for Life Laboratory, Stockholm.

Quality trimming of sequences. SeqPrep 1.2 [54] was used to remove Illumina adapters from the sequences. This was followed by removal of any leftover PhiX sequences by mapping the reads against the PhiX genome (NCBI Reference Sequence: NC_001422.1) using bowtie2 2.3.4.3[55]. Trimmomatic 0.36 [56] was used to quality trim reads with the following parameters: LEADING:20 TRAILING:20 MINLEN:50. Final quality of the trimmed reads were checked with FastQC 0.11.5 [57] and MultiQC 1.7 [58].

Taxonomic annotation. SSU rRNA sequences were extracted from the DNA and RNA sequence data with SortMeRNA 2.1b [59] followed by annotation using Kraken2 2.0.7 [60]. After ribosomal depletion RNA sequencing still yielded on average 89% rRNA sequences per sample (range 86–92%). We therefore decided to also taxonomically classify 16S rRNA sequences. Kraken2 was run with a paired-end setup against the small-subunit SILVA [61] (database downloaded 1 March 2019) and NCBI NT for eukaryotic 18S rRNA sequences (database downloaded 12 March 2019). The Kraken2 reports were combined into a biom-format file using the python package kraken-biom 1.0.1 (with the following setup:—-fmt hdf5 -max D—min S). The python package biom-format 2.1.7 [62] was then used to convert the biom-table to a text table. In the taxonomy table chloroplast sequences were removed, and data was normalized as relative abundance (%) and analyzed in the software Explicet 2.10.5 [63].

Protein classification of functional genes and RNA transcripts. Paired-end DNA and RNA sequences were merged using PEAR 0.9.10 [64], and non-rRNA sequences were used to extract using SortMeRNA/2.1b. Protein annotation was conducted by aligning sequences against the NCBI NR database (e-value threshold < 0.001, database downloaded 2 April 2 2019) using Diamond 0.9.10 [65] in conjunction with BLASTX [66]. MEGAN 6.15.2 [67] was used to analyse the taxonomy and protein classification of the output diamond files using default LCA parameters and software supplied databases (taxonomy: prot_acc2tax-Nov2018X1.abin, and InterPro protein database: acc2interpro-June2018X.abin). To distinguish between AMO and pMMO sequences reads classified against the AMO/pMMO protein family was extracted from MEGAN. The extracted sequences were classified against the UniProtKB database

(2019 July version) using BLASTX 2.7.1+ with an e-value threshold of 0.001. Data was normalized among samples as counts per million sequences (CPM; relative proportion × 1 000 000).

Statistics. Alpha diversity (Shannon's H) was conducted using the 2018 16 rRNA data (for all taxonomic classifications, as shown in Additional File 2: Data S1) in the software Explicet after sub-sampling to the lowest sample size (8 636 018 counts) and bootstrap × 100. Non-metric multidimensional scaling (NMDs) based on the Bray-Curtis dissimilarity index of the relative abundance was analyzed in the software past 3.22 [68]. Shapiro-Wilk tests were used to test for normal distribution of the taxonomy data, and SPSS 26 was used to test for differences among stations using non-parametric Kruskal-Wallis tests or One-Way ANOVAs with post hoc Tukey HSD tests for parametric data. Correlations between variables were conducted with Spearman correlations using data from all stations (two-tailed). Correlation between CH_4 nM and the relative abundance of Methylococcales in the sediment was done by using the same nM value for CH_4 for each replicate sediment core, as CH_4 was measured in the water surface. Acoustic data of CH_4 seeps was correlated with water depth using Pearson correlation in the software MATLAB 2017a. Differences in metabolic functions (InterPro classifications of RNA transcripts data) were tested with the R package edgeR 3.24.3 [69]. In more detail, the perl script "run_DE_analysis.pl" supplied with Trinity 2.8.2 [70] was used to run the analysis. The script inputs raw read data, and normalize read counts, and analyze differential gene expression using edgeR. Statistic significances were indicated by false discovery rate (*FDR*) values < 0.05. See Additional File 2: Data S4 for full details of sequence counts before and after quality trimming, average read lengths, extracted 16S rRNA sequences etc.

Declarations

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of data and materials

The raw sequencing data supporting the conclusions of this article is available in the NCBI BioProject repositories, PRJNA541421 (DNA data) and PRJNA541422 (RNA data).

https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA541421

https://www.ncbi.nlm.nih.gov/bioproject/?term=PRJNA541422

Competing interest

The authors declare that they have no competing interest.

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Author contributions

EB designed the study, sampled in the field, conducted laboratory work, bioinformatics, data analyses, and drafted the manuscript. XS measured CH_4 during field sampling. CS collected acoustic data during field sampling and analysed acoustic data. SB analysed NH_4^+ samples in the laboratory. MG designed and developed the WEGAS system. AN sampled in the field. CH assisted with measurements of CH_4 during field sampling. FJNA designed the study and contributed to drafting the manuscript. All authors gave feedback on the manuscript and gave final approval for publication.

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Tables

Table 1 List of the station numbers ("off" denotes offshore stations (5, 7, 10, 13), and inshore stations are denoted solely by their station number 11, 12, 15, 16), the amount of sediment cores collected and sliced (0−2 cm surface) for DNA/RNA extraction or pore water NH_4^+ analyses, sampling dates during 2017 and 2018, latitude, longitude, and water column depth.

Station	DNA/RNA extraction (n)	NH_4^+ analyses (n)	2017 date	2018 date	Lat. (dd)	Long. (dd)	Depth (m)
off-5	3	-	Jun 4	-	59.8319	23.29566	45
off-7	3	3	Jun 4	Sep 23	59.8430	23.28035	37
off-10	3	3	Jun 4	Sep 20	59.8559	23.26695	36
11	3	3	Jun 4	Sep 20	59.8521	23.25475	18
12	3	3	Jun 4	Sep 22	59.8521	23.24495	10
off-13	3	3	Jun 4	Sep 22	59.8620	23.25615	40
15	3	3	Jun 5	Sep 20	59.8602	23.25155	28
16	3	3	Jun 5	Sep 22	59.8613	23.24387	10

Table 2 CH_4 measured in the 0−0.5 m surface water (nM), pore water NH_4^+ concentrations (μM), RNA transcripts attributed to proteins affiliated with the Photosynthesis GO category, whole microbial community AMO/pMMO proteins, and all proteins affiliated with the Methanogenesis GO category (CPM values). Offshore stations are denoted with the label "off", and dashes in the table denote no data collected.

Station	CH$_4$ 2018	NH$_4^+$ 2018	Photosynthesis 2018 RNA	InterPro AMO/pMMO			Methanogenesis		
				2018 RNA	2018 DNA	2017 DNA	2018 RNA	2018 DNA	2017 DNA
off-5 A	-		-	-	-	1552	-	-	676
off-5 B		-	-	-	-	1536	-	-	656
off-5 C			-	-	-	1539	-	-	659
off-7 A	16.2	336	8402	6763	1455	1494	464	643	644
off-7 B		417	7254	7250	1450	1485	411	650	646
off-7 C		385	8131	9018	1445	1479	363	642	631
off-10 A	15.8	297	5210	12 738	1460	1495	330	619	689
off-10 B		267	5190	11 562	1528	1499	260	674	643
off-10 C		251	3838	11 117	1486	1482	352	653	648
11 A	40.6	193	9187	1842	1519	1477	346	612	642
11 B		199	8137	2156	1523	1429	351	605	607
11 C		147	6128	2241	1500	1454	413	638	619
12 A	29.8	194	16 818	1635	1458	1460	485	625	651
12 B		161	15 792	1804	1457	1441	505	679	668
12 C		170	13 684	2010	1502	1456	363	636	702
off-13 A	23.4	276	5247	12 530	1652	1459	362	648	678
off-13 B		253	5222	12 990	1605	1488	652	667	683
off-13 C		286	6100	10 165	1634	1504	378	649	709
15 A	23.4	281	5244	2709	1510	1533	338	590	572
15 B		283	4376	3285	1497	1503	246	587	661
15 C		254	5699	2577	1478	1548	292	571	701
16 A	23.6	152	21 523	1855	1491	1477	381	574	631
16 B		156	24 214	1606	1494	1500	385	631	619
16 C		167	11 963	1674	1482	1453	435	654	628

Figures
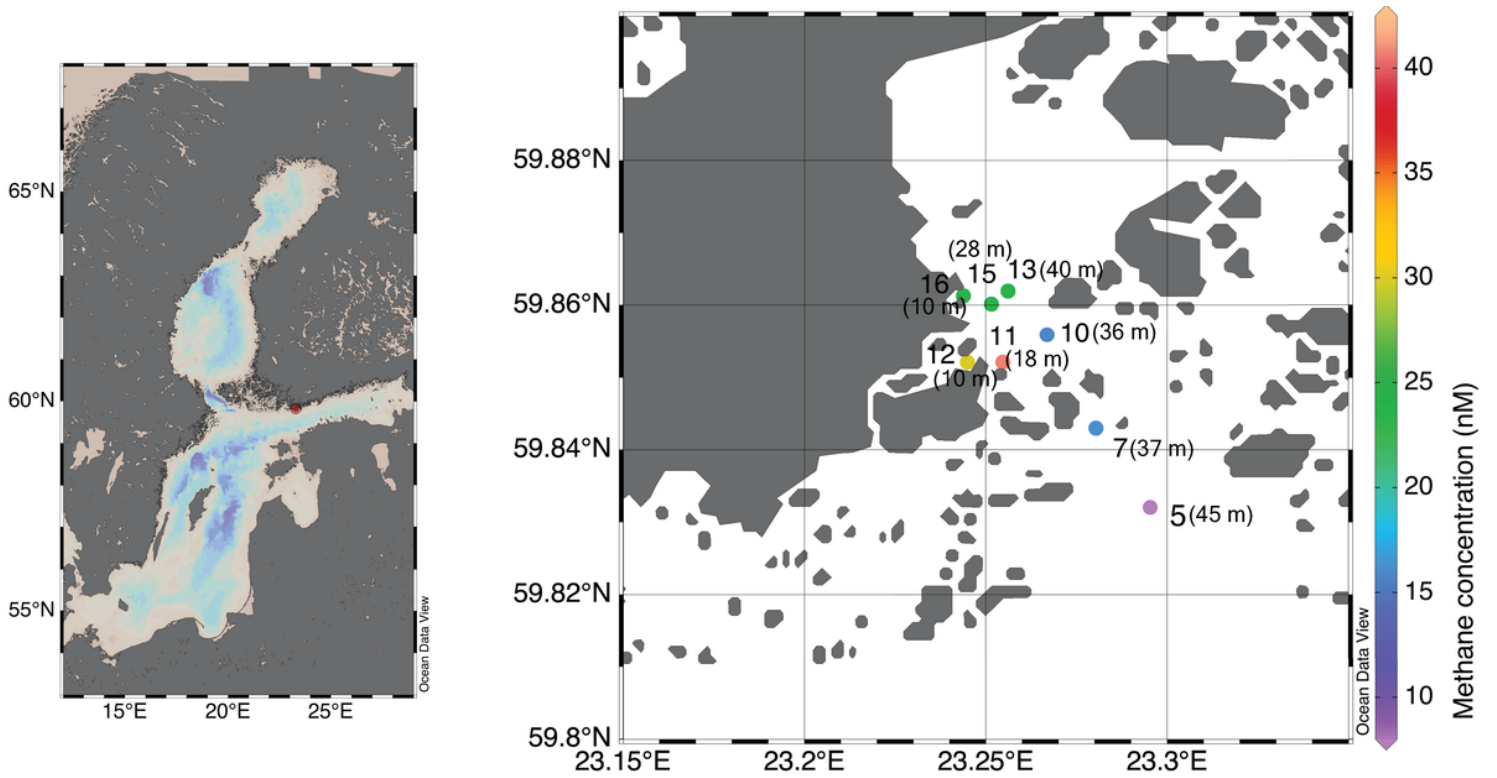
Figure 1

The map shows the Baltic Sea and the location of the study area in the Western Gulf of Finland. Sediment was collected (top 0−2 cm surface) during June 2017 and September 2018 from the eight sampling stations in the Storfjärden bay (n = 3 per station). The colour legend shows the methane concentration in the surface water for each station (0-0.5 m), while the parenthesis between each station number shows the water column depth (m).
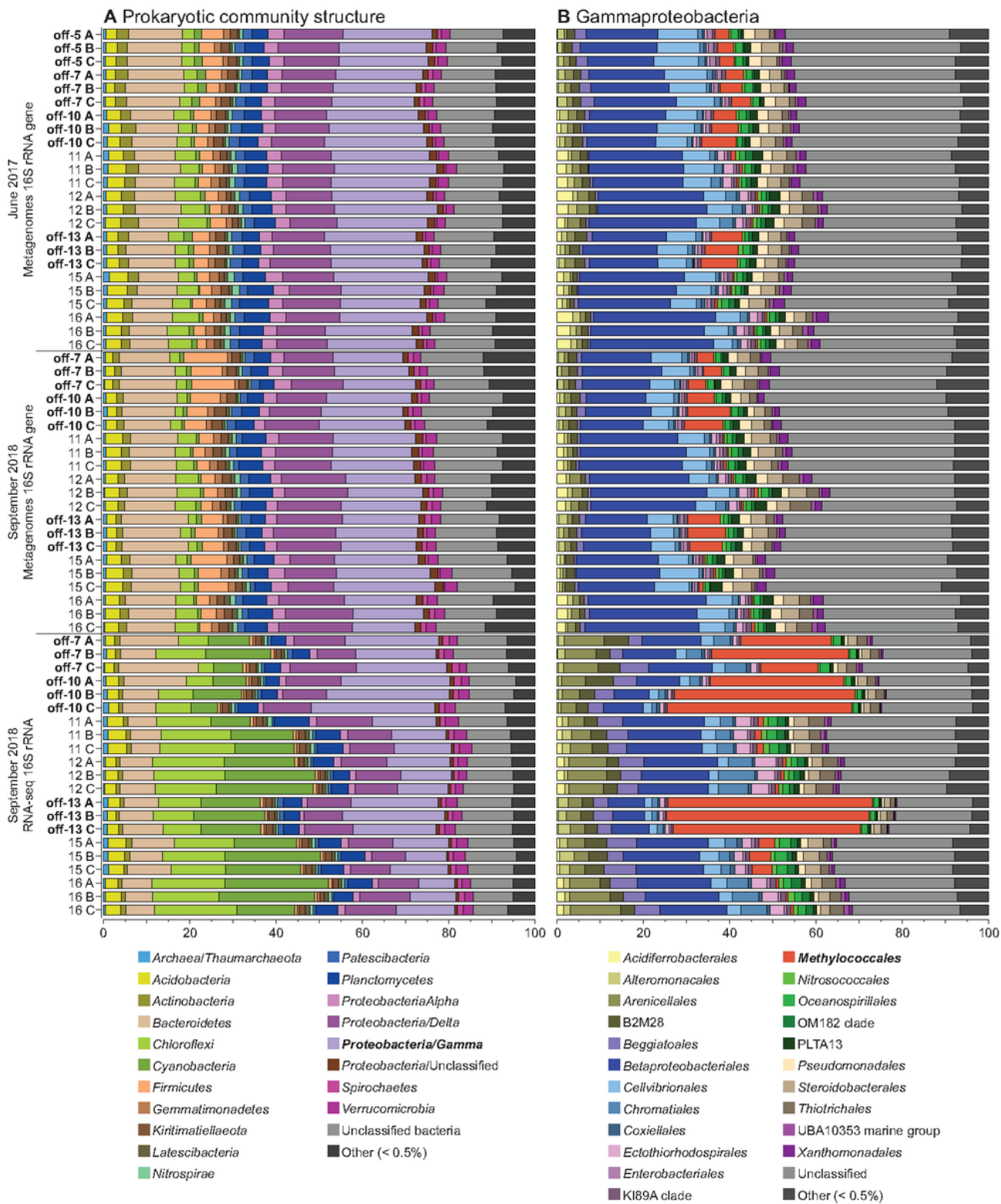
Figure 2

The stacked bars show relative abundance (x-axis %) of A) prokaryotic phyla and Proteobacteria classes, and B) Gammaproteobacteria orders. The dataset was delimited to taxonomic groups > 0.5 % (average of all samples), and bold text denote Gammaproteobacteria (in A) and methanotrophic Gammaproteobacteria (in B). The y-axis shows the sampling year, sample type (DNA or RNA), and station

names and replicate samples indicated by letters A, B, and C. Offshore stations are indicated on the y-axis with the label "off".
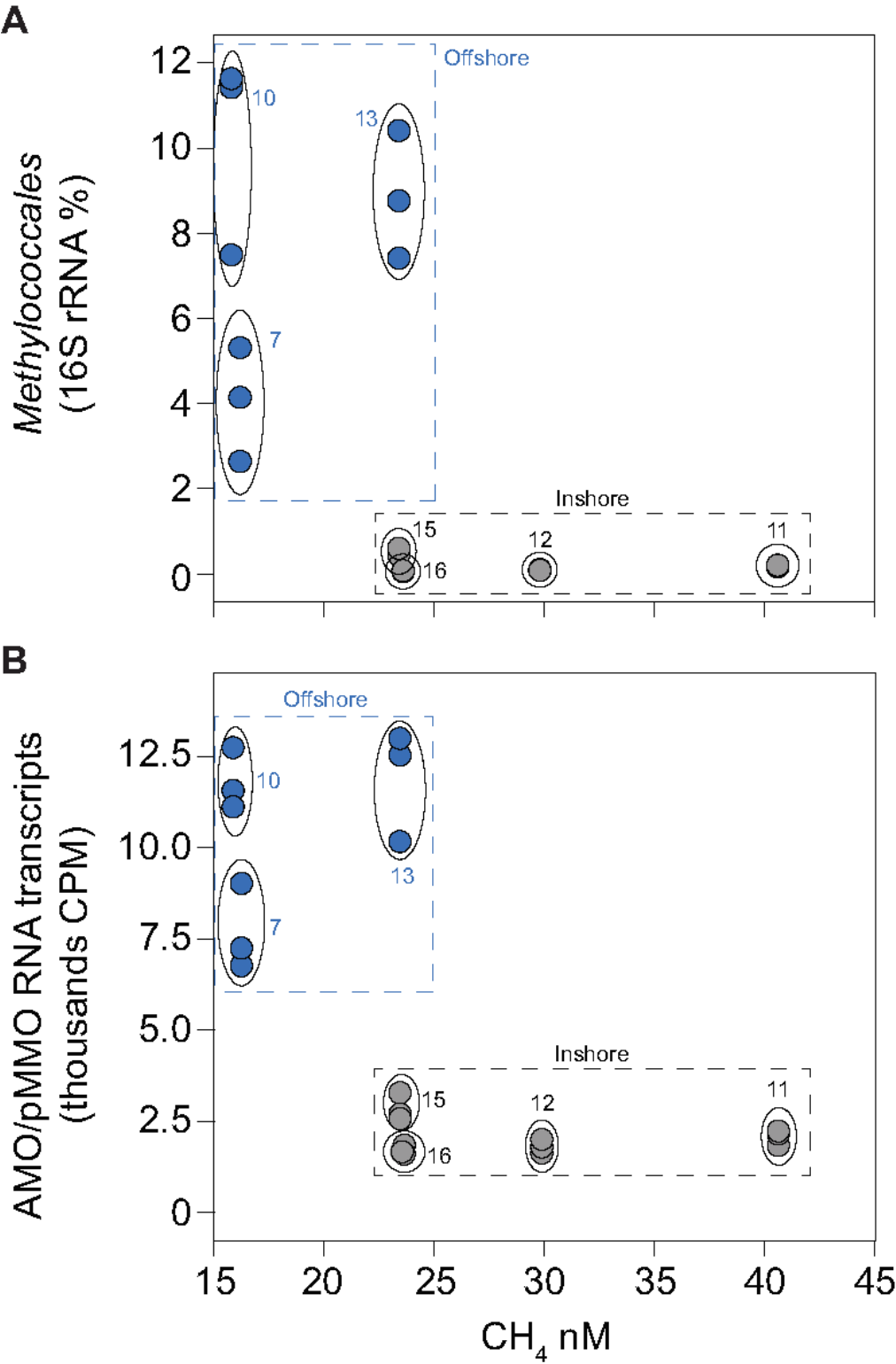
Figure 3

A) The relative abundance of the methanotrophic Gammaproteobacteria order Methylococcales in the 0–2 cm sediment surface (y-axis, n = 3 per station) and measured CH4 in the water column (x-axis, n = 1 per station). The relative abundance of Methylococcales shown is based 16S rRNA sequences extracted from

the total 2018 RNA-seq data, while CH4 was measured in the water surface (0.5-1.0 m water depth). B) RNA transcripts of the whole prokaryotic community classified as AMO/pMMO against the InterPro database. Values shown are normalized sequence counts (counts per million sequences; CPM). Red filled circles denote stations further away from the coast, and the outlining circles show the sampling stations for each cluster of data points.
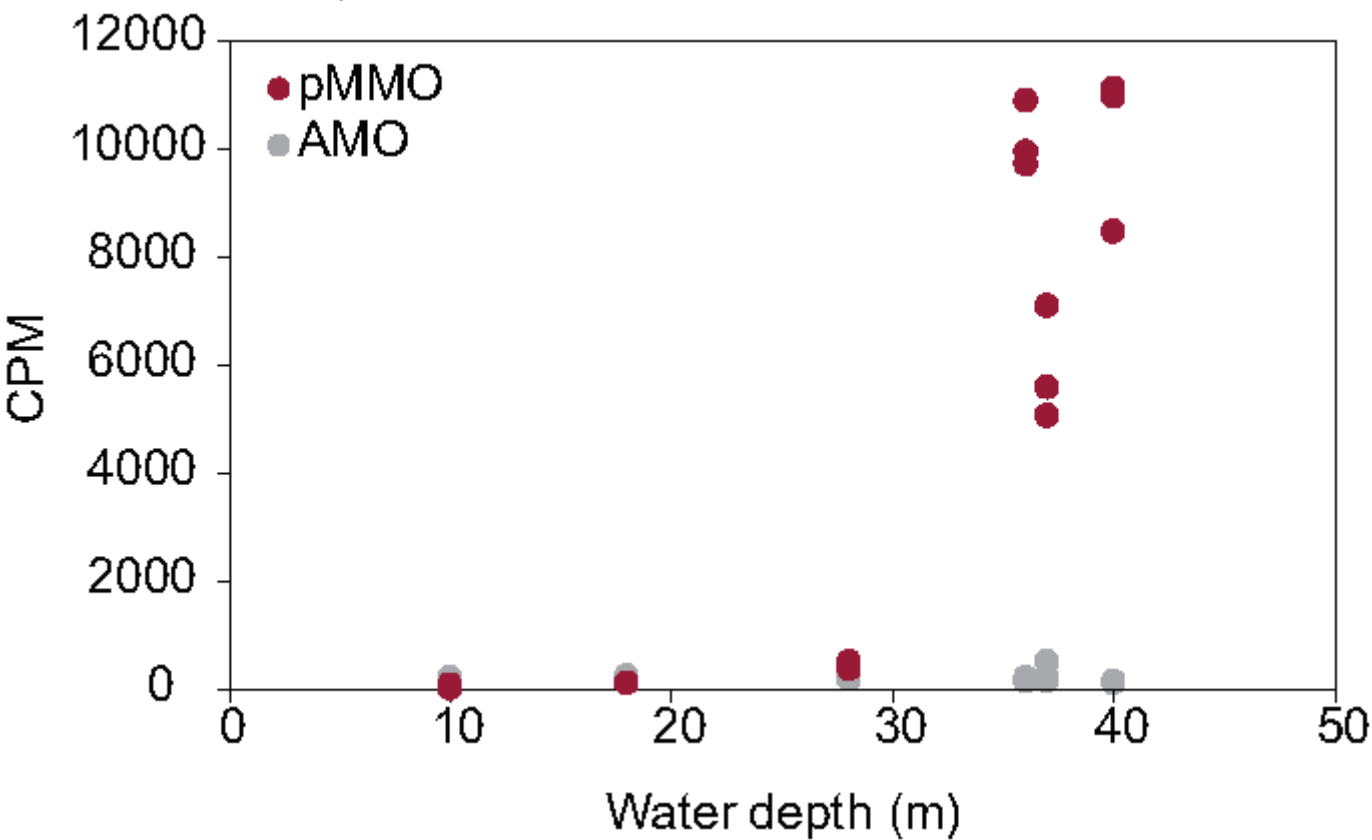
Figure 4

Sequences annotated to the InterPro AMO/pMMO family was classified against the UniProtKB database and compared to the water depth (m) of the stations (x-axis). CPM values shown are based on all proteins classified against the InterPro database (y-axis). Each circle in the graph denotes RNA transcripts derived from the 0−2 sediment surface from individual sediment cores. Dark red circles denote pMMO and light grey circles denote AMO. All pMMO proteins were classified to the reference species Methylococcus capsulatus in the UniProtKB database.
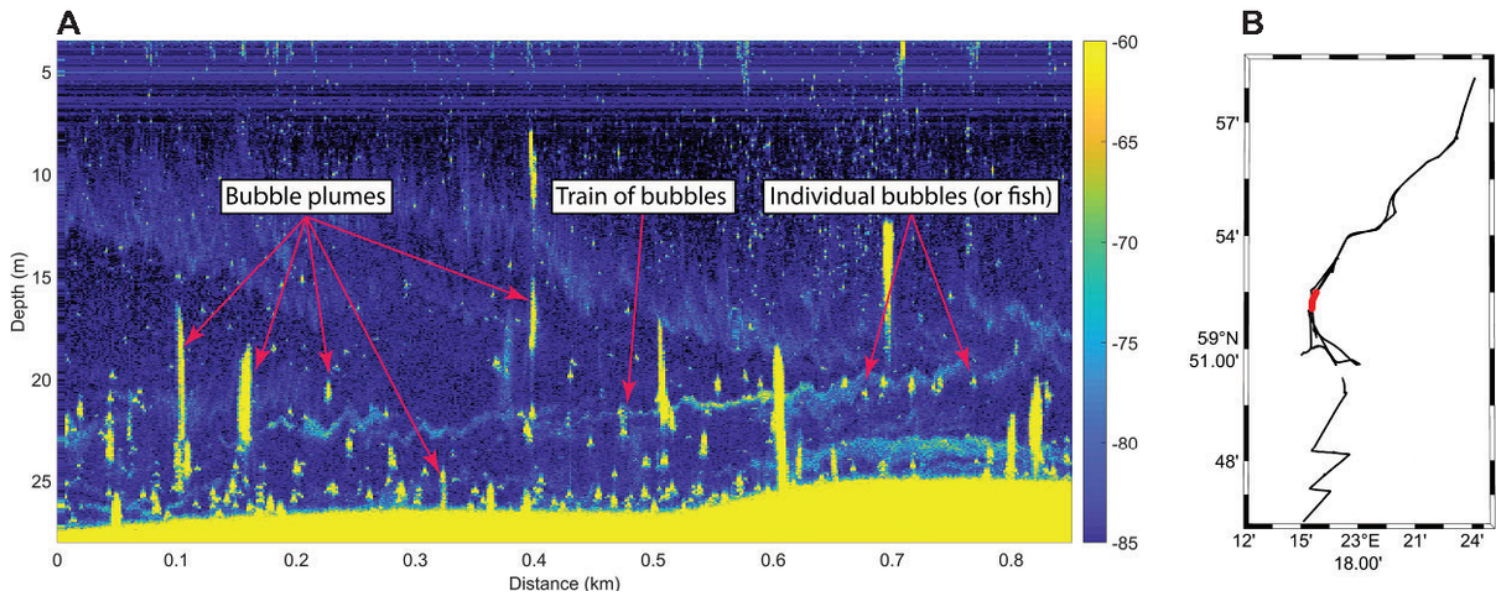
Figure 5

Onboard the research vessel acoustic data (EK80 wide band transceiver) was collected from the southern, central, and northern Storfjärden bay. A) Example of echogram with seeps from the seafloor and within the water column. The right-side y-axis shows target strength (dB) as a function of horizontal distance (x-axis) and water depth (left-side y-axis). B) Map of the Storfjärden bay showing the data track from all the acoustic data that were collected (black) and the track shown in the echogram in A (red).
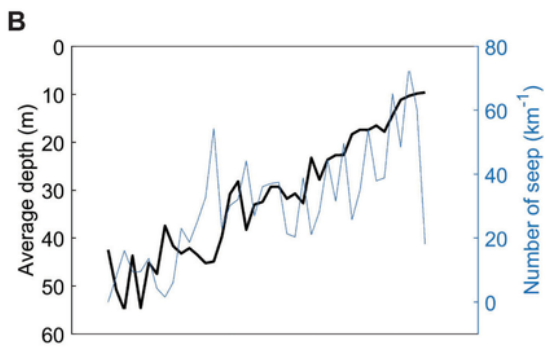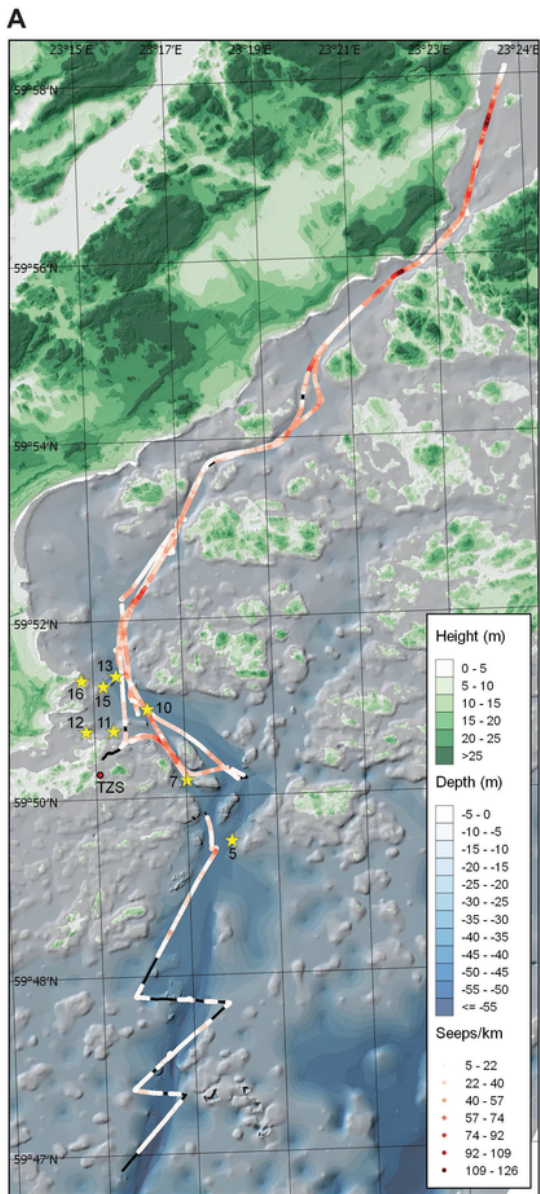
Figure 6

A) Shows the cruise track and amount of CH4 seeps km-1 (red colour gradient) observed with acoustic data in the bottom water above the sediment. Black lines on the cruise track denotes no CH4 seeps observed. The blue gradient denote water depth (m) and the green gradient terrestrial land height (m). B) Water depth (black line, y-axis increasing with lower water depth) compared to the amount of CH4 seeps

observed (blue line, y-axis increasing with more CH4 seeps). Stations were sediment were sampled are denoted by star symbols.
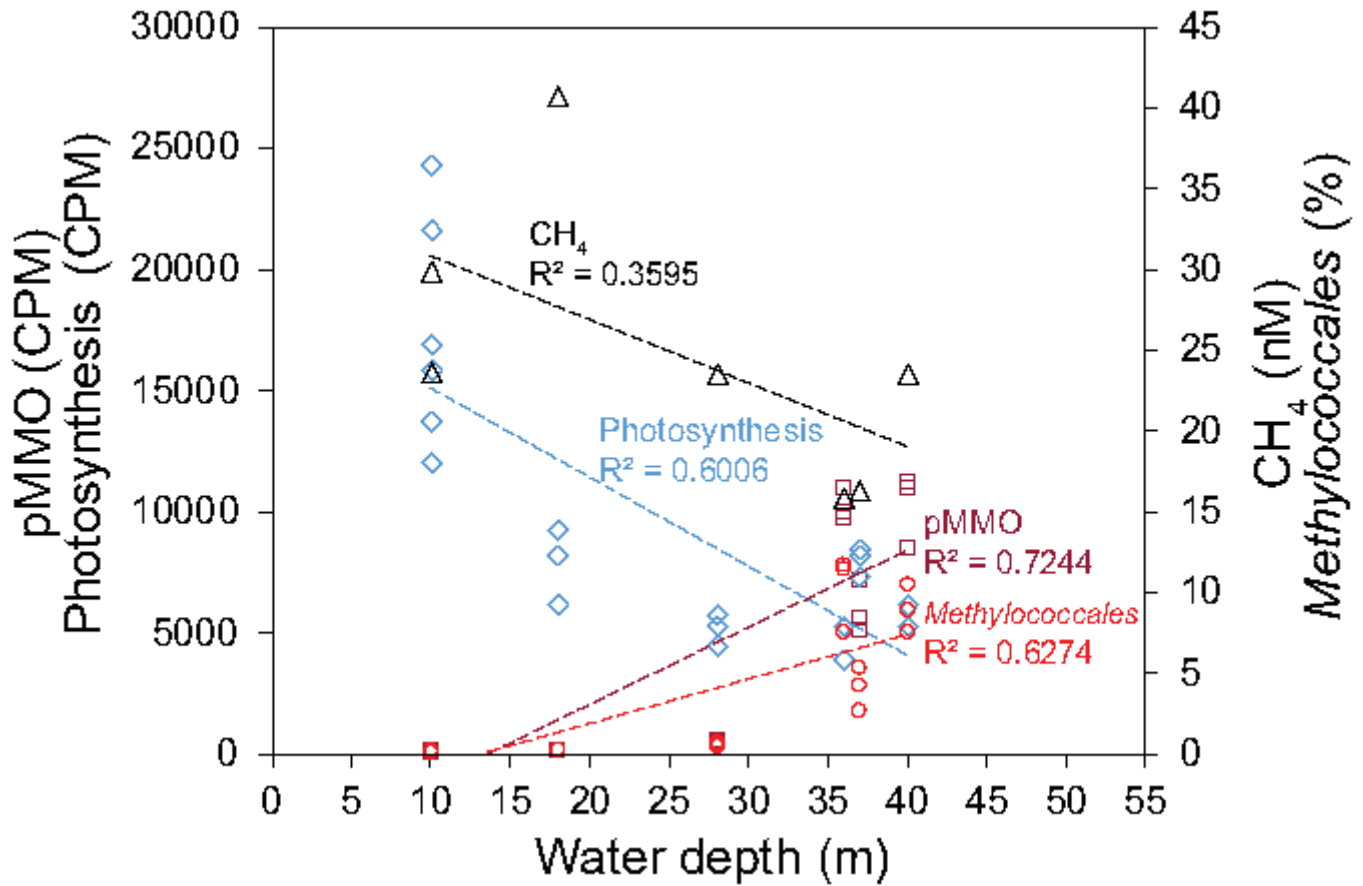
Figure 7

The relationship between water depth (x-axis) and CH4 concentrations in the surface water (black symbols and trend line), the relative abundance (%) of Methylococcales (red), the enzyme pMMO (CPM, dark red), and the sum of proteins belonging to the GO category photosynthesis (CPM, light blue).