A short heat wave causes a shift in bacterial composition in *Stauroneis constricta* monocultures

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Increasing temperatures cause physiological responses of species populations and thereby change species interactions across and within trophic levels. In marine environments, heat stress can alter algal metabolism, change micro algal community structures and increase bacterial activity. In this study we investigated the effect of a short realistic heat wave of 26 days on monocultures of the diatom *Stauroneis constricta*, one of the dominant producers of the Western Baltic Sea coastal area, and its associated bacteria. Biomass, EPS and bacteria samples were taken during and after the heat wave with a maximal recovery time of 30 days. *S. constricta* total biovolume decreased significantly during the heat wave while bacterial numbers increased. After the heat wave, algal total biovolume slowly increased but pre-heat wave values were never recovered. Bacterial communities in both heat wave and control treatment changed over time. Out of five identified species, two disappeared during the heat wave treatment (*Sulfitobacter* sp. DG885 and *Limnobacter litoralis* KPI-23/18/19) and were not found in the successive sampling times, indicating that the heat wave caused an established shift in bacterial dominance.

Key words: *Stauroneis constricta, climate change, algal-bacterial interaction, 16s rRNA, bacterial composition, monocultures, EPS.*

Introduction

Heat waves, extended periods of unusually high atmosphere-related heat stress (Robinson 2000), have a significant impact on humans (Kalkstein 1991; Semenza *et al.* 1996; Tan *et al.* 2006), terrestrial- (Jolly *et al.* 2005) and marine ecosystems (Garrabou *et al.* 2008; Munari 2011). Assessing the impacts of such extreme weather conditions on ecosystems is important, because in a future warmer climate with increased mean temperatures, heat waves are predicted to become more intense, longer lasting, and/or more frequent (Meehl and Tebaldi 2004).

Heat waves induce physiological responses that influence growth and survival of individuals, which may lead to changes in community structures (Hooper *et al.* 2005; Cardinale *et al.* 2006). This has for example been demonstrated for vegetation growth in the Swiss Alps (Jolly *et al.* 2005), cyanobacterial blooms in eutrophic, shallow lakes (Huber *et al.* 2012), benthic community structure of the Comacchio Saltworks in Italy (Munari 2011) and in Northwestern Mediterranean rocky benthic communities (Garrabou *et al.* 2008).

Consequences of extreme climatic events on ecosystems are difficult to predict because of species-specific interactions both within and between trophic levels. It is therefore crucial to investigate effects of extreme temperatures on food-web dynamics to understand and predict changes in populations and communities.

Effect increased temperature: algae and bacteria

Diatoms are important primary producers in coastal waters and can contribute up to 50% of the total primary production in marine ecosystems (Underwood and Kromkamp 1999). Heterotrophic bacterioplankton are the major organisms responsible for the decomposition of dissolved organic carbon (DOC; Fuhrman 1992), which constitutes >90% of the organic carbon pool in the ocean (Piontek *et al.* 2009).

Elevated temperatures affect these organisms by altering metabolism: a complex network of biochemical reactions that are catalyzed by enzymes, allowing the concentrations of substrates and products and the rates of reactions to be regulated (Brown *et al.* 2004). Increasing temperatures can accelerate enzymatic reactions

as long as they do not cause damage or denature proteins (Piontek *et al.* 2009).

The reaction of an organism to temperature changes is given with the Q10 value. This value shows the respiration rate and therefore activity for each 10°C rise in temperature (Shiah *et al.* 2000). The higher the Q10 value, the higher the respiration rate. For microalgae, Q10 values range between 1.88 and 2.30 for optimal growth (Eppley 1972). For heterotrophic bacteria, these values range around 3 (Pomeroy and Wiebe 2001). Bacteria therefore show higher activity at increased temperatures, and have a higher temperature optimum than phytoplankton.

Marine bacteria and algae are thought to closely interact in the 'phycosphere', which is the micro-zone surrounding algal cells (Bell and Mitchell 1972). In the phycosphere, bacteria may be free-living (Blackburn *et al.* 1998), or attached to the algal surface (Kogure *et al.* 1981). Bacteria can affect diatom growth in both stimulating and inhibiting manners. They can stimulate algal growth by nutrient regeneration, vitamin production and N2 fixation (Cole 1982). Bacteria inhibit growth by using up the supply of dissolved oxygen by aerobic respiration, actively killing the algal cell by lysis, win the competition for nutrient uptake (Cole *et al.* 1982) and by the degrading of extracellular polymeric substances (EPS) (Wotton 2004). EPS are the building blocks of three-dimensional bio films build by benthic diatom communities in which cells are embedded in a self-produced matrix (Decho 1990).

EPS biofilms are made of glucose and other monomers (Bruckner *et al.* 2008) and have several functions: e.g. attaching sessile diatoms, building the adhesion matrix for diatom colonies (Hoagland et al 1993), and stabilizing soft sediment by increasing its critical erosion threshold (de Brouwer *et al.* 2000, Paterson *et al.* 2000). EPS are a major carbon source for diatom associated heterotrophic bacteria (Middelburg *et al.* 2000; Giroldo *et al.*, 2003; Grossart & Simon, 2007; Haynes *et al.* 2007) and temperature induced increases in bacterial activity can therefore create unfavorable and destabilizing conditions for diatoms. Moreover, elevated temperatures also increase the activities of sugar- and protein-degrading extracellular enzymes (Piontek *et al.* 2009) which may limit EPS concentrations even further by increasing EPS degradation.

We studied effects of a heat wave on the benthic diatom *Stauroneis constricta* and its associated bacteria. S*tauroneis constricta* is a solitary mobile benthic diatom and the dominant biomass producing microalgae in rocky-shore periphyton communities (mixtures of algae in aquatic systems that are attached to the sediment surface or to aquatic, macrophyte vegetation) in the southern Baltic Sea (Matthiessen *et al.* 2010, Eggers *et al.* 2012). An earlier study demonstrated that a heat wave caused bacteria to increase while diatoms decreased, and that this shift in community structure was stable up to 10 days after the heat wave (Helen Moore 2010). It was suggested that initially the microalgae suffered from inefficient metabolic rates, enzyme malfunction and destabilization due to increased EPS degradation; and that subsequent recovery of the diatoms was inhibited due to the persisting high abundance of bacteria. We hypothesized that the permanent shift in community towards bacteria dominance was mediated by changes in bacterial species composition leading to changed competitive hierarchies favoring bacteria over diatoms; by for example an intensified nutrient depletion by changes in bacteria nutrient uptake rates. In this thesis, we tested if a heat wave induced changes in community structure over longer times that included a shift from diatom to bacteria dominance, as well as changes in bacteria community composition. We did this by exposing *S. constricta* cultures to a heat wave and following recovery for 30 days. We hypothesized that:

1. The heat wave shift community structure to an alternative state with a permanently lower diatom abundances and permanent higher bacteria abundances.

2. The heat wave cause a change in the species composition of the bacteria community that accompany the increase in bacteria abundances.

Materials and methods

Culture conditions

Monocultures of *S. constricta* diatoms were isolated from periphyton samples of the Kiel Fjord. These cultures were kept in filtrated Baltic Sea Water (16 psu) enriched with N:P:Si concentrations up to the Redfield ratio, comparable with the early spring nutrient conditions in the Kiel Fjord. All cultures were kept at a 18-8 light/dark regime at a constant light intensity of 50 µmol photons s^{-1} m^{-2}. Temperature and light conditions were controlled by a computer program (CID-PRO 4.01) and recorded using Tinytalks that were checked regularly.

Experimental setup

36 monocultures of *S. constricta* were created by subculturing from the same stock culture, with an inoculum of 9.72 x 10^9 *S. constricta* biovolume/flask (each flask contained 40 ml. medium). All cultures were given new medium every third day: 20 ml of the old medium was carefully removed from the top layer and replenished with 20 ml new medium.

Cultures were randomly assigned to four climate cabinets to decrease cabinet effect. Half of the cultures (18) were subjected to simulated heat wave (a condensed version of the natural heat wave of 2003 in the Baltic Sea), while the other half (18) were set as the control group. The two control cabinets were set to a constant temperature of ±16°C, each containing nine cultures. The other two cabinets were set to simulate a heat wave. Both heat wave and control program ran for 61 days continuously. The heat wave consisted of 6 days of 16°C, followed up by a step-by-step raise in temperature from day 7 to day 27, where the temperature rose from 16°C to 27°C. At day 27, the temperature gradually moved back to 16°C (day 31). From this day forward, the temperature was set to 16°C until the last sampling day (day 61).

Samples were taken four times (figure 1): the day after the heat wave reached the highest temperature (T1), the first day after the heat wave (T2), 10 days after the heat wave (T3) and 30 days after the heat wave (T4). Samples at T1, T2 and T3 were taken in quintuplicate while T4 samples were taken in triplicate.

Sampling

Each sampling time we took sampled for analyses of bacterial DNA, RNA and biomass, EPS concentrations and *S. constricta* biomass. Before sampling, the algae were carefully and thoroughly scraped from the bottom area and the bottle was shaken roughly 3 times. After shaking, the bottles were turned over 2 times to homogenize. This homogenization step was repeated between every sample. Sampling took place in the sterile environment of the flow hood.

Bacterial DNA (bacterial composition) *and RNA* (bacterial activity) samples (8-10 ml and 5 ml respectively) were filtrated over 2.0 µm polycarbonate filters to capture the attached bacteria (Crump *et al.* 1999). The filtrate was captured on a 0.2 µm polycarbonate filter to capture the free-living bacteria. Filters were folded once, placed in a safelock Eppendorf cup, snap-frozen in liquid nitrogen and stored at -80°C

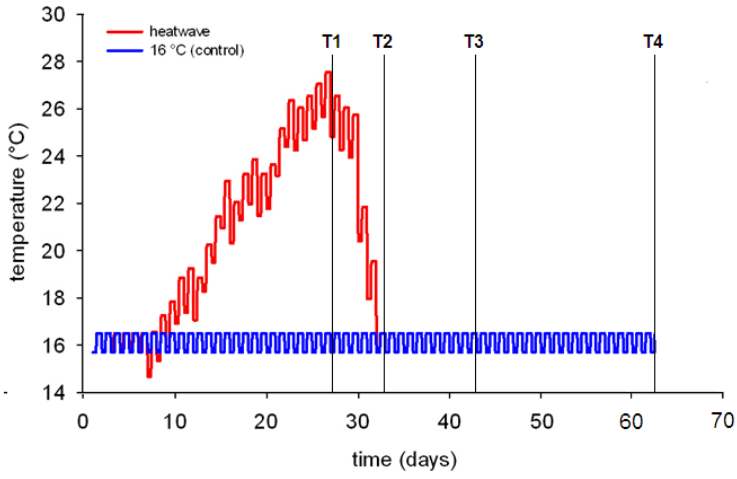

Fig. 1: *Temperature curve of the heat wave (red) and control (blue) treatment. Samples were taken after highest temperature (T1), directly after the heat wave (T2), 10 days after the heat wave (T3) and 30 days after the heat wave (T4)*

until further analysis.

EPS was sampled by capturing 4 ml of each culture in white cap Plexiglas tubes and EPS was extracted according to de Brouwer & Stal, (2002) and Wolfstein & Stal. (2002) (*appendix I-II*).

Bacterial biomass was sampled by capturing 4 ml culture into 5 ml kryotubes. 200 µl 37% formalin was added, the tubes were snap frozen in liquid nitrogen and stored at -80°C until further analysis. No exact bacterial counts were made due to flow cytometer malfunctions, and bacterial growth was monitored with microscope viewings.

Algal biomass was sampled by storing the remaining culture fixed in acid Lugol (10 ml = 150 µl) in a small brown glass bottle. All Lugol bottles were stored at 4°C in a box to keep the samples in the dark.

Molecular analysis

DNA was extracted using the standard cetyltrimethylammonium bromide method (Doyle & Doyle, 1987) (*appendix V*) and stored at -80ºC. For PCR the variable V6 region of the 16S rRNA gene was amplified with the universal bacterial primers U968f-GC forward primer, containing a GC clamp at the 50 end, and the U1401R reverse primer (Nübel *et al.*, 1996). A touchdown PCR program was used for gene amplification (*appendix VI*). 2 µl PCR products were mixed with Orange G loading dye and separated by DNA gel-electrophoresis on a 1% agarose gel stained with ethidium bromide (*appendix VII*). Products were visualised with UV light and amplication yield was estimated by comparing the bands with a DNA Smart Ladder (O'GeneRuler 1kb DNA Ladder). PCR products of expected size and quantity were subjected to DGGE analysis (*appendix VIII-XIV*): 80 ng of each amplicon were run on DGGE with 20-70% denaturing gradients. 14 of the most visual bands were selected for sequencing (triplicates) and cleaned using EXOSAP (*appendix XV*) and Sephadex G-50 (*appendix XVI*). 14.4 ng of the PCR products were analysed using Sanger sequencing (ABI-3130XL) (*appendix XVII*). Sequences were checked manually using CHROMAS v. 2.01 and the best result from each triplicate was chosen for finding the corresponding microorganism using NCBI BLAST (http://www.ncbi.nlm.nih.gov/BLAST).

RNA was extracted using TRIzol (*appendix XVIII*) and stored at -80ºC. Due to lack of time RNA could not be further analysed.

EPS analysis

EPS was analysed according to the Phenol/H2SO4 assay after Underwood *et al.* (1995) , Liu *et al.* (1973) and Dubois *et al.* (1956) (*appendix I-IV*). Each sample was compared to a glucose dilution series by measuring duplicates of the samples with a spectrophotometer (Wallac 1420 Multilable Counter Victor^{TM}) at 485 nm. The amount of glucose (µg ml^{-1}) was calculated and corrected for the glucose standard.

Biovolume analysis

Algal biovolume was calculated from microscopy cell counts. The lugol fixed samples were placed in an Uthermöhl counting chamber (total area 531 mm^2, volume 2.973 ml). Cells were counted with an inverted microscope (ZEISS) at 40x magnification (phase contrast). A grid (0.091991 mm^2 at 40x magnification) was used to count. Both alive and dead cells were counted.

Total algal biovolume per area (µm^3 cm^{-2}) was calculated according to Hillebrand *et al.* (1999), with a set biovolume of 5809,17 µm^3 per cell. Also empty silicate shells were counted for both treatments to indicate dead, and therefore lost, algal biovolume.

Statistics

Factorial ANOVA's were used to test the main effects of the heatwave over time on biovolumes and EPS (temperature and time was used as crossed fixed factors in the analyses). Assumptions of homogenous variances were tested with the Cochran C. test and the Fisher LSD post hoc test was used to determine which pairs differed. Alive biovolume and EPS were square-root transformed.

Results

Biovolume

The biovolume of *S. constricta* decreased from the heat wave and was significantly lower in the heat wave treatment compared to the control treatments throughout the experiment (Main effect of heat wave: $F=172.96$, $p<0.01$; Fig. 2, Fig. 4, upper graph). In addition, there was a significant interaction effect between heat wave treatment and time ($F=3.48$, $p<0.05$). At 16 degrees, *S. constricta* increased continuously with time: biovolume was significantly higher at T4 compared with T1 (Fishers LSD test: $p<0.01$) (Fig. 4, upper graph). In the heat wave treatment *S. constricta* first decreased (T1 was significantly higher than T3; Fishers LSD test $p<0.01$) then increased (T4 significantly higher than T1-3; Fishers LSD test $p<0.01$),

Dead biovolume did not change significantly during the heat wave treatment, but showed a significant interaction effect in the control treatment and time ($F=82.07$, $p<0.01$). In this treatment, dead biovolume accumulated over time with a significant increase between all sampling times (Fisher LSD test: $p<0.01$ for each T).

The dead biovolume exceeded the total (alive) biovolume at T1, T2 and T3, but due to cell growth at T4 the alive and dead biovolume reached about the same levels. The lack of increase in dead biovolume is directly correlated to the lack of alive biovolume.

EPS

EPS concentrations did not change significantly over time for both the heat wave and the control treatment. Effect of heat wave was only visible when comparing between the two treatments: from T2 to T4 the heat wave treatment showed significantly less EPS than the control treatment (Fisher LSD post hoc test, $p<0.01$ (T2 and T3), $p<0.05$ (T4)).

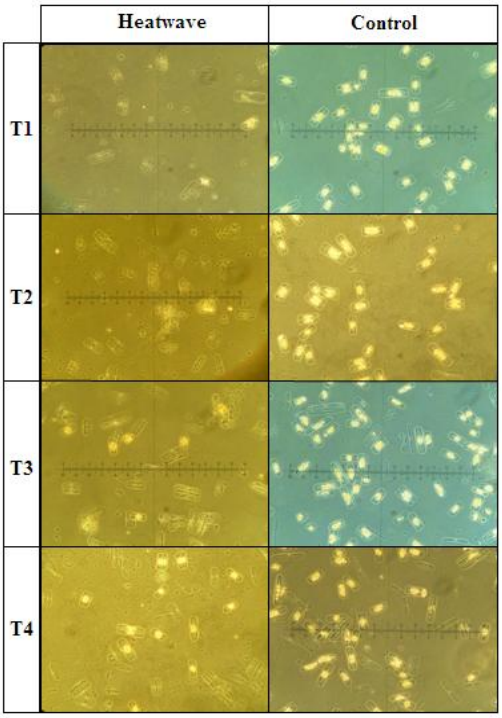

***Fig. 2**: Microscope pictures (40 x magnification) from S. constricta over time.*

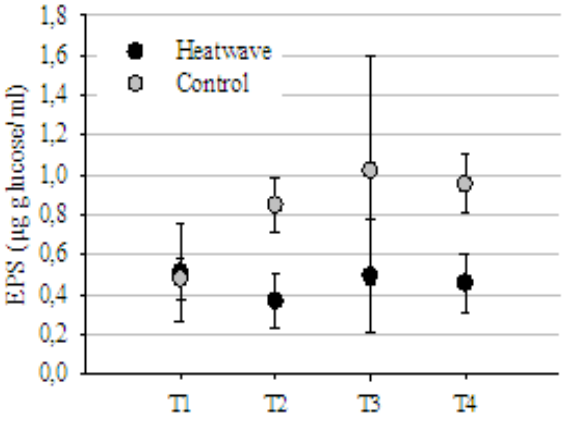

***Fig. 3**: EPS concentration (µg glucose/ml) with standard errors for the heat wave (black) and control (grey) treatment for each sampling time (T).*

Bacterial composition

DGGE analysis of the 16S rRNA gene showed differences in bacterial composition between the heat wave and the control treatment (fig. 5). A total of five bacterial species were identified in the control treatment by extracting DNA from the gels, while the heat wave treatment contained only three species. Other bands represented *S. constricta* chloroplast DNA. Moreover, the control treatment only contained free-living

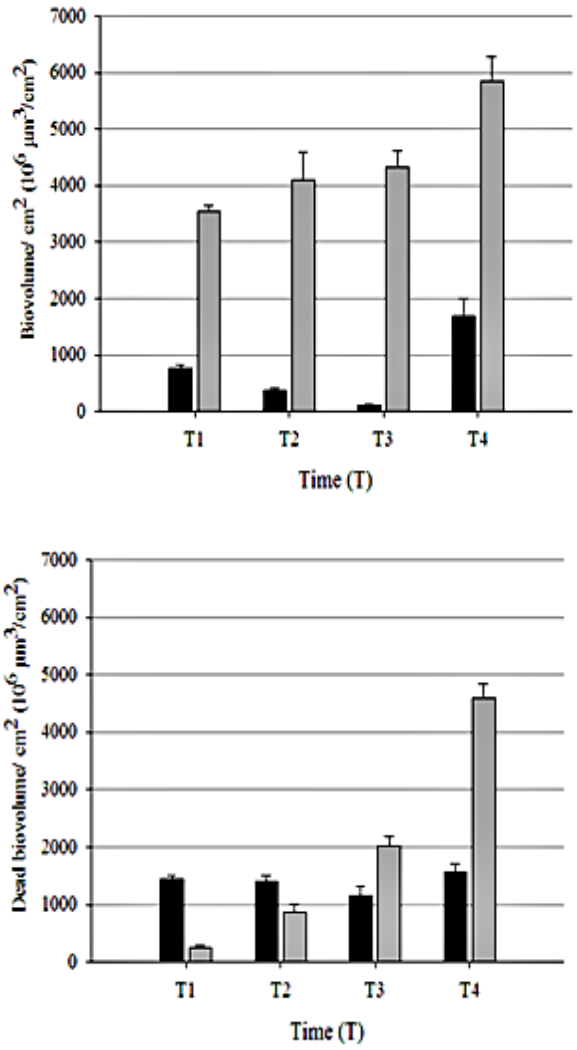

Fig. 4: *Development of biovolume for both alive (upper graph) and dead cells (lower graph) of Stauroneis constricta over time in the heat wave treatment (black) and the control treatment (gray). Plain bars indicate total algal biovolume and striped bars indicate dead algal biovolume. Error bars denote SE.*

bacteria while the heat wave treatment contained both free-living and attached bacteria. The fact that sometimes only one or two species were found made it impossible to perform dominance or diversity calculation. Therefore dominance was indicated manually from the DGGE gel results (fig. 5, graphs). Both treatments showed differences in band thickness over time, indicating fluctuations in bacterial biomass.

The following species were found:

(1) *Loktanella* sp. RCC2403, an α-proteobacteria of the order Rhodobacteraceae (Garrity *et al.* 2005), an order that contains approximately 70 recognized genera from which over two-thirds of the species originate from marine environments (Kim *et al.* 2010).

(2) *Limnobacter litoralis* KPI-23/18/19, a gram-negative, aerobic, heterotrophic bacteria (Lu *et al.* 2011) of the class β-proteobacteria.

(3&4) *Marinobacter* B. and FO-NAM9**,** a nitrifying and de-nitrifying bacteria that is capable of carrying out the transformation $NH_3 \rightarrow NO_2^-$ or $NO_2 \rightarrow NO_3^-$.

(5) *Sulfitobacter* sp. DG885, a gram-negative, aerobic, α-proteobacteraceae, sulfite-oxidating bacteria of the order Rhodobacteraceae.

All 5 species were represented in the temperature control treatment (fig. 5, right graph). *Sulfitobacter* sp. and *Limnobacter litoralis* biomass did not seem to fluctuate much over time, while *Loktanella* sp. and the two *Marinobacter* species showed changes in total biomass. The two *Marinobacter* species behaved in the same manner, with high total biomass at sampling time T1 and lower total biomass in the successive sampling times. *Loktanella* sp. showed highest total biomass at sampling time T2 and the lowest total biomass at T1.

In the heat wave treatment (fig. 5, left graph), *Limnobacter litoralis* and *Sulfitobacter* sp. disappeared already during the heat wave. The total biomass of the three remaining species fluctuated over time. The two *Marinobacter* species behaved in the same manner, with the lowest total biomass at T1 and the highest at T3. *Loktanella* sp. showed highest total biomass at T1 and T4.

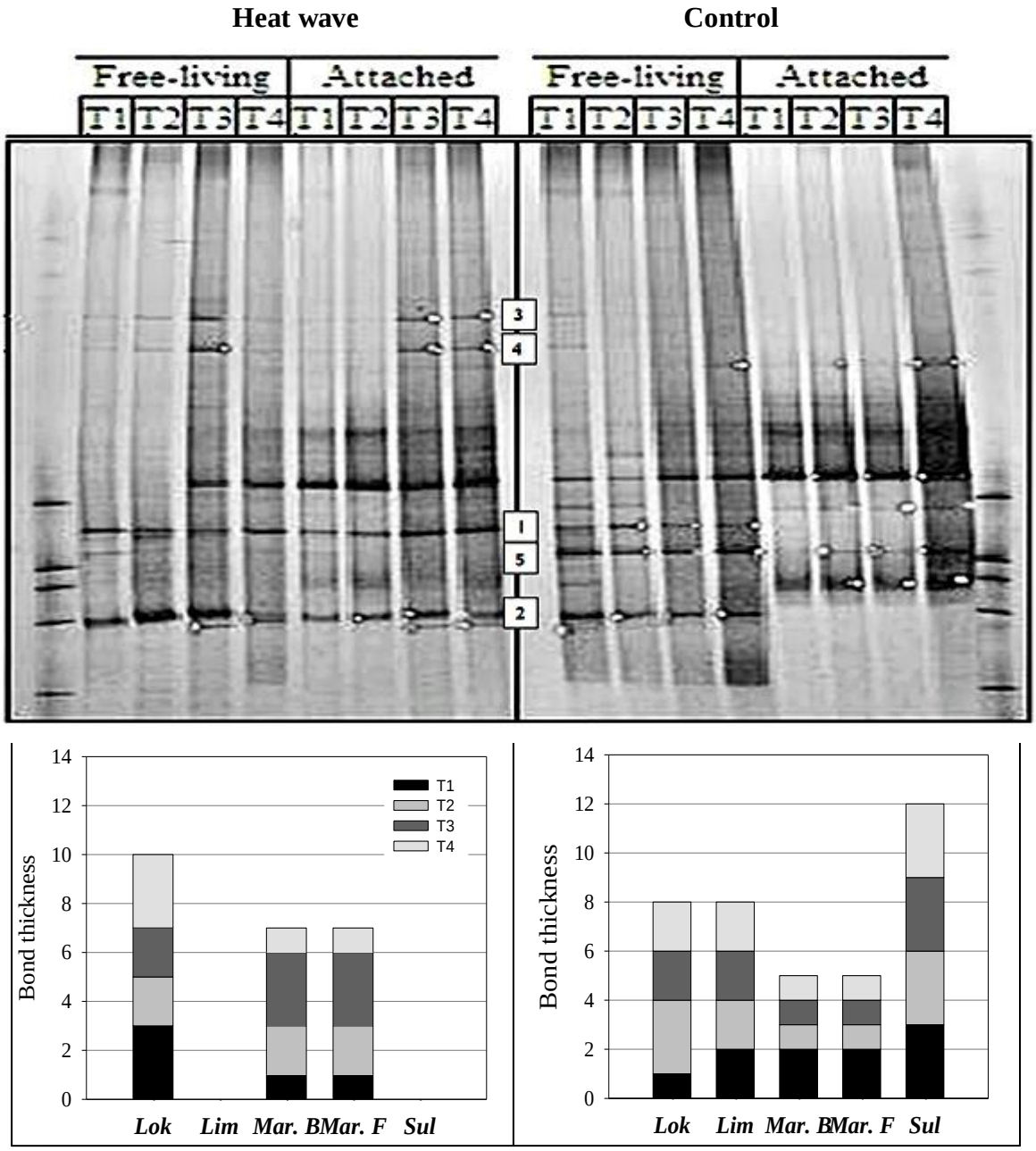

Fig. 5: *DGGE results and estimated biomass of (1) Loktanella sp. (**Lok**), (2) Limnobacter litoralis (**Lim**), (3) Marinobacter B (**Mar.B**), (4) Marinobacter FO-NAM9 (**Mar.F**) and (5) Sulfitobacter sp. (**Sul**). The graphs (left: heat wave, right: control) were composed from estimated band thickness. The thicker the band, the higher the bacterial biomass at that given sampling time. Thickest bands were given 3 points, thinnest 1 point and bands in between 2 points. The graphs do not show actual amounts.*

Discussion

The heat wave changed the structure of the experimental periphyton community by promoting bacteria and suppressing diatoms. This change was still evident after 30 days of recovery, suggesting that the heat wave had shifted the community to an alternative stable state – supporting hypotheses 1. The shift in community structure also had significant effects on the function of the periphyton community, as EPS levels decreased by 50 % after the heat wave.

The results support previous findings of a possible alternative state (Helen Moore 2010) and indicate that *S. constricta* is unable to fully

recover after a heat wave due to increased bacterial activity and changes within the bacterial community.

Total algal biovolume

There was no full recovery, as total biovolume only reached about 1/3 of the carrying capacity seen in the control treatment at the end of the experiment (resp. 5,854 ± 729, 1,686 ± 539 10^6 $\mu m^3/cm^2$). Moreover, total biovolume levels at T4 (heat wave treatment) never reached up to control biovolume levels found at T1, where *S. constricta* of the control experiment had been growing for 26 days from inoculation until T1, and had reached stationary phase with a stable carrying capacity at 3,547 ± 243 10^6 $\mu m^3/cm^2$. This incomplete recovery could be due to photosystem/metabolism damage after the temperature shock, the presence of bacteria consuming EPS and preventing the diatoms from recapturing their stable condition or a combination of these factors. Moreover, the bacteria compete with the diatoms for nutrients, and their large numbers give them an advantage in obtaining these nutrients. The heat wave treated *S. constricta* cultures may have reached an alternative state.

An explanation for the lack of differences in dead biovolume would be the massive cell death seen directly after the highest temperature (T1). Since the silicate shells of the diatoms stay behind after the diatom dies, these shells are re-counted at each sampling time. The lack of more total biovolume loss indicates that there are not that many cells left to die. Although a significant decline of total "alive" algal biovolume from T1-T3 would suggest a significant increase of dead biovolume as a result, this was not found. This could be explained through dissolution of the silicate skeletons. The high numbers of bacteria could have increased the silicate turnover rate. Bacteria accelerate silica dissolution in the sea by colonizing and enzymatically degrading the organic matrix of diatom frustules (Bidle and Azam 2001). Also, it has been suggested that the rate of bacterial attack on diatom films may control the rate of dissolution of the skeletons to some degree (Kamatani 1982). Finally, a drop in pH can contribute to a faster dissolving rate of the empty diatom shells which could be caused by increased bacterial activity.

EPS

EPS levels did not show significant change over time. For the heat wave treatment this is somewhat peculiar, since bacterial numbers increase over time and therefore EPS levels are expected to decline due to EPS consumption. This somewhat stable EPS concentration could be explained by intracellular polysaccharides (IPS) that were released from the many lysed diatom cells (Baldi *et al*. 1997) and caused an overload of polysaccharides.

EPS levels slightly increased over time in the control experiment and showed about the same trend as the total algal biovolume, which is expected since more diatom cells cause higher EPS concentrations. The high variation at T3 is due to two outliers which are possible measurement errors. The low EPS concentration at T1 (control treatment) cannot be explained.

Bacteria

Bacterial abundance

Bacteria increased during the heat wave (microscope findings) and showed immense numbers directly after the heat wave (T2). This confirms the bacterial temperature advantage over the diatom *S. constricta*. Bacterial increase due to increased temperatures has been shown in previous studies (Kirchman *et al*. 2005, López-Urrutia *et al*. 2006) when nutrients are resupplied regularly (O'Connor *et al.* 2009). Microscope samples show a decrease in bacteria from T3, which is most likely a combined factor of EPS and temperature decrease. When diatom numbers are low and EPS production decreases, bacteria become limited by carbon (Liu *et al.* 2012) . The decrease in bacterial biovolume has a positive effect on *S. constricta*: the diatoms encounter less competition and can regain their growth. Although EPS limitation is a plausible reason why bacterial numbers start decreasing, EPS measurements did not underline this statement. Therefore, the decrease of bacteria after the heat

wave (T3 and T4) is most likely temperature and therefore Q10 related.

Bacterial composition

The shift in community structure was accompanied by a dramatic change in bacteria species composition – supporting hypothesis 2.

Four reasons could explain why so little species were found. First, only few bacterial species were interacting with the *S. constricta* monocultures and the five species found represent the actual bacterial community. Second, some species were missed during sampling: the filtration method used, the DNA extraction method used or rare species were missed during PCR. Third, species could have been missed due to overshadowing of other DNA. This last reason is very plausible: From all 14 bonds that where sequenced, only 5 represented bacteria. All other (sometimes rather thick) bonds represented *S. constricta* chloroplast DNA. The fourth reason could be because of missed bands in the DGGE gel. Only the clearly visible bands were picked for sequencing and less abundant species (represented by hardly visible bands) were left out.

An explanation why attached bacteria were found in the heat wave but not in the control treatment is that these bacteria got trapped in empty diatom silicate shells (*microscope findings, fig. 6*). In the first filtration step (2 µm) bacteria should pass through the filter while the diatoms were caught. Bacteria trapped in the empty shells were probably captured on the filter and wrongly classified as attached bacteria.

If this is the reason why attached bacteria were found in the heat wave treatment but not in the control, a lack of empty shells (dead diatoms) would be expected in the control experiment, but this is not the case. Why the bacteria in the control experiment did not get trapped and no attached bacteria were found is not clear. An explanation could be that there were only a few bacteria present in the control samples, reducing the chance that they would become trapped. But microscope samples did show trapped bacteria in the control treatment (fig. 6), disproving this hypothesis.

Fig. 6*: Bacteria trapped in an empty S. constricta shell. Picture taken at T2 (control treatment).*

Bacterial role in the monocultures

Unfortunately, only little information about the uncovered bacterial species could be found, but their main role within the marine ecosystem was recovered. With the increase of the two *Marinobacter* species the process of nitrification (sequential oxidation of ammonia (NH_4^+) to nitrate (NO_3^-) via nitrite) or denitrification (nitrate (NO_3^-) reduced to nitrogen gas (N2)) also increases. This increase of nitrate might cause eutrophication and could improve *S. constricta* growth. On the other hand, nitrification leads to acidification by internal H^+ production (van Miegroet & Cole 1984), which causes more rapidly dissolving of the silica walls of diatoms (Lewin 1961). If the nitrate levels did indeed increase is not known, and nitrate measurements are needed to confirm this. Also, pH measurements could give more information about the process of nitrification and could explain if acidification played a role in both recovery and the dissolving of empty silicate shells (as less empty shells were found at T3 than expected).

Marinobacter is also able of denitrifying: under anoxic conditions certain heterotrophic bacteria are stimulated into utilising nitrates and nitrites as final electron acceptors for cellular respiration in place of oxygen (Ketchum, 1988; Cappuccino and Sherman, 1992) and denitrification takes place by nitrate and nitrite reduction. This actively removes nitrate from the water, creating a undesirable environment for the diatoms and impeding recovery. Nitrate and oxygen measurements are necessary to indicate if denitrification took place and if the environment has turned anoxic.

The disappearance of the two sulfur processing bacterial species (*Sulfitobacter* sp. and *Limnobacter litoralis*) indicate that these species cannot survive high temperatures. Another explanation for the disappearing of these two species is that they are outcompeted by other species which have an advantage at higher temperatures. At least one of the "lost" species, *Sulfitobacter* sp., is a dominant species in the control experiment. The other species, *Limnobacter litoralis*, also plays a large role in the control samples. When these two rather dominant species disappear during the heat wave, the three other species become more abundant. If this increase is a direct result from the disappearance of *Sulfitobacter* sp. and *Limnobacter litoralis*, or if their disappearance is due to the increase of the other three species is not known.

The lack of thiosulfate ($S_2O_3^{2-}$)- and sulfite (SO_3^{2-})- oxidizing bacteria in a system means no turnover of sulfur compounds in the monocultures. Sulfur plays an important role as sulfate (SO_4^{2-}) or sulfide (HS^-) are the sulfur sources used by algae for amino acid construction (Mason and Kelly, 1988). The inability found in heat wave treated *S. constricta* monocultures to grow back at control-equal values might be explained by the (co-)factor of reduced or even removed sulfate in the system. If these species disappeared completely is hard to tell from the sequenced DNA and conclusions should be drawn carefully. Beside DNA samples, RNA samples were taken to get more knowledge about the bacterial activity over time. Sequencing these RNA samples could give more information about whether or not *Sulfitobacter* sp. and *Limnobacter litoralis* have disappeared during the heat wave. RNA might also reveal possible 'missed' species that have been overshadowed by chloroplast DNA. Unfortunately there was no time to analyse the RNA, but sequencing RNA would probably give more information about the bacterial activity over time.

Bacterial role in vivo

Comparing monocultures with *in vivo* metacommunities is difficult. The Baltic sea metacommunities are complex, with many more players in the photosynthetic role. On one hand, the incapability for *S. constricta*, the dominant producer in this ecosystem, to fully recover after a heat wave could mean a great loss of algal biomass. On the other hand there is the possibility for other species to take over and become the dominant species, compensating *S. constricta* biomass loss.

In a previous research on metacommunities (de Boer *et al.* submitted) this possible compensation has been investigated. De Boer found *S. constricta* to be the main driver of ecosystem processes and found that its loss during a comparative heat wave was not compensated by the second dominant diatom *Amphora coffaeiformes*, neither by the other eight species of the metacommunity. Furthermore, she found that cultures only containing *S. constricta* were the most resilient to a heat wave (with regard to biomass). When looking at recovery, de Boer *et al.* (submitted) found no recovery in all metacommunities ten days after the heat wave.

We found the same result in our monocultures, and as in de Boer *et al.* (submitted), *S. constricta* biomass had decreased even more instead of recovering. In our research we gave the *S. constricta* monocultures twenty days more to recover, and recovery occurred somewhere between the 10^{th} and 30^{th} day after the heat wave. This shows that *S. constricta* is able to recover, but low cell numbers after 30 days at 16℃ shows that the alternative stable state suggested by Moor (master thesis), with a shift from algal to bacterial dominance, is plausible. Concerning the microbial community and its change during our experiment it is important to consider what this change could mean for *in vivo* ecosystems.

Marine microbes are very important for marine carbon re-mineralisation and maintaining. Microbes account for more than 95% of the respiration in the oceans (Del Giorgio & Duarte 2002). They control global utilisation of nitrogen through N_2 fixation, nitrification, nitrate reduction, and denitrification, and drive the bulk of sulfur, iron, and manganese biogeochemical

cycles (Kirchman 2008; Whitman *et al*. 1998). It is widely accepted that shallow coastal sediments are important sites for the mineralisation of organic matter (Jorgensen 1982; Lomstein & Blackburn 1992). These transformations are mediated principally by bacteria and the resulting gradients of nutrients result in their release to the overlying water column or adsorption and burial in deeper sediment layers (Herbert 2006). A shift in dominance and decrease or disappearance of two species due to a heat wave, the sulfite oxidizing *Sulfitobacter* sp. and thiosulfate-oxidizing *Limnobacter litoralis*, could cause a possible change in sulfur turnover in the Western Baltic Sea.

Fortunately, sulfate reduction in the coastal areas of the Baltic Sea is carried out by many species of the eubacteria, archaebacteria and chemolitothropic sulfur oxidising bacteria (Widdel 1988; Trüper 1975; Kuenen *et al*. 1992). Therefore it is unlikely that the loss or decrease of *Sulfitobacter* sp. and *Limnobacter litoralis* would have a large impact on the sulfur cycle. An increase in nitrification due to increased numbers of the nitrifying *Marinobacter* species could cause eutrophication and acidification of the Baltic Sea, but decreased numbers in our two nitrifying species 30 days after the heat wave indicate that nitrification returns back to pre-heat wave levels when temperatures are normal again.

Although bacterial composition had changed due to the simulated heat wave, there seemed to be no persisting bacterial dominance. Bacterial numbers decreased after the heat wave (microscope findings) and *S. constricta* slightly recovered. Instead, the findings showed an alternative state with a shift in bacterial composition and lower total algal biomass, without a clear dominant organism. It is likely that the changes in bacterial composition played an important role in *S. constricta* recovery. The disappearance of sulfate and/or decrease in nitrate could explain this slow and incomplete recovery.

It is unsure if the findings indicate an irreversible alternative state and RNA analysis could give more information on the subject. Even if this shift in bacterial composition is indeed irreversible and *S. constricta* recovery is directly correlated to bacterial composition, it is unlikely that this shift has a large effect on the Kiel Fjord ecosystems. There are many more players in the microbial community and it is doubtful that all species accountable for important processes like sulfate reduction disappear after a heat wave.

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Reference list

BACC, the BALTEX Assessment of Climate Change for the Baltic Sea basin. Regional climate studies ISSN 2008 Springer-Verlag Berlin Heidelberg (pp 59)

Baldi F, Minacci A, Saliot A, Mejanelle L, Mozetic P, Turk V, Malej A. 1997. Cell lysis and release of particulate polysaccharides in extensive marine mucilage assessed by lipid biomarkers and molecular probes. Mar Ecol Prog Ser Vol. 153: 45–57

Bannerman JA, Gillespie DR, Roitberg BD. 2011. The impacts of extreme and fluctuating temperatures on trait-mediated indirect aphid–parasitoid interactions. *Ecol. Entomol.* Vol. 36, 490–498

Bell WH and Mitchell R. 1972. Chemotactic and growth responses of marine bacteria to algal extracecllular products/. Biol. Bull. Vol. 143: 265-277

Bidle KD and Azam F. 2001. Bacterial control of silicon regeneration from ddiatom detritus: significance of bacterial ectohydrolase ad species identity. Limnol. Oceanogr. Vol. 46: 1606-1623.

Blackburn N, Fenchel T, Mitchel J. 1998. Microscale nutrient patches in planktonic habitats shown by chemotactic bacteria. Science Vol. 282: 2254–2256

Brown JH, Gillooly JF, Allen AP, Savage VM, West GB. 2004. Toward a metabolic theory of ecology. *Ecology* Vol. 85(7): 1771–1789

Bruckner CG, Bahulikar R, Rahalkar M, Schink B, Kroth PG. 2008. Bacteria Associated with Benthic Diatoms from Lake Constance: Phylogeny and Influences on Diatom Growth and Secretion of Extracellular Polymeric Substances. Applied and

environmental microbiology. Vol. 74(24): 7740–7749

Cappuccino JG, Sherman N. 1992. Microbiology; A Laboratory Manual (3rd edn.). Rockland Community College, Suffern: New York.

Cardinale BJ, Srivastava DS, Duffy JE, Wright JP, Downing AL, Sankaran M, Jouseau C.2006. Effects of biodiversity on the functioning of trophic groups and ecosystems. Nature Vol. 26;443(7114): 989-92

Ciais P, Reichstein M, Viovy N, Granier A, Ogée J, Allard V, Aubinet M, Buchmann N, Bernhofer C, Carrara A, Chevallier F, De Noblet N, Friend AD, Friedlingstein P, Grünwald T, Heinesch B, Keronen P, Knohl A, Krinner G, Loustau D, Manca G, Matteucci G, Miglietta F, Ourcival JM, Papale D, Pilegaard K, Rambal S, Seufert G, Soussana JF, Sanz MJ, Schulze ED, Vesala T, Valentini R. 2005. Europe-wide reduction in primary productivity caused by the heat and drought in 2003. Nature. Vol. 22;437(7058): 529-33

Cole JJ. 1982. Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics*, Vol. 13: 291-314

Crump BC, Armbrust EV, Baross JA. 1999. Phylogenetic analysis of particle-attached and free-living bacterial communities in the Columbia river, its estuary, and the adjacent coastal ocean. *Applied and environmental microbiology*. Vol. 65(7): 3192-204

de Boeck HJ, Dreesen FE, Janssens IA, Nijs I. 2011. Whole-system responses of experimental plant communities to climate extremes imposed in different seasons. New Phytol. Vol. 189(3): 806-17

de Brouwer JFC, Bielic S, de Deckere EMGT, Stal LJ. 2000. Interaction between biology and sedimentology in a mudflat (Biezelingse Ham, Westerschelde, the Netherlands). Cont. Shelf Res Vol. 20: 1159–78

de Brouwer J FC and Stal LJ. 2002. Daily fluctuations of exopolymer in cultures of the benthic diatoms *Cylindrotheca closterium* and *Nitzschia* sp. (Bacillariophyceae). J. Phycol. Vol. **38**: 464-472

Decho AW. 1990. Microbial exopolymer secretions in ocean environments: Their role(s) in food webs and marine processes. Oceanogr. Mar. Biol. Annu. Rev. Vol. 28: 73–153

del Giorgio PA, Duarte CM. 2002. Respiration in the open ocean. Nature Vol. 420: 379–84

Doyle JJ & Doyle JL. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. Phytochem Bull Vol. 19: 11–15

Dubois M, Gilles KA, Hamilton JK, Rebers PA, Smith F. 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. Vol. 28: 350-356

Eggers SL, Eriksson BDHK, Matthiesen B. 2012. A heat wave and dispersal cause dominance shift and decrease biomass in experimental metacommunities. Oikos Vol. 121(5): 721-733

Eppley RW. 1972. Temperature and phytoplankton growth in the sea. Fishery bulletin Vol. 70(4): 1063-1085

Fuhrman JA (1992) Bacterioplankton roles in cycling of organic matter: the microbial food web. In: Falkowski PG, Woodhead AD (eds) Primary productivity and biogeochemical cycles in the sea. Plenum Press, New York, p 361–383

Garrabou J, Coma R, Bensoussan N, Bally M, Chevaldonne P, Cigliano M, Diaz D, Harmelin JG, Gambi MC, Kersting DK, Ledoux JB, Lejeusne C, Linares C, Marschal C, Pérez T, Ribes M, Romano JC, Serrano E, Teixido N, Torrents O, Zabala M, Zuberer F, Cerrano C. 2009. Mass mortality in Northwestern Mediterranean rocky benthic communities: effects of the 2003 heat wave. Global Change Biology Vol. 15(5): 1090–1103

Giroldo D, Vieira AAH, Paulsen BS. 2003. Relative increase of deoxy sugars during microbial degradation of an extracellular polysaccharide released by a tropical freshwater Thalassiosira sp. (Bacillariophyceae). J. Phycol. Vol. 39: 1109–1115

Grossart HP and Simon M. 2007. Interactions of planktonic algae and bacteria: effects on algal growth and organic matter dynamics. Aquat. Microb. Ecol. Vol. 47: 163–176

Grossart HP, Tang KW, Kiørboe T, Ploug H. 2007. Comparison of cell-specific activity between free-living and attached bacteria using isolates and natural assemblages. FEMS Microbiol Lett Vol. 266: 194–200

Haynes KTA, Hofman C J, Smith AS, Ball GJ, Underwood C, Osborn AM. 2007. Diatom-derived carbohydrates as factors affecting bacterial community composition in estuarine sediments. Appl. Environ. Microbiol. Vol. 73: 6112–6124

Hillebrand H, Dürselen CD, Kirschtel DB, Pollingher U, Zohary T. 1999. Biovolume calculation for pelagic and benthic microalgae. J. Phycol. Vol. 35: 403–424

Hoagland KD, Rosolvski JR, Gretz MR, Roelner SC. 1993. Diatom extracellular polymeric substances: function, fine structure, chemistry, and physiology. J Phycol Vol. 29: 537-566

Hooper DU, Chapin FS, Ewel JJ, Hector A, Inchausti P, Lavorel S, Lawton JH, Lodge DM, Loreau M, Naeem S, Schmid B, Seta H, Symstad AJ, Vandermeer J, Wardle DA. 2005. Effects of biodiversity on ecosystem functioning: a consensus of current knowledge. Ecological Monographs Vol. 75(1): 3–35

Huber V, Wagner C, Gerten D, Adrian R. 2012. To bloom or not to bloom: contrasting responses of cyanobacteria to recent heat waves explained by critical thresholds of abiotic drivers Oecologia Vol. 169: 245–256

Jentsch A, Kreyling J, Beierkuhnlein C. 2007. A new generation of climate-change experiments: Events, not trends. *Frontiers in Ecology and the Environment* Vol. 5: 365–374

Jorgensen B. 1982. Ecology of the bacteria of the sulphur cycle with special reference to anoxic–oxic interface environments. *Philos T Roy Soc London* Vol. 298: 543–61

Kalkstein LS. 1991. A New Approach to Evaluate the Impact of Climate on Human Mortality. Environ. Heal. Persp. Vol. 96: 145-150

Kamatani A. 1982. Dissolution Rates of Silica from Diatoms Decomposing at Various Temperatures Marine Biology Vol. 68: 91-96

Ketchumpa. 1988. Microbiology: Concepts and Applications. John Wiley & Sons, Inc.: Canada.

Kirchman DL. 2008. New light on an important microbe in the ocean. Proceedings of the National Academy of Sciences Vol. 105: 8487-8488

Kogure K, Simidu U, Taga N. 1981. Bacterial attachment to phytoplankton in sea water. Journal of Experimental Marine Biology and Ecology Vol. 56(2-3): 197-204

Kuenen JG, Robertson LA, Touvinen OH. 1992. The genera Thiobacillus, Thiomicrospira, and Thiosphaera. In: Balows A, Triiper HG, Dworkin M, Harder W. Schleifer KH (eds) The prokaryotes, 2nd edn. Springer, Berlin, p 2638-2657

Lewin JC. 1961. The dissolution of silica from diatom walls. Geochimica et Cosmochimica Acta Vol. 21(3-4): 182-198

Liu D, Wong PTS, Dutka BJ. 1973. Determination of carbohydrate in lake sediment by a modified phenol-sulfuric acid method. Water Research Vol. 7(5): 741–746

Lomstein B, Blackburn TH. 1992. Sediment nitrogen cycling in Aarhus Bay, Denmark. Danish Environmental Protection Agency, Copenhagen

Mason J, Kelly DP. 1988. Thiosulfate oxidation by obligately heterotrophic bacteria. Microbiol Ecol Vol. 15: 123-134

Matthiesen B, Mielke E, Sommer U. 2010. Dispersal decreases diversity in heterogeneous metacommunities by enhancing regional competition. Ecology Vol. 91(7): 2022-33

Middelburg JJ, Herman PMJ, Boschker HTS, Barranguet C Moens T, Heip CHR. 2000. The fate of intertidal microphytobenthos: an in situ 13C labelling study. Limnol. Oceanogr. Vol. 45: 1224–34

Meehl GA, Tebaldi C. 2004. More Intense, More Frequent, and Longer Lasting Heat Waves in the 21st Century. Science Vol. 305: 994

Munari C. 2011. Effects of the 2003 European heatwave on the benthic community of a severe transitional ecosystem (Comacchio Saltworks, Italy). Mar Pollut Bull Vol. 62(12): 2761-70

Nübel U, Engelen B, Felske A, Snaidr J, Wieshuber A, Amann RI, Ludwig W & Backhaus H. 1996. Sequence heterogeneities of genes encoding 16S rRNA in Paenibacillus polymyca detected by TGGE. J Bacteriol Vol. 178: 5636–5643

Paterson DM, Tolhurst TJ, Kelly JA, Honeywill C, de Deckere EMGT, Huet V, Shayer SA, Black KS, de Brouwer JFC, Davidson I. 2000. Variations in sediment stabil ity and sediment properties across the Skeffling mudflat, Humber estuary, UK. Coast. Shelf Sci. Vol. 20: 1373–96

Piontek J, Händel N, Langer G, Wohlers J, Riebesell U, Engel A. 2009. Effects of rising temperature on the formation and microbial degradation of marine diatom aggregates Aquat Microb Ecol. Vol. 54: 305–318

Pomeroy LR, Wiebe WJ. 2001. Temperature and substrates as interactive limiting factors for marine heterotrophic bacteria. Aquat Microb Ecol Vol. 23: 187–204

Robinson PJ. 2001. On the Definition of a Heat Wave. Journal of applied meteorology Vol. 40: 762-775

Semenza JC, McCullough JE, Flanders WD, McGeehin MA, Lumpkin JR. 1999. Excess Hospital Admissions during the July 1995 Heat Wave in Chicago. Amer. J. Prevent. Med. Vol. 16: 269-277

Shiah FH, Gong GC, Chen TY, Chen CC. 2000. Temperature dependence of bacterial specific growth rates on the continental shelf of the East China Sea and its potential application in estimating bacterial production. Aquat Microb Ecol Vol. 22: 155–162

Smith DS. 2011. The ecological role of climate extremes: current understanding and future prospects. Joumal of Ecology Vol. 99(3): 651-655

Tan J, Zheng Y, Guixiang S, Kalkstein LS, Kalkstein AJ, Tang X. 2007. Heat wave impacts on mortality in Shanghai, 1998 and 2003. Int J Biometeorol Vol. 51: 193–200

Thibault KM, Brown JH. 2008. Impact of an extreme climatic event on community assembly. Proc Natl Acad Sci U S A. Vol. 105(9): 3410-5

Truper HG. 1975. The enzymology of sulfur metabolism in phototrophic bacteria-a review. Plant Soil Vol. 43: 29-39

Tylianakis JM, Didham RK, Bascompte J, Wardle DA. 2008. Global change and species interactions in terrestrial ecosystems. Ecol lett. Vol. 11(12): 1351-63

Underwood GJC, Kromkamp J. 1999. Primary production by phytoplankton and microphytobenthos in estuaries. Adv Ecol Res Vol. 29: 94–153

Underwood GJC, Paterson DM, Parkes RJ. 1995. The measurement of microbial carbohydrate exopolymers from intertidal sediments. Limnol Oceanogr. Vol. 40: 1243–1253

van Miegroet H. and Cole DW. 1984. The impact of nitrification on soil acidification and cation leaching in a red alder ecosystem. Journal of Environmental Quality Vol. 13: 586-590

Whitman WB, Coleman DC, Wiebe WJ. 1998. Prokaryotes. The unseen majority. Proceedings of the National Academy of Sciences of the United States of America Vol. 95(6): 578–6,583

Widdel F. 1988. Microbiology and ecology of sulfate- and sulfur-reducing bacteria. In: Zehnder AJB (ed) Biology of anaerobic microorganisms. Wiley & Sons, New York, p 469-587

William MJ, Dobbertin M, Zimmerman NE, Reichstein M. 2005. Divergent vegetation growth responses to the 2003 heat wave in the Swiss Alps. GEOPHYSICAL RESEARCH LETTERS, Vol. 32

Wolfstein K and Stal LJ. 2002. Production of extracellular polymeric substances (EPS) by benthic diatoms: effect of irradiance and Temperature. Marine Ecology Progress Series Mar Ecol Prog Ser. Vol. 236: 13–22

Wotton RS. 2004. The ubiquity and many roles of exopolymers (EPS) in aquatic systems. Sci. Mar. Vol. 68(1): 13-21