Effects of increased pCO2 and terrestrial organic matter on phytoplankton and bacterioplankton activity and dynamics

A microcosm experiment on natural microbial assemblages

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Abstract

In the light of predicted climate change scenarios, atmospheric and water CO$_2$ partial pressure (pCO$_2$), as well as precipitations, are expected to increase during the next decades. High-latitude water masses, such as the Baltic Sea, are more susceptible to be affected by the increase of these two environmental factors due to the high solubility of CO$_2$ in cold water and the greater input of terrestrial matter from the rivers. We carried out a microcosm experiment in order to test the single and combined effects of increasing CO$_2$ and terrestrial organic matter (TOM) on natural phytoplankton and bacterioplankton assemblages present in the seawater from a coastal area in the Gulf of Bothnia. Our results suggest no interaction between pCO$_2$ and TOM, but strong effects of TOM on microbial dynamics. On the other hand, pCO$_2$ did generally not show effects on either trophic group, suggesting a less important role compared to organic matter inputs under climate change scenarios. We also observed an uncoupling between phytoplankton and bacterioplankton dynamics among all the treatments.

Key words: Climate change, CO$_2$, TOM, phytoplankton, bacterioplankton
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1. Introduction

Different environmental factors have been studied during the last decades under the light of predicted climate change scenarios. Nowadays, the entire scientific community is aware of the exponential increase in the atmospheric CO₂ partial pressure throughout the Earth, which has an important effect on the acidification of marine water masses as a consequence of the absorption of CO₂ by the oceans (IPCC, 2007). High-latitude water masses are more susceptible to be affected due to the high solubility of CO₂ in cold water (Steinacher et al. 2009). Among the predicted biological consequences, the reduction of calcification due to an increased dissolution of the calcium carbonate is one of the most visible effects, as this structural compound is part of many biological structures (e.g., corals, coccolithophores, crustaceans, or mollusks) (National Research Council 2011). Furthermore, recent researches have also studied the effect of higher CO₂ concentration in seawater (pCO₂) on the basic levels of the food-web, showing in some cases effects on phytoplankton and bacterioplankton activities (see Joint et al., 2011 for a review). However, in most of the studies, CO₂ concentrations were achieved by addition of CO₂-saturated water (e.g. Pointek et al. 2013, Motegi et al. 2013), CO₂-enriched water (e.g. Brussaard et al. 2013, Silyakova et al. 2013), or HCl and NaHCO₃ (e.g. Ray et al. 2012), and not by the exposure of the water to a high CO₂ concentration atmosphere, which is the natural scenario. Despite the susceptibility of the Baltic Sea to acidification, the effect of increasing CO₂ levels has been overlooked in this region.

In addition, increased precipitations in northern Europe is other consequence expected from climate change scenario (Meier 2006), which would ultimately lead to increasing dissolved allochthonous organic carbon inputs due to greater runoffs. Discharge of dissolved organic carbon (DOC) into aquatic systems has been found to promote bacterial metabolism due to its bioavailability, allowing them to play a more prominent role at the basal trophic level (Cole et al. 1988). This switch from phytoplankton-based to bacterial-based production has been described to reduce the efficiency of pelagic food webs by dissipating energy as it is transferred to higher trophic levels (Sommer & Stibor 2002). Furthermore, the input of allochthonous DOC has been suggested to be an important cause of uncoupling between phytoplankton and bacterioplankton activities (e.g., Andersson et al., 2013). The reason is again the high bioavailability that these compounds for bacterioplankton, allowing them to perform independently from the DOC produced by phytoplankton (exudates). This independency leads ultimately to the uncoupling of the carbon transfer between these two trophic levels (Larsson and Hagström 1979).

The individual effects of pCO₂ and DOC on phytoplankton and bacterioplankton reported throughout the literature are diverse and strongly dependent on the biological parameter in question (production, abundance, biomass, enzymatic activity, community composition, etc) and on interactions with other environmental factor (Reul et al. 2014; Sobrino et al. 2014). For instance, the effect of pCO₂ on phytoplankton activity has been suggested to be mainly positive, but neutral and negative results have been also described (e.g., Gao et al. 2012 and references therein). In regard to bacterial activity, pCO₂ has been described as a positive factor for some parameters such as growth rate (e.g., Grossart et al. 2006), enzymatic activity (e.g., Pointek et al. 2010) or bacterial production (e.g., Coffin et al. 2004; Montegi et al. 2013), whereas no significant effects were found in other studies on either the same or different bacterial parameters (e.g., Allgaier et al. 2008; Grossart et al. 2006). Observations on the effects of DOC on phytoplankton have usually been reported to be negative mainly due to two factors (Andersson et al. 2013): a) brownification of waters experimenting inputs of humic-rich substances, which commonly implies high DOC concentration and a light intensity decline; and b) stimulation of bacterial activity by bioavailable DOC. Finally, as
discussed above, DOC has been extensively observed to be an important source of stimulation of bacterial metabolism.

As the effects of individual environmental variables on the trophic food-webs become more established, new experimental approaches are necessary to understand the multiple interactions between such factors and their effects on different trophic levels, which can be either additive (synergistic or antagonistic) or independent (Folt et al. 1999). To our knowledge, the combination and interactions between pCO₂ and DOC have never been tested in the Baltic Sea. In that respect, we carried out a microcosm experiment in order to test the single and combined effects of increasing CO₂ and terrestrial organic matter on natural phytoplankton and bacterioplankton assemblages present in seawater from a coastal area in the Gulf of Bothnia. We hypothesize an increase of phytoplankton activity following increased CO₂ concentration, with a concomitant release of DOC from phytoplankton and, as a consequence, an increase of bacterial activity. The input of terrestrial organic matter may hamper the phytoplankton-bacterioplankton coupling, likely due to a stimulation of bacterial activity under high DOC concentrations.

2. Methods

Study area and experimental design

Water from 1 m depth was collected and screened (200 µm mesh) in May 2014 at the station B7 (63° 31’ 50” N, 19° 48’ 49” E) near the coast of Umeå (Fig. 1), and then placed into a climate room at 4-5 °C with a light intensity of 40 µE s⁻¹ m⁻². Approximately 12 hours after collection, the water was transferred to 90 1L culture bottles (VWR Polycarbonate 1000 ml 32 oz) and divided into 6 different treatments (i.e. 15 bottles per treatment): Control: untreated water; S: soil extract addition; 1xCO₂: bubbling by 800 ppm pCO₂; 2xCO₂: bubbling by 1500 ppm pCO₂; 1xCO₂-S: combined treatment of soil addition and bubbling by 800 ppm pCO₂; and 2xCO₂-S: combined treatment of soil addition and bubbling by 1500 ppm pCO₂. The bottles were filled with 800 mL water leaving a headspace of 200 mL. The addition of DOC was carried out using a natural humic rich solution created by mixing water with soil collected from a creek bank close to the Öre river (and thus also close to the sampling site in this study) as described in Lefébure et al. (2013). The soil solution was added at a
concentration of 2.4 mg C/L, representing a 36% increase in DOC from the present levels (Eriksson-Hägg et al. 2010). After the soil addition into the corresponding bottles, they were sealed by a rubber stopper and the bubbling was carried out. To perform this procedure, the pertinent bottles were bubbled using two different gas mixtures containing 21% O₂, N₂ as background gas, and CO₂ in concentrations of 801 ppm and 1510 ppm, respectively. Thus, the water was bubbled for 5 min through a circulating system, which was then detached from the bottles to hence leave the microcosms utterly sealed. Finally, the microcosms were incubated for 18 days under a light-dark cycle representative of the season (18 hours day length). Since the microcosms only could be sampled once (when opened, the elevated CO₂ treatment would be lost due to degassing), we instead sampled at 5 different sampling occasions (triplicate bottles per treatment and sampling occasion): 3, 7, 11, 14 and 18 days after the collection of the bulk water.

**pCO₂ measurements**

Samples for analysis of pCO₂ in both headspace and water were collected before opening the sealed microcosms by inserting a long needle attached to a syringe through the rubber stopper. Twenty mL of air from the headspace were taken, allowing a weak under-pressure to form, and then injected into an infrared gas analyzer (IRGA EGM-4, PP-Systems). The pCO₂ in water was measured using a headspace technique consisting in the collection of 40 mL of sample water by a 60 mL syringe, after which 20 mL of atmospheric air (pCO₂ previously determined) was injected as a headspace. The syringe was closed and then thoroughly shaken for 1 minute in order to reach the gas-water equilibrium state. The resulting gas contained in the headspace was analyzed by an IRGA as above and the water pCO₂ was finally calculated using Henry’s law and the fugacity-pressure relationship described by Weiss (1974). The temperature of the sample water was measured directly in the syringe just after the shaking procedure. The IRGA was periodically calibrated during the measurements by using 4 different CO₂ concentrations: 0, 801, 1510, and 8080 ppm. In addition, the pressure of the system was constantly auto-calibrated by the IRGA itself.

**DOC, DIC and nutrients**

Samples of 50 mL from each microcosm were filtered through a 0.2 µm filter (syringe filter Acrodisk) according to Norrman (1993) for DOC analysis. These samples were then acidified by 375 µl of 1.2 M HCl and stored at -20°C for less than 1 month. DOC was measured by the high-temperature catalytic oxidation method using a Shimadzu TOC-5000 instrument with platinum-coated Al₂O₃ granulates as a catalyst.

Total dissolved ammonium, nitrite, nitrate, and phosphate were measured by filtering 10 mL of water from each microcosm. The samples were frozen at -20 °C for 4 weeks and then analysed using a 4-channel auto-analyzer (QuAAtro marine, Bran & Luebbe®) according to the Swedish standards Institute and HELCOM (Grasshoff et al. 1983).

Samples for dissolved inorganic carbon (DIC) analysis were prepared by injecting 4 mL of water from the microcosms into 20 mL glass vials that were previously flushed with nitrogen gas (99.999% chemically pure) and sealed. The vials contained 100 µL of 1.2 M HCl in order to ensure a final pH of approximately 2 and to drive the carbonate system towards CO₂ (g). The headspace gas thus created was analysed using a gas chromatograph (Clarus 500 mass detector, PerkinElmer, U.S.A.), which was calibrated during every analytical process against 4 different standard carbonate solutions.

**Chlorophyll-a concentration**

Each sample consisted of 100 mL of water that was filtered through a 25 mm GF/F filter (Whatman, Kent, UK) by using a filter adapter connected to a syringe. Blanks were also prepared using Milli-Q water. The filters were frozen at 80°C for no longer than 4 weeks and
then transferred into 15 mL plastic Falcon tubes containing 4 steel balls of 5 mm diameter. A volume of 10 ml of 95% ethanol was added to each tube, and the stoppers were securely tightened. In order to smash the filters, the tubes were placed in a rack and thoroughly shanked using a large amplitude linear shaker at approximately 200 rpm for 5 min. Then the tubes were kept overnight in the dark and were centrifuged (after a brief hand-shaking) at 3500 rpm for 10 min using a Heraeus Instruments Labofuge 400 (Hanau, Germany). The fluorescence of the samples was analysed on a Perkin Elmer LS 30 spectrofluorometer (Waltham, MA, USA) operating at excitation and emission wavelengths of 433 and 673 nm, respectively. The chlorophyll-α content was calculated using a calibration constant obtained in a previous calibration.

**Primary production**

Primary production was measured by the 14C method (Gargas 1975). A sample of 90 mL from each microcosm was collected and mixed with 90 µl Na14CO3 (DHI, specific activity = 100 µCi/mL), to a final activity of 9 µCi. Then, 4 replicates of 20 mL each were made from that solution, 3 of them being incubated in light (in situ radiation) and 1 in dark. In order to analyse the true radioactivity added, 5 mL from the solution were immediately mixed with 15 mL scintillation cocktail (Optiphase HiSafe 3). The incubation was carried out for 4 hours, after which 1 mL formalin was added to each vial. These samples were stored in dark for 2 months. Next, a sub-sample of 5 mL from each vial was mixed with 150 µL 6M HCl. After 30 min of bubbling, 15 mL scintillation cocktail were added and thoroughly mixed and the samples were stored until analysis in a scintillation counter (Beckman Coulter LS 6500/Packard Tri-Carb 1600 TR). Net phytoplankton primary production was calculated using a slightly modified version (Larsson et al. 2001) of the equation in the National Research Council guidelines (HELCOM 2009).

**Bacterial production**

Bacterial net production was measured using the [3H-methyl]-thymidine incorporation method (Fuhrman & Azam 1982). Two 1 mL replicates and one killed control from each microcosm were incubated in dark at in situ temperature (4-5 °C) with [3H-methyl]-thymidine (80 000 Ci mol⁻¹), at a final concentration of 24 nM. The incubation was stopped by the addition of 100 µL of 50% TCA (trichloroacetic acid) to the live replicates and all samples were then centrifuged in cold at 13 000 rpm for 10 minutes. The resulting pellet was washed with 5% TCA and, after adding 1 mL of scintillation cocktail, the samples were analysed in a scintillation counter (Beckman Coulter LS 6500/Packard Tri-Carb 1600 TR). Bacterial production was calculated using a conversion factor of 1.4 × 10¹⁸ cells mol⁻¹ (Smith & Azam 1992, Wikner & Hagstrom 1999).

**Phytoplankton biomass and abundance**

A 50 mL sample for phytoplankton identification and counting was collected from each microcosm and fixed in acid Lugol’s solution. The samples were analysed after settling in sedimentation chambers (25 ml) for 24 h by using an inverted microscope (Nikon Eclipse TE 300/ Wild M40). In order to analyse microplankton (20 to 200 µm), the samples were analysed at 100x magnification, while 400x magnification was used for nanoplankton (2 to 20 µm). Cell density was high enough to analyse only one transect across the chamber. Thus, phytoplankton were counted and measured for estimations of biovolumes and biomass using the equations and size classes described for the Baltic Sea (HELCOM 2014).

**Microbial counting**

Samples for both bacteria and phytoplankton were preserved in 0.1% glutaraldehyde (final concentration) and frozen at -80°C as described by Marie et al. (1997). The samples were thawed in a 30°C bath and filtered through a 50 µm mesh. They were then counted using a
BD FACSVersetm flow cytometer (BD Biosciences) fitted with 488 nm laser (20 mW output) and a 640 nm laser (40 mW output). For phytoplankton analysis, the flow rate was set to 120 µL min⁻¹, with an acquisition time of 5 min. Microspheres of 3 µm and 10 µm (Fluoresbrite plain YG, Polysciences) were added to each sample and used as internal standards to discriminate between phytoplankton size groups. Forward light scatter (FSC), side light scatter (SSC), and red fluorescence from chlorophyll-α (783 ± 28) were measured. For bacterial counting, the samples were stained with SYBR Green I (Invitrogen) to a final concentration of 1:10 000 (Marie et al. 2005). Samples were diluted with Milli-Q water to avoid coincidence despite a relatively low flow rate of 30 µL min⁻¹ and an acquisition time of 2 minutes were used during the flow cytometric analysis. As an internal standard, 1 µm microspheres (Fluoresbrite plain YG, Polysciences) were added to each sample. Forward light scatter (FSC), side light scatter (SSC) and green fluorescence from SYBR Green I (527 ± 15) were measured to estimate bacteria abundance. Bacterial biomass was then calculated using a conversion factor of 20 fg C cell⁻¹ according toLee & Fuhrman (1987).

Statistical analysis

Two-factor (treatment and time) permutational multivariate analysis of variance (PERMANOVA; PRIMER software) was used to test the significance between the means of the different treatments from some of the environmental and biological parameters in study. The statistical significance of the comparisons was tested using 9999 permutations of residuals with a chosen significance level of α=0.05. Pairwise Pearson tests (Microsoft Excel software, StatPlus tool package) were carried out to assess the significance of correlations between the different parameters (α=0.05 as significance level).

3. RESULTS

Chemical parameters

After enclosure of the water and treatment manipulation, the level of dissolved nutrients (phosphorous and nitrogen) decreased in all the non-soil microcosms (Fig. 2E, F). This reduction in nutrient concentration was especially remarkable for total dissolved phosphorous (P). Therefore, the environment enclosed in the non-soil microcosms should be considered as a nutrient limited system.

The bubbling carried out on the samples, the average water pCO₂ at day 3 (one day after the bubbling) was 370 (control; C), 676 (soil addition; S treatment), 589 (800 ppm bubbling; 1xCO₂-S), 922 (1500 ppm bubbling; 2xCO₂-S), 630 (soil-800 ppm combined treatment; 1xCO₂-S), and 1025 (soil-1500 ppm combined treatment; 2xCO₂-S) µatm, whereas the pCO₂ in the bulk water was ca. 480 µatm (Fig. 2). These values decreased and fluctuated throughout the experiment, generally reaching the lowest values around days 11 and 14, and then slightly rising again. Overall, only 2xCO₂ and 2xCO₂-S treatments showed significantly higher CO₂ levels compared with the control water (Table 1), which can be also observed in figure 2A. On the other hand, no significant differences on 1xCO₂ - 1xCO₂-S (p = 0.35) and 2xCO₂ - 2xCO₂-S (p = 0.96) were found, suggesting that soil addition did not contribute to the rise in pCO₂ levels. The pCO₂ levels in the head space of the culture bottles also varied during the experiment, presenting a significant positive correlation with the pCO₂ values in water (r = 0.77, p < 0.001). This positive correlation indicates a constant balance of the pCO₂ level between water and air.

Average DIC concentration also showed fluctuating values over time, reaching the highest values on day 14 and then dropping at the end of the experiment. While S treatment, as well as both bubbling treatments (1xCO₂ and 2xCO₂), did not present significantly different values
than the control samples (p > 0.05; both overall and throughout different days), the soil extract seemed to have an additive effect to the bubbling on the DIC concentration, since the combined treatments (1xCO$_2$-S and 2xCO$_2$-S) showed higher DIC values compared to the control water (p = 0.021 and p = 0.003, respectively) (also see Fig. 2A). As expected, the pH showed an opposite trend when compared to DIC, with a significant negative correlation (r = 0.63, p < 0.001; Fig. 2C). DOC concentration strongly increased after the addition of the soil extract (S, 1xCO$_2$-S, and 2xCO$_2$-S) and then progressively decreased until the end of the experiment (Fig. 2D). DOC values were much lower in C, 1xCO$_2$, and 2xCO$_2$ treatments, where the concentration slightly increased until day 11 and then slowly decreased. In fact, these 3 treatments showed very similar trend throughout the experiment (Fig. 2D) with no significant differences in DOC content (Table 1).

![Figure 2](image)

**Figure 2.** Comparison of (A) CO$_2$ partial pressure, (B) dissolved inorganic carbon, (C) pH, (D) dissolved organic carbon, (E) total dissolved phosphorous, and (F) total dissolved nitrogen. Average and SD of all microcosms are shown.

The addition of the soil extract also raised the concentration of dissolved nutrients in the water. In the same way as with DOC, P strongly increased after the addition of the soil extract, showing a significant correlation with DOC (r = 0.89; p < 0.0001). P gradually decreased throughout the experiment, showing very similar trends and values in all the soil treatments (Fig. 2E). In the case of control and single treatments, P decreased rapidly and remained below the detection limit until the end of the experiment. Total dissolved nitrogen (N) also showed a marked increase after the soil extract addition (significant correlation with DOC; r = 0.89; p < 0.0001), and then a strong drop after the first sampling occasion (day 3), remaining with more stable values during the rest of the experiment (Fig. 2F). The trend of N
in the control and single treatments presented less fluctuating values, with a slight increase from the first sampling occasion.

**Phytoplankton activity**

The general trend of different phytoplankton parameters is pictured in figure 3A, B and C. Primary production (PP) and chlorophyll-α concentration (Chl-α) show a conspicuous peak around the middle of the experiment in the soil treatment (S, 1xCO₂-S and 2xCO₂-S), whereas the non-soil treatments (C, 1xCO₂, and 2xCO₂) do not exhibit remarkable variations throughout the experiment. On the other hand, the data from flow cytometry (phytoplankton abundance, PA) do not suggest in general peak events, showing a gradual increase in phytoplankton abundance from the initial level toward the end of the experiment. Figure 3A, B and C also reveals the distinction of different phases based on the experimental manipulations (soil and CO₂ addition) and the temporal development of PP and Chl-α. Moreover, the microcosms can be clustered into two different groups according to the characteristics displayed during each of the phases: soil (S, 1xCO₂-S, and 2xCO₂-S) and non-soil (C, 1xCO₂, and 2xCO₂) treatments. Thus, the phases occurring in the non-soil treatments can be generally defined as:

Phase 1 (0d to 3d): application of the treatments (bubbling), after which a decrease in PP and an increase in Chl-α concentration can be observed. Phase 2 (3d to 11d): slight increase in PP over the previous values (except 2xCO₂ microcosms) and slight decrease in Chl-α followed by a rise above the values in phase 1. Phase 3 (11d to 18d): general decrease in PP, showing the lowest values. C samples showed higher values at 14d (still below the initial values) and decreased again toward the end of the experiment. Chl-α content generally decreased from the previous values, except in 1xCO₂ microcosms, where Chl-α timidly increased by the last sampling occasion.

In contrast to the slight variations found in the non-soil treatments, the microcosms including soil addition showed more extreme fluctuations in PP and Chl-α, displaying more conspicuous peak events:

Phase 1 (0d to 3d): application of the treatments (bubbling and soil addition). General drop in PP, being especially notable in 2xCO₂-S microcosms. Contrary, Chl-α showed a strong increment from the bulk value. Phase 2 (3d to 11d): pronounced rise in PP, reaching the highest values and followed by a new drop (peak-shape phase). As PP, Chl-α showed a very clear bloom trend, displaying strongly a similar pattern across the 3 different soil treatments. Phase 3 (11d to 18d): although the particular behavior differs throughout the different soil treatments, in general PP reached its lowest values during this phase. However, PP increased again by the last sampling occasion in S and 2xCO₂-S treatments, although never above the bulk levels. Chl-α increased overall during this phase, reaching its maximum in 2xCO₂-S microcosms.

Other phytoplankton and chemical parameters (such as PA and DOC content, respectively) also suggest a clear division of the treatments into such two groups (Fig. 3C and 2D). In addition, significant correlations were found between DOC and PP ($r = 0.56; p = 0.002$), Chl-α ($r = 0.89; p < 0.001$), and PA ($r = 0.93; p < 0.001$), as well as between Chl-α and P ($r = 0.76; p < 0.0001$), which suggests a causal relationship of soil addition and phytoplankton activity. As pointed out above and contrary to PP and Chl-α, PA shows a constant increasing trend toward the end of the experiment, reaching in general the highest values in phase 3. The differences between the two groups of treatments mainly lie again in the extent of the increment, where the soil treatments show significant higher values in PA (Table 1), especially from the outset of phase 2.
### Bacterial activity

Bacterial production (BP) showed fluctuating values throughout the experiment, with the most stable values taking place in the control microcosms (Fig. 3D). Overall, BP decreased from the bulk level during the initial days and then peaked around day 11. After this peak, there was a decrease at day 14 and the values raised again toward the end of the experiment. This general trend is observed in most of the treated microcosms and the controls, with the highest and most extreme values happening in the treated microcosms (Fig. 3D). However, the S treatment shows a different pattern, presenting the highest values during the initial days and again in the end of the experiment. This latter treatment, together with 1xCO2 and 2xCO2-S, presents the highest BP values overall, with no significant differences between each other (see p-values in table 1).

Bacterial biomass (BB) generally increased from the initial value only in the S treatments (Fig. 3E). Apparently, the bubbling treatment did not have a visible effect, since the corresponding microcosms (1xCO2 and 2xCO2) do not differ significantly from the control microcosms (Table 1). Thus, although BP does not show neither a partitioning of the treatments into different groups nor a common pattern among them, it can be inferred from figure 3E a general trend in BB throughout the different types of microcosms and across the different phases described above. Phase 1 is hence characterized by a pronounced increase in the soil treatments, whereas the non-soil treatments show similar or lower values than the bulk water. In phase 2, it can be observed an overall drop in all microcosms, reaching in
general the lowest values during the experiment. Finally, BB slightly increased toward the end of the experiment (phase 3), and more particularly in the soil treatments.

The distinction between soil and non-soil treatments in BB is also supported by the significant correlations found between this parameter and DOC (r = 0.52; p = 0.01), P (r = 0.63, p = 0.0002) and N (r = 0.54, p = 0.002).

Table 1. PERMANOVA results of differences in the parameters pCO2, DIC, DOC, PP, Chl-a, PA, BP, BB, BP:PP ratio between the different treatments. The two parameters involved in every comparison are specified in the two initial rows. Significant differences of p ≤ 0.05 (derived by permutation analysis) are indicated in bold.

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<td>0.0003</td>
<td>0.0003</td>
<td>0.2636</td>
<td>0.4917</td>
<td>0.6694</td>
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</tr>
<tr>
<td>BP:PP</td>
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<td>0.1388</td>
<td>0.3452</td>
<td>0.0149</td>
<td>0.0843</td>
<td>0.4328</td>
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<td>0.8363</td>
<td>0.5629</td>
<td>0.0665</td>
<td>0.5323</td>
</tr>
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</table>

4. Discussion

Enclosure effect and grazing pressure

As Mercado et al. (2014) pointed out, artificially enclosed environments and removal of higher trophic levels could potentially constitute important experimental artifacts affecting the natural behavior of microbial communities. The general bubbling effect was initially tested in pilot experiments, which showed no significant differences between bubbled and control samples in regard to phytoplankton integrity (data not shown). Furthermore, the fact that the values on day 3 from different phytoplankton parameters (such as PP, PA and Chl-a; Fig. 3A, B, C) did generally not show significant differences between the bubbled (1xCO2 and 2xCO2) and control microcosms (p < 0.05; see also Fig. 3A, B, C) also suggests no important effects on the integrity of phytoplankton. The same interpretation can be also applied on bacterial parameters (BP and BB, Fig. 3D, E).

Some studies focused on phytoplankton-bacteria activity and coupling (Kluijver et al. 2010, Neale et al. 2014, Mercado et al. 2014, Reul et al. 2014) have suggested a substantial effect on phytoplankton community dynamics due to the pre-screening (200-300 µm pore mesh) of the water in study and the subsequent removal of meso- and macrozooplankton. This leads to a trophic cascade triggered by a relaxation of the grazing pressure on phytoplankton and microzooplankton. At the same time, this relaxation of zooplankton grazing also leads to a reduction in dissolved organic matter excretion (Nagata and Kirchman 1991), which is an important source for bacteria. Such effects need to be considered in broad environmental studies of natural assemblages of organisms, nonetheless the present study mainly focused on bottom-up control at the lowest trophic levels and more specifically on the effects of organic terrestrial matter and pCO2.
Effect of treatments on biological parameters

In this study we explored the single and combined effects of pCO2 and terrestrial organic matter on aquatic pelagic food webs. After the bubbling and soil addition, the water pCO2 was set up to range from the present CO2 concentration to future levels predicted for the end of this century (IPCC 2007): 370 – 1025 µatm. As observed in figure 2A, there were clear differences in pCO2 among different treatments at the outset of phase 2, distinguishing between 3 groups of microcosms: 1) C; 2) S, 1xCO2, and 1xCO2-S; and 3) 2xCO2 and 2xCO2-S. However, those differences were not significant through the whole experimental time (Table 1), and overall only the microcosms bubbled with the highest pCO2 (2xCO2 and 2xCO2-S) showed significantly higher pCO2 levels compared with the control and the other treatments. This suggests a minor contribution of soil addition to the increase of CO2 in water. On the other hand, the addition of soil extract led to clear differences in DOC concentration between two groups of microcosms: soil (S, 1xCO2-S, and 2xCO2-S) and non-soil (C, 1xCO2, and 2xCO2) treatments (Fig. 2D). The high discrepancy between two groups based on DOC concentration is also observed in nutrient concentration (P and N; figure 2E and F, respectively) and seems to be further reflect in some biological parameters such as PP, Chl-a, PA, and BB (Fig. 3A, B, C, and E, respectively). On the contrary, the differences in pCO2 levels among treatments do not seem to reflect the differences in the mentioned biological parameters, since 2xCO2 treatment was not significantly different from the control microcosms (Table 1). All these observations suggest a more conspicuous effect of soil addition over CO2 addition (bubbling) on the dynamics of different biological parameters.

Phytoplankton activity and treatments

Increased pCO2 did not seem to have a direct effect on phytoplankton, as supported by the absence of correlation between PP or Chl-a and either CO2 or DIC. This lack of direct effect of pCO2 on phytoplankton activity has been reported in other articles (Tortell et al. 2000, Sobrino et al. 2014, Mercado et al. 2014, see also Riebesell and Tortell 2011 for a review), but is in contrast to some studies showing enhanced photosynthetic activity in natural marine phytoplankton communities as a result of high CO2 concentrations (Tortell & Morel 2002, Egge et al. 2009, Engel et al. 2013). One of the explanations supporting an enhanced PP under elevated CO2 levels is the higher diffusion rates of CO2 through the cell membrane under increased pCO2 (Sobrino et al. 2014). This higher, passive diffusion reduces the energy needed in the transfer of C from the external environment to Rubisco, enabling the use of the remaining energy for other processes such as cell growth or primary production (Raven 1991). At the same time, Sobrino et al. (2014) attributes the absence of PP increase to interactions among the other environmental drivers, such as nutrients concentration and light intensity. In fact, soil addition appears to have a more direct effect on some phytoplankton parameters in the present study, as supported by the significant correlations found between DOC and PP, Chl-a and PA (p < 0,005). Although PP generally decreased in all treatments during phase 1 (probably due to the initial enclosure effect), it markedly raised during phase 2 in the soil treatments (Fig. 3A), where DOC concentration was significantly higher compared to non-soil treatments (Fig. 2D and table 1). However, no direct link between PP and DOC can be ensured since the soil addition also led to a notable input of dissolved phosphorous and nitrogen (Fig. 2E and F). Contrary to PP, Chl-a increased in phase 1 in all treatments, but more conspicuously in the soil treatments. This increase after soil addition coincided with the strong rise in DOC, as well as in P and N. In fact, the significant correlation between Chl-a and P (p < 0,0001) suggests an important direct effect of P on phytoplankton activity (Benitez-Nelson 2000). At the same time, the strong correlation between DOC and P (p < 0,0001) shows a straight link between the input of P from soil addition and an increased phytoplankton response in the soil treatments. The positive relationship between Chl-a and DOC could also be partially attributed to the shade effect usually observed under elevated DOC concentrations (Andersson et al. 2013). This shade effect can promote the synthesis of chlorophyll-a to compensate the reduced light-
harvesting capacity (Neale et al. 2014). However, such a scenario ought to be discarded in this study since the addition of soil extract did not lead to a marked light attenuation (data not shown).

Bacterial activity and treatments

Neither pCO2 nor DOC seemed to act as significant factors driving BP in the present study. Although BP was significantly higher in some of the treatments compared to control microcosms (S, 1xCO2 and 2xCO2-S; see table 1 and figure 3D), no significant relationship was found between BP and DOC. One the other hand, while the bubbling treatments (and pCO2 values) did not show a visible effect on BB (no significant differences of 1xCO2 and 2xCO2 when compared with control; table 1), the microcosms can also be clustered into soil and non-soil treatments according to significantly higher BB values observed in microcosms with soil addition (table 1). This is supported by the significant correlation observed between DOC and BB (p < 0.01). However, as explained above for PP and Chl-a, the effect of soil addition on nutrients concentration must be considered in order to distinguish between the effect from DOC on the one hand, and nutrients on the other, on bacterial activity. In fact, BB showed positive correlations with both P and N (p < 0.05). Although significant, these correlations are relatively weak, suggesting an interactive effect of multiple factors on BB (Kluijver et al. 2010). Some studies have also found positive effects on bacterial activity after nutrient addition (Kluijver et al. 2010, Mercado et al. 2014).

Phytoplankton-bacterioplankton coupling

Phytoplankton carbon exudation constitutes a direct link between phytoplankton and bacterial performance (Larsson and Hagström 1979, Fogg 1983, Ducklow 2008, Kluijver et al. 2010, Piontek et al. 2013). Some studies describe a high dependency of bacteria on phytoplankton throughout experimental phases. Kluijver et al. (2010) and Piontek et al. (2013) pointed out a succession of increased phytoplankton activity followed by increased bacterioplankton performance. They did not always find a temporal covariation of this relationship, but rather the succession could consist of a lag of only a few days to weeks (Ducklow et al. 1999, Kirchman 2009b). On the other hand, some studies have shown uncoupled phytoplankton-bacterioplankton dynamics. In a study considering several environmental factors (including pCO2), Neale et al. (2014) suggest a rather weak coupling between phytoplankton and bacterioplankton abundances. Montegi et al. (2013) also described an absence of coupling between phytoplankton biomass (i.e., Chl-a) and bacterial cell production. These latter observations are in line with the results found in the present study, as no significant covariation or correlation were found between phytoplankton and bacterioplankton parameters. The dynamic observed in both trophic levels can be observed in figure 3, which does not show any evident lag as described above. Kluijver et al. (2010) pointed some possible explanations for this uncoupling (see references therein for more details).

Phytoplankton can actively release DOM under nutrient limitation, which may have occurred in the non-soil treatments. However, no remarkable DOC increase was observed in such treatments during the experiment (Fig. 3D), so this scenario ought not be considered as a reliable interpretation in this study. Ducklow et al. (2008) provided another possible explanation suggesting a state of dormancy in which a great proportion of bacterial species are found in marine environments under nutrient and/or energy starvation. Again, if this were the main factor responsible for the uncoupling, we would observe coupled dynamics in the soil treatments. A last possibility given by Kluijver et al. (2010) considers the “hypothetical existence of non-dividing subpopulations of cells”, which would produce the uncoupling of productions but may be in any case noticed in an increasing biomass and a reduced abundance, as well as in a change in size structure specially in phytoplankton. Preliminary examination of species composition by microscopy suggests a change in community composition from the bulk water, as well as a positive correlation between
phytoplankton biomass and abundance from the outset of phase 2 on (data not shown). This appears to show actively dividing phytoplankton populations, in contrast to the non-dividing subpopulations hypothesis. However, this conclusion must be taken with caution until completion of analyses. Finally, a more plausible explanation that may apply to the present study is pointed out by Andersson et al. (2013): allochthonous dissolved organic carbon, which in natural conditions is at high concentration in the Baltic Sea waters compared to other marine systems (Deutsch et al. 2012), can become an important factor uncoupling phytoplankton and bacterioplankton dynamics. This is due to the bioavailability of allochthonous DOC to bacterioplankton, which hence allows them to grow and/or produce independently of the DOC released by phytoplankton. This process can lead to the dominance of bacterioplankton over phytoplankton as basal producers. Although BP:PP values fluctuated throughout the experiment (Fig. 3F), its overall increase from the bulk value in all microcosms may support this hypothesis.

**Concluding remarks**

Our results generally showed a lack of clear interactions between the two environmental stressors studied (increased pCO2 and TOM) on natural assemblages of phytoplankton and bacterioplankton from the Gulf of Bothnia. We found strong effects of TOM addition on microbial dynamics, especially on phytoplankton performance. On the other hand, no significant effects were observed from pCO2 alone, indicating a less relevant role of increased CO2 levels in future climate change scenarios in which important TOM inputs have been predicted. Furthermore, we suggest the high allochthonous DOC concentrations present in our experiment as the main driver of uncoupled activities of phytoplankton and bacterioplankton.

The extrapolation of the findings from this study should be taken with caution in respect to marine systems. However, this study provides a fundamental understanding of bottom-up control of basal trophic levels under increased pCO2 and TOM, which constitutes an essential prerequisite to further investigate the interaction with top-down controls and develop experimental setups including higher trophic levels and longer experimental times.

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6. References


