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Effects of historical perturbations on aquatic bacterial communities

A microbial journey into the 4th dimension!

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Degree project in biology, Master of science (2 years), 2012
Examensarbete i biologi 45 hp till masterexamen, 2012
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Abstract

This project investigates whether historical spatial and environmental mechanisms are of importance in determining bacterial community composition (BCC). The study was performed in rock pools at the Swedish Baltic Sea Coast. The sampling was done in 48 hours intervals in a time series over a 9 day period. I tested whether bacterial community composition at the end of the sampling period correlated stronger with past environmental conditions compared to present conditions, using partial redundancy analysis and Mantel tests. The results showed that spatial factors were generally not important and that salinity was the most important structuring environmental factor. Correlations between salinity and BCC demonstrated that higher correlations were achieved with past compared to contemporary salinity conditions. This demonstrates the existence of an interference of past perturbation events on present bacterial communities in a natural environment. My results also highlight the necessity of conducting sampling for bacterial community analysis in time series in order to acquire a greater understanding of the historical effects and the timespan during which they affect bacterial communities.

Introduction

Bacteria are key players for the functioning and stability of all ecosystems on Earth as a result of their cosmopolitan distribution, abundance and metabolic capabilities (Whitman et al., 1998; Hallin et al., 2009). This creates a great incentive to understand the diversity and global biogeography of bacterial communities. However, due to the simple fact that bacteria are microscopic (and therefore hard to observe and manipulate), our understanding of bacterial communities is less complete compared to those of macro organisms. Technological development and adaptation of previous metacommunity research (a metacommunity is defined as a set of local communities that are linked by dispersal of multiple interacting species (Wilson, 1992)) has nonetheless allowed for significant advances of our understanding of the biogeography of bacteria communities over the last decade (Zinger et al., 2011; Lindström & Langenheder, 2012).

The three main perspectives used in BCC (bacteria community composition) research that are derived from metacommunity research are Species sorting (Leibold 1998), Mass effect (Shmida & Wilson 1985) and Neutral theory (Hubbell 2001). Species sorting explains the community as an effect of species competition and species adaptations to the local environment. Essentially the species sorting perspective assumes that species have different niche requirements and can thrive under different abiotic and biotic conditions and that this difference is what determines if a species can exist in a locality. This was the first ecological concept to be established for bacterial communities, beginning with Baas-Becking"s famous statement "everything is everywhere, but the environment "selects". The idea is that bacteria are vastly abundant with a high level of plasticity and at the same time easily spread as a result of their minute size. This was interpreted as that all species of bacteria must be spread globally. The presence or absence of bacterial species is then explained as a pure product of environmental selection (Baas-Becking, 1934). Despite originally being based on logic rather than empirical studies the theory still holds a lot of recognition today (Finlay & Clarke, 1999; Fenchel & Finlay, 2004). Species sorting is in fact the perspecive that most often significantly explains BCC (Lindström & Langenheder, 2012), although most researchers nowadays acknowledge that there are limitations in bacterial dispersal (Schauer et al., 2010) in contrast with Baas-Becking"s statement.

The Mass effect perspective predicts that declining populations of a species can be stable due to regular inflow of migration. Regarding bacterial communities the Mass effect is similar to species sorting in the way that it predicts that there is/can occur a vast and continuous distribution of bacteria in the environment. The Mass effect perspective then predicts that as a result of massive fluxes of bacteria, high abundances of bacteria commonly end up in environments that they are poorly adapted to. According to the Mass effect perspective these species can remain in the community as a result of constant immigration and the balance between the decline and the rate of the immigration would then set the abundance level of that species (Shmida & Wilson, 1985; Mouquet & Loreau, 2002). This perspective could also explain why bacterial communities correlat so poorly with factors that other organisms are strongly dependent on, as Mass effects decouples bacterial communities from their environment. If bacteria can be stable in an environment that actually should cause them to decline, it would make a researcher that samples this environment incline to believe that the environmental parameters have no effect. Recent research has casted doubts regarding if Mass effect is a likely event to occur in a natural bacterial communities (Logue & Lindström, 2010; Lindström & Östman 2011)

Neutral theory is a relatively new theory in the field of metacommunity research and has sparked a lot of interest and controversy. Neutral theory states that the difference between similar species on the same trophic level is irrelevant or neutral to their chance of success (Hubbell, 2001). Furthermore it assumes that randomly occurring events in nature are the actual determining factors for a species abundance and biodiversity in general. For example: Assume four similar species of flowers that exist as seeds in a forest. When spring comes three out of the four sprouts, the fourth dies as a result of landing in a *Sphagnum* area. Out of the remaining three only one is able to produce seeds as the two other seeds happened to land in a shady area and did not receive the necessary energy to produce offspring. During the following year the two species in the shady area have grown larger and can now reach the sunlight. The third species has now nine individuals this year. As a result the third species now has more opportunities to create offspring this year. Hence, the third species will always be producing more offspring from this point forward and thus become the dominating species. Eventually, if there is no migration and so forth, the third species will outcompete the other flower species (as an effect of it having more opportunities to succeed) and with time be the only remaining flower in the forest. This means that the biodiversity and abundance among the flowers in the forest is all a result of the original seed by chance being blown by the wind to a suitable spot even though the difference between the flowers was “neutral”.
Neutral theory is often referred to as a null theory to other ecological metacommunity concepts, including mass effect and species sorting (Bell, 2001). Neutral theory has been adapted to bacterial community research and seems to be increasingly more approved as several studies shows patterns similar to that predicted by the neutral model (Sloan et al., 2006; Keymer et al., 2009; Drakare & Liess, 2010; Östman et al., 2010).

Additionally are the influences of bacterial dispersal limitation and priority effects are often emerging in the discussions of bacterial community assembly. Dispersal limitation simply states that bacteria are subjects of geographical limitations. These patterns have also been observed in bacterial community studies (Schauer et al., 2010). This is of great importance for bacterial community research as several theories are based on the fact that there is a limitation in bacterial dispersal. Priority effects (or founder effect) are comparable to the saying “first come, first served”. The organism that can establish itself first in an emerging patch has a competitive advantaged towards late-comers, either as result of reducing the available amount of a limiting resource or by changing the environment in a way that makes in unsuitable for

other species. This effect has been observed among coral reef fish (Almany, 2003) and plant communities (Fukami et al., 2005) but has not yet been investigated for bacterial communities.

The common standpoint in current metacommunity research is that all theories mentioned are relevant for the understanding of metacommunities but that none of them gives a complete answer for a given community. Despite substantial progress within these concepts there is still a great gap remaining in our understanding of the variation seen in bacterial communities, commonly leaving 60-80% of the seen variation among locations in a metacommunity unexplained (Langenheder & Ragnarsson 2007; Van der Gucht et al., 2007; Zinger et al., 2011). A constant lack for all concepts mentioned above is the influence of historical events. Historical events are frequently discussed as one of the factors that could influence the results of metacommunity research (Leibold & Mikkelson, 2002; Langenheder & Ragnarsson, 2007) but which have yet to be thoroughly tested for bacteria communities. The ideas behind historical factors are similar to those of other fields of ecology, with many explanatory factors being influenced by usually unknown events that have occurred in the past. In this study I will look at the influences that historical events might cause for the interpretation of how environmental and spatial factors influence bacterial community composition. Other concepts such as Neutral theory and Priority effects are also likely to be heavily influenced by historical factors, the specific investigation of Priority effects and Neutral theory are, however, outside of the scope of this study.

Historical environmental effects on BCC can be fairly straightforward. For example consider a situation where a strong but momentary change in pH has recently wiped out a significant fraction of the species within a bacterial community. The pH quickly returns to its original state, while lack of immigration (or other factors) prevents the bacterial community to go back to its previous state. As a result the correlation between environmental values and BCC becomes low, which then falsely would be interpreted as the environment having a minor effect. This type of event will henceforth be referred to as "historical interference". In addition to historical interference I want to mention "present interference" and delayed response time. Present interference is when a long term community adaptation in combination with an environmental perturbation causes a decrease in correlation between the environment and the community. For example, consider a situation where a researcher collects bacterial samples from two lakes with similar bacterial communities. One of the lakes recently had a substantial drop in oxygen; the bacterial community is temporarily coping with the stress of the low oxygen levels. When the researcher later correlates oxygen levels towards BCC the correlation gets incorrectly low as the two lakes have similar BCC but very different oxygen levels. Thus, whereas historical interference changes the bacterial community but leaves no measurable trace; present interference does not leave an impact on the bacterial community but changes the measured parameters. In reality it would not be possible to separate the two from one another if the sampling was not done in a time series as both results in a none-traceable decrease of correlations between environmental conditions and BCC.

Finally is there the delayed response time of the community, which is the difference between initial and "complete" response of a community to an environmental or spatial fluctuation. For example, consider that there is a bacterial community with 50 000 different species within it. A relatively minor change in environmental conditions causes 1% of the species to get extinct, 69% of the bacteria can resist the environmental change but have to use a substantial part of their metabolism to resist the stress while 30% of the species will not get noticeably

affected. The initial measurable response would thus be that 1% of the bacteria are lost. However, if the environmental increase remains an increasing share out of stressed bacteria might also get extinct. This could then get further complicated by the fact that the competitive balance between species will begin to move as an effect of the stress and thus initiate a species sorting process (the recovery time from a perturbation is an additional factor). This will result in a large difference between the initial and final effect of the fluctuation. Baho et al. (2012) found strong alterations in BCC still ongoing two weeks after a pulse of salinity was added to the community. This shows that the gap between the initial and final response does exist and that this effect can be ongoing a long time after the perturbation, therefore increasing the likelihood of this being an issue when conducting field studies.

Historical spatial effects can be a large variation of events; rain, wind or temporary immigration bridges and so forth. Historical spatial effects pose similar issues for BCC research by possibly blurring results of correlation tests. One could argue that the testing of mass effects is in some regards are tests of historical spatial effects (i.e. mass effect immigrations caused by spatial events). There are, however, numerous other possible historical spatial events, e.g. previous long term connections, mixing of multiple communities or creating opportunities for founder effects.

There are issues verifying that historical mechanisms occur in bacterial communities in their natural environment. A historical mechanism would be calculated as following: Impact of historical mechanism $X = X_{\underline{past}} - X_{\underline{present}}$. There are few logical reasons to assume that the past consistently and greatly would affect the bacterial community to a higher degree then the present. Firstly, there is no specific reason to believe that the conditions that lie close in the past would be vastly different from the current conditions, i. e. they will often be autocorrelated. Secondly, if the community has been affected by a sudden pulse of "change" and thereafter resumed its original state there is reason to assume that the bacterial community would start to recover with time. This would be an effect of the present conditions being a selecting factor on the community, thus always pushing the community towards the most ideal adaptation to the present environmental state.

If there has not been a sudden change or if the change has remained until the present point, the past and present are identical and there is no reason to separate the two ($X = 1 - 1 = 0$). As a result of this the power of historical factors is likely to be significantly lower than the factor itself in most cases. The only scenario where a historical effect is more important than the present factor itself is if the event in the past has more than twice the effect as the same factor in the present (Past = 2.05, Present = 1 (Historical factor $X = 2.05 - 1 = 1.05$. $1 < 1.05$)). It is fair to assume that this is an unlikely scenario. If historical effects are on average weaker compared to present factors this is an issue in particularly when working with BCC in field studies, where known explanatory factors often only explain a minor fraction of variation in community composition. As a result of being dependent on previously known factors, the historical aspects are not likely to revolutionize the degree of explanation we observe for each given factor.

On the other hand does the history influence the patterns of all theories explaining BCC. Therefore, the addition of historical aspects to a factor could contribute to a somewhat higher degree of explanation. This would be of particular importance if several different theories are used for explaining observed variance in a community. In most cases the historical effects are likely to remain an unknown factor, affecting the outcome of the observed pattern to an unidentified extent. By investigating the likelihood and magnitude of historical impacts in the

different metacommunity perspectives we can start to assess the relevance of missing historical effects and thereby learn how to better approach the issue when conducting research. Studies of historical factors will also contribute to other areas of understanding of bacterial communities, such as bacterial resistance and response time to environmental perturbations.

The possibilities and impact of historical events on BCC depend on several factors. Firstly, bacteria must have a limitation in their dispersal ability. If bacteria species would be able to instantly establish in any suitable environment the effect of historical events would be none. The resilience of bacterial species would also impact the significance of historical events. If species of bacteria can survive in harsh conditions for a period of time, or only reduce their abundance, it would infer that temporary disturbances would have a relatively small effect on BCC. The response rate of the bacterial community to changes is also of importance as the time period needed for observing the effect would be decided by this variable.

In this study I implemented a field study with the aim to test if historical mechanisms occur in bacterial communities in their natural environment. The bacterial communities were located in rock pools, *i.e.* a small bedrock depression filled with either fresh or brackish water. Rock pools are suitable objects for metacommunity research as they present an opportunity to look at a large number of separated bacterial communities in small "areas". The rock pools are also very heterogeneous in environmental conditions, in particular with regard to salinity and water colour. Moreover, pool volume is varying greatly, ranging from $1m^3<$ to $<0.05\ m^3$, depending on the rock depression and weather conditions. Their small size implicates that rock pools are likely to experience strong variability in environmental conditions over time and thus makes them an ideal system to study effects of historical events. The purpose here was to test how much of the variation in BBC among pools can be related to differences in present environmental conditions as well as to environmental conditions measured multiple times during a 9 day period prior to the sampling event.

Specifically, the aim of the project was to investigate the following questions:

1) Can historical environmental events affect BCC in their natural environment?

2) Can historical spatial events affect BCC in their natural environment?

3) What is the time period during which historical impacts on BCC become observable?

Material and Methods

Field sampling

Samples were taken from twenty rock pools located along the Baltic Sea coast in the province of Uppland in central Sweden (60° 29′ 54″ N, 18° 25′ 45 E). Each rock pool was sampled five times, starting at August 3rd 2011 and ending at August 11th 2011, with a 48h interval between each sampling occasion. At each sampling occasion the following factors were measured in the field: pool width, length, depth, salinity and temperature. Length, width, and depth of the pools were measured using a yardstick and measuring tape. Salinity and water temperature were measured using a WTW Conductometer LF 191. Additionally, water samples were collected for measurements of BCC, bacterial abundance, total-phosphorous

concentration, chlorophyll-a concentration, absorbance and abundances of zooplankton and flagellates. In addition, samples for BCC and bacterial abundance were also collected from air and rain samples with 3 samples of each at each sampling occasion. For this a set of traps was constructed. The traps consisted of a plastic bucket with a smaller sterilized plastic container within it. In order to prevent larger particles to get into the sample a net was placed on the top of the bucket. In the air traps a solid lid was also placed above the net, with approximately 4 cm gap between the lid and the bucket, in order to prevent rain bacteria from contaminating the air sample. The traps for air bacteria were filled with 100 ml Mili-Q water, whereas the rain traps did not contain any water. Water was collected in 1L sterilized plastics bottles. Upon arrival to the laboratory the water samples were processed further (see below). Geographical coordinates for pools were recorded using a GPS unit. Weather data was collected from a local weather station belonging to the Swedish Meteorological and Hydrological Institute (SMHI).

Laboratory measurements

All measurements mentioned below were carried out 5 times per pool, resulting in a total of 100 samples/values for each environmental parameter. For the analyses of chlorophyll-a concentration 100–500 mL water were filtered onto 47-mm glass fiber filters (Whatman GF/C). Chlorophyll-a was then extracted by submerging the filter in 95% ethanol for 1 minute. The extract"s absorbance was measured at 665nm and 770nm wavelength and the final value was corrected for pheophytin interference. Total phosphorus concentration were analysed according to Mezel & Corwin (1965). Water colour was obtained by measuring absorbance at 436 nm in a 5 cm cuvette. For bacterial abundance measurements the samples where preserved in filter-sterilized formaldehyde and stored in a 4 ^{o}C dark room. Bacteria cells were counted using a flow cytometer (CyFlow space, Partec, Germany). To determine flagellate abundance 2 ml formaldehyde-preserved water were filtered onto a 0.8 μm polycarbonate filter and stained with DAPI (final concentration 100 μg ml^{-1})**.** Counting was then performed with an epifluorescence microscope by counting all flagellates in a pre-determined area on the filter surface (25 mm × 100 μm).

For zooplankton abundance and identification animals were collected by filtering water (2L) through a plankton net and were then sorted and kept in a 50 mL polypropylene test tube (Falcon) with a 50% ethanol solution and were stored in a 4^{o}C room. The zooplankton was counted and identified using an Olympus SZ61 microscope.

Pool volume was calculated from the length, width and depth presuming that each pool had the shape of an inverted pyramid. Locations and distances between pools on the first day was calculated with ArcGIS 9.2. Due to issues with the ArcGIS software the calculation of changes in the closest neighbor distance (as pool volume and flow connection changed with the rain) was done "manually" in Excel. Here I calculated for all sampling occasions, the distances between the GPS points with the reduction in distance that was added as a result of the increasing areas of the pools.

BCC was determined twice, once at the starting point (day 1) and then at the final sampling (day 9). BCC was analysed with the T-RFLP method (terminal restriction fragment length polymorphism) (Liu et al. 1997). DNA was extracted from the sample using the Soil DNA isolation kit according to the instruction manual (MOBIO Laboratories). The 16S rRNA gene was then amplified using PCR (Polymerase chain reaction) with the fluorescently labelled bacteria-specific forward primer 8F-HEX and the universal reverse primer 519r. One 50 μL PCR reaction was carried out for each sample with the following mixture:2 μM of each

primer, 2.5 mM of each dNTP, 50mM $MgCl_2$ in 1 × NH4 buffer -and 0,5µL 5 U/µL Biotaq DNA polymerase. PCR products were then concentrated and purified with a PCR purification kit (Qiagen). Quantification of the purified products was done with the Quant-iT^{TM}PicoGreen® dsDNAReagent kit. Fluorescence measurements were performed with a Tecan ultra evolution microplate reader (TECAN – Ultra 384). DNA was afterwards diluted with Mili-Q water in order to achieve a concentration of 4 ng/µL of purified DNA. The restriction enzymes Hha I and Hae III (New England Biolabs, Ipswich, Massachusetts, USA) were used for 2 separate digests, each sample having a duplicate and incubated at 37 ^{o}C for 18 hours. Restriction fragments were then separated using an ABI3730XL DNA Analyzer in the Rudbeck laboratory in Uppsala.

The analysis of the T-RFLP data was performed using GeneMarker (Version 1.95). All peaks smaller than 50 base pairs and less than 0.5% of the total signal were removed from the analysis. Peaks closer than 0.5 base pairs were merged in order to account for the differences in running time between different samples. Each peak that remained after these modifications was considered an operational taxonomical unit (OTU). For statistical analyses, only the Hae III-digested samples were used since there were more successful runs within those samples.

Statistical analyses

Prior to the testing all data was transformed to a logarithmic scale (log (x+1)) to achieve a normal distribution of the data. To determine correlations between BCC and spatial and environmental factors, partial redundancy analysis (pRDA) was used. The pRDA procedure enables the determination of the independent effects of each explanatory factor on BCC as well as shared effects due to co-variation. For these calculations CANOCO 4.5 was used using Chord transformations of the species data to make it conform to a linear gradient, which is a requirement for the use of RDA (e.g. Legendre & Birks, 2012). In all models significance testing was done using Monte Carlo permutation tests with 999 permutations

The statistical analysis was done in following steps:
1) A standard RDA was performed to test the correlation of all environmental factors (total-P, chl-a, absorbance, zooplankton and flagellates) and BCC at day 9 separately for all 5 sampling days. Then the same procedure was performed with the spatial factors volume and closest neighbor values. Volume was used as spatial factor since it relates to merges of rock pools, over-all decreases in distance between pools, and increase of water movement. Environmental factors were significantly correlated with BCC at day 9 at all sampling occasions while none of the spatial factors gave a significant result at any sampling point. Hence, no further calculations were performed with the spatial data. 2) Forward selection of the environmental factors. Forward selection of environmental variables was implemented as described in Blanchet et al (2008) using two-cut off values to determine whether or not environmental variables made a significant contribution to explaining variation in BCC: (a) a p-value < 0.05 and the adjusted R^2 value (Peres-Neto et al., 2006) of the global model calculated in step 1. Hence, for all sampling days, salinity was the only environmental factor that was included into the model. 3) pRDAs were performed using salinity at the 5 sampling days and BCC in order to see which day had the highest degree explanation. This was also done in order to test if the patterns of explanation would remain the same with co-variation being included and to further to identify trends in the degree of BCC that can be explained by salinity variations over time. For example the effect of salinity at day 1 on BCC at day 9 was tested with salinity at day 3 as co-variable, next the effect of salinity at day 1 on BCC at day 9 was tested with day 5 salinity as co-variable and so on, with all 20 possible combinations being tested.

Finally correlations between salinity at the different sampling dates and BCC were also tested with partial Mantel tests, which were implemented using the Excel add-on XLSTAT. Similarity matrices were created for all variables after they had been $\log_{10}$ transformed and for the BCC matrix Bray-Curtis dissimilarity was used, for salinity data matrices Euclidian distances were used.

Terminology

- BCC always refers to BCC at day 9 (final sampling) if nothing else is mentioned.

Results

Weather data was retrieved for a 18 day period, 9 days prior to the sampling and 9 days during the sampling (Table 1). The weather conditions had 3 distinct stages that were of importance, the first stage being the 12 opening days, 8 days prior to the sampling plus the 4 initial days of sampling. This period was characterized by dry weather with a remarkably constant temperature. The second stage was on day 3-5 of the sampling period, during which the area received intensive rainfall. This event increased the average volume of the pools by more than 100%, which in turn triggered a number of environmental responses (Table 2). The third stage, constituting the 4 last days, was characterized again by dry weather and also by a drop in water temperature and more homogenous conditions among pools. The substantial change in environmental conditions that was initiated during the second stage remained.

BCC patterns among pools at days 1 and 9 were weakly, but significantly, correlated (Mantel test, $p<0.01$, $R_M = 0.28$). Only approximately half of all observed OTUs (47%) were, however, found at only one of the two sampling occasions. With regard to samples taken for community analyses of rain and air bacteria only two (one air and one rain) of the six samples were successful in both the PCR and T-RLFP process. Hence no statistical testing was possible. When the rain and air sample were included in an NMDS analysis with the pool samples they were very remote from the cluster of pool samples (Fig. 1).

Table 1 Daily mean values and standard deviation of air and water temperature (°C) and amount of precipitation (mm). „Before rain" refers to the period 9 days before sampling and at day 1-3 of sampling. „During rain" refers to the period between days 3-5 during the sampling period and „After rain" to the period between days 5-9.

Weather	Before rain	During rain	After rain
Average air temp ± SD	18.1 ±1.21	20.4 ±3.26	16.4 ±1.62
Average water temp ± SD	25.2 ±2.01	23.0 ±1.16	20.1 ±0.88
Rain (mm)	2	19.2	0.8

Table 2. Mean values ± standard deviations (SD) of environmental variables in the rock pools before and after the rain period. Before: day 1and day 3. After: day 5 and day 7.

Environmental factors	Sampling before the rain period	Sampling after rain period
Salinity average ± SD (psu)	2.85 ±4.58	0.49 ±0.76
Absorbance average ± SD (436nm)	71.3 ±47.6	39.7 ±21.4
Total-P average ± SD (µg/l)	10.3 ±13.6	4.73 ±4.86
Clh-a average ± SD (µg/l)	8.05 ±14.2	4.4 ±7.3
Volume average m^3 ± SD	0.14 ±0.11	0.34 ±0.25

Results from pRDAs (Table 3) and partial Mantel tests (Table 4) show that initial environmental conditions, in particular those found at day 1, 3 and 5, often explained more of the variation in (or were more strongly correlated to) BCC than more recent environmental conditions, *i.e.* those observed at days 7 or 9. Without accounting for co-variation, salinity at day 1 and day 3 had a higher correlation to BCC than salinity at days 5, 7 and 9 has, both in Mantel and pRDA tests (Tables 3 and 4). With co-variation among sampling days taking into account, the pattern remained the same although slightly harder to perceive. Salinity at days 1 and 3 had a total of 9 significant correlations with BCC (both Mantel tests and pRDAs) while days 5, 7 and 9 only had 4 significant correlations in total. Notably, salinity at any sampling date was never significant when co-variation with days 1 and 3 was taken into account. Days 1, 3 and 5 were on the other hand significantly correlated to BCC in 10 out of 12 cases also when co-variation of day 7-9 was taken into account (Table 3 and Table 4). Finally, in only 3 out of the 40 tests was the salinity at a specific day significantly influencing BCC when co-variation to salinity to a sampling point closest to it in either direction of time was included.

Table 3. Results from pRDA where salinity at the 5 different sampling points was correlated to BCC at day 9. The values are the percentage of variation in BCC explained. The first row is the total variation in BCC explained by salinity at the individual day. The following rows are the explained variance with co-variance with other sampling days withdrawn from the degree of explained variance.

Salinity-BCC correlation pRDA	Day 1	Day 3	Day 5	Day 7	Day 9
R^2_a without co-variation	0.12	0.12	0.11	0.10	0.10
R^2_a day 1 with co-variation	-	ns	ns	ns	0.05
R^2_a day 3 with co-variation	ns	-	ns	0.04	0.05
R^2_a day 5 with co-variation	ns	ns	-	0.04	0.04
R^2_a day 7 with co-variation	ns	ns	0.04	-	ns
R^2_a day 9 with co-variation	ns	ns	ns	ns	-

Table 4. Results from Mantel test showing correlations (R_M) between salinities at the 5 different sampling points and BCC at day 9. First row is the salinity the individual day compared to BCC. The following rows are explained variance with co-variance with-drawn. Note that the magnitude of values cannot be compared to the pRDA tests.

Salinity-BCC correlation mantle	Day 1	Day 3	Day 5	Day 7	Day 9
R2 day 1 without co-variation	0.598	0.611	0.536	0.528	0,521
R_M day 1 with co-variation	-	ns	0.316	0.333	0.344
R_M day 3 with co-variation	ns	-	0.353	0.365	0.383
R_M day 5 with co-variation	ns	ns	-	ns	0.212
R_M day 7 with co-variation	ns	ns	ns	-	ns
R_M day 9 with co-variation	ns	ns	ns	ns	-

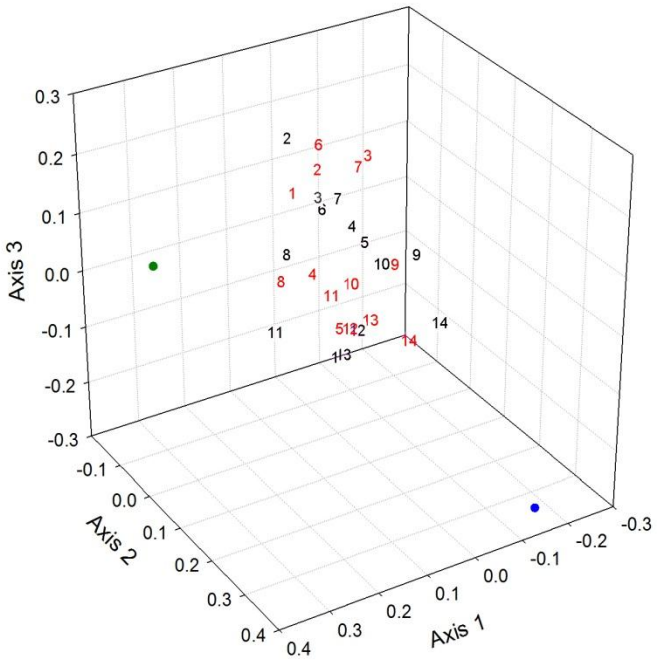

Figure 1. Non-metric multidimensional scaling (NDMS) plot showing BCC from rain and air in comparison to the BCC of the rock pools (Bray Curtis distances). The green dot is the rain sample and the blue dot the air sample. The black numbers are from day 1 and red numbers are from day 9. Only 14 pools are included due to the necessity of paring the samples.

Discussion

This research project aimed to explore the impact of historical mechanisms on bacterial communities. The most prominent result of the study is that BCC was to a greater extent determined by salinity conditions in the past compared to contemporary salinity conditions. This confirms first of all the importance of salinity as an key structuring factor of bacterial communities (Lozupone & Knight 2007; Tamames et al., 2010), and, more importantly demonstrates the impact that historical effects have on natural bacterial communities. My

results also display the need of studying changes in BCC and their regulating factors over time since it will enhance our ability to link specific alterations with the responses of bacteria. This will promote a better understanding of the attributes regulating bacterial communities. For spatial mechanisms there was no correlation between spatial factors and BCC observed regardless of the day. BCC between the two time points (day 1and day 9) were only weakly correlated and 47% of all observed OTUs were observed at only one of the two time points showing that an alteration of the community is likely to have occurred during the sampling period.

There were three different weather periods during the sampling; (1) an initial period with dry and warm weather, (2) two days with intense rain (3) and four days of dry but slightly colder weather. The intense rain period turned out to be a pivotal point of the study as numerous environmental and spatial parameters greatly shifted (Fig. 2) as a result of the changes in water volume, which on average increased by 140%. These great fluctuations made the study ideal for testing the importance of historical environmental effects as well as potential dispersal events. The rain had additionally a number of homogenization effects on the metacommunity. Firstly there was a homogenization of most environmental parameters, likely a result of the total range of values getting reduced by the dilution. Secondly, it would be reasonable to assume that the decreasing distance to closest neighbouring pool (some pools even merged temporarily) and an increase of the overall water movement would lead to an increase of migration between the pools, thus reducing heterogeneity. No significant correlation was, however, found between spatial parameters and BCC, and as a consequence no spatial historical effects could be found, thus rejecting the second hypothesis addressed in this study. If one does consider rain as a path of bacterial immigration into the rock pools this should also be an important mechanism for homogenization, considering that 60% of the average pool volume in the rock pools recently had originated from the rain at the point when BCC was analyzed. This has an interesting connection to the research conducted by Lindström et al. (2006) which showed that 85-95% similarity in BCC between inlet and epilimnion of lakes had a retention time of less than 100 days. The rock pools should presumably have average retention times well below 100 days. This leads to the assumption that there should be a high level of similarity between rain and rock pools if the rain was an important pathway of bacterial migration. The complete lack of this pattern, in my study cast doubts if this actually is the case (Fig. 1) and confirms previous studies that could show that dispersal from atmospheric bacteria is of relatively minor importance (Jones & McMahon, 2009).

Support was found for the hypothesis that historical environmental events can affect BCC in natural environments. Salinity was significantly correlated to BCC at all sampling days and more notably salinity that was measured further away (in time) more strongly correlated to BCC than salinity at closer sampling points. The most likely explanation is that the constantly dry and warm weather during a 12 day period, prior to the rainfall, resulted in an equally stable environment in the rock pools, -which included the first two sampling points (days 1 and 3). The higher degree of explanation at the first sampling points may therefore indicate that there has been an adaptation in the bacterial community to the more stable environmental conditions prior to the start of the sampling period. Although the exact salinity conditions prior to the sampling are unknown it is reasonable to assume that they were higher and more constant than salinity measurements at the later stages of the sampling. This seems likely since this was the case at the first two sampling points and because the weather at those points was similar to the weather prior the start of the sampling. It is important to note again that 47% of all OTUs changed during the sampling, which means that the perturbations following

the rainfall had a considerable effect on the community. What is more essential for this study was that past salinity conditions had stronger correlation compared to contemporary salinity conditions even after these considerable perturbations. This demonstrates the substantial effect historical mechanisms can have in bacterial communities. To summarize, variation in BCC at day 9 could be explained to a greater extend by salinity measurement further away in time indicating that the response of the bacterial community is „lacking behind" the change in environmental conditions.

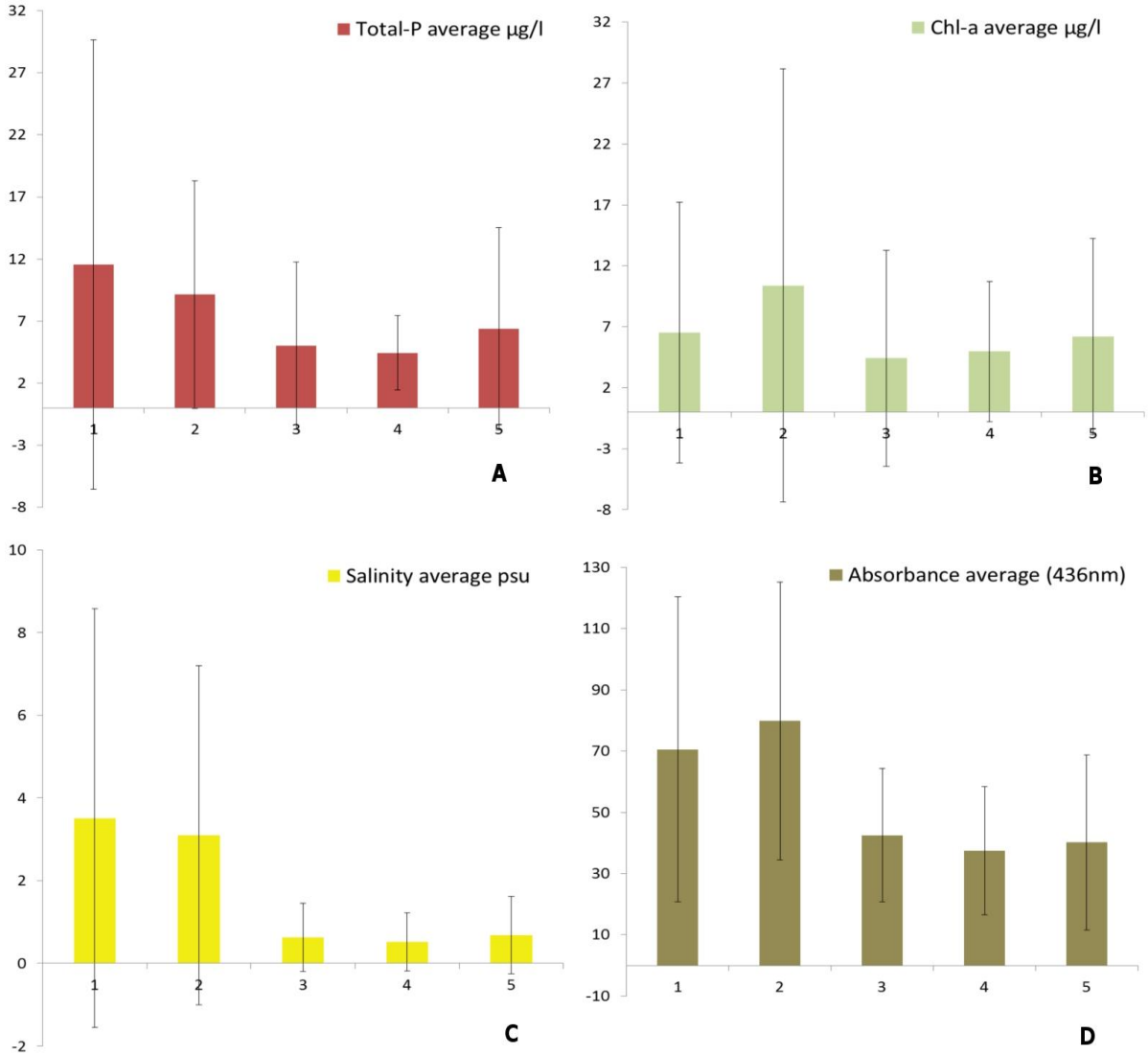

Figure 2. Environmental fluctuations in the rock pools at five sampling points. A: Average total phosphorus (µg/l). B: Average chlorophyll-a concentrations (µg/l). C: Average salinity (psu: practical salinity unit). D: Average absorbance (436nm) with SD bars at the five sampling points. Error bars refer to standard deviations from the mean in all cases (N = 5).

This result is notably similar to a laboratory study conducted Baho et al. (2012) where a salinity "pulse" was used as a perturbation and where the authors still found a noticeable effect on the bacterial community two weeks after the end of the pulse, also in samples were re-colonization was possible due to migration. It is also conform with another recent experimental study that also showed that BCC starts to respond to environmental changes

with a delay of a few days (Berga et al., 2012). Although the salinity change in my study was rather a press than a pulse disturbance, effect and time lengths were similar to what was observed in their laboratory experiment. To conclude, my results point towards the necessity to better understand historical factors and the advantages of conducting bacterial research in time series.

Connected to this I tried to find a suitable length of time gap between the presence and the past when testing historical impacts on BCC. The total duration time of the study was 9 days and each sampling point was separated by 48 hours. The initial idea for choosing this sampling interval was that it should be long enough to allow for several bacterial generations to elapse. Another consideration was that the time gap should not be long enough for the community to recover from effects of historical events. For future projects I would consider using slightly longer time gaps between each sampling point since bacterial communities responded at a slower rate than anticipated, with only 3 out of the 40 measured salinity values being significant when correlated towards points closest in time in either direction. Additionally, I think it is important to point out that the extent of the environmental fluctuations within this field study was rather exceptional for a 9 day sampling period, indicating that longer time periods might be more appropriate in a study where changes in environmental conditions are slower and less drastic. Regarding the total length of sampling time I would increase this period as well for similar reasons as mentioned for the time gaps. There are, however, shortcomings and benefits with both longer and shorter time periods, thus should the aim of the study and the characteristics of the sampling area be taken into consideration when deciding the length of time period used. If sampling is performed in a field study with larger water bodies I would encourage using longer time periods than was done in this study.

There are a few additional methodological issues and additional suggestions for improvements that arose from the present study. Firstly, I would consider sampling less explanatory factors and focus on increasing the number of sampled localities and sampling points. There are several reasons for this, the main issue is the fact that sampling conducted in time series will quickly give an unmanageable amount of samples if one does not reduce the number of factors if one aims to increase the number of localities and time points. The low number of localities does also influence the power of statistical tests, in particular that of multivariate analyses such as the RDAs, limiting the conclusion that can be drawn. As stated previously it is also harder to find historical mechanisms of a factor compared to proving the factor itself. A factor that is rarely or generally only slightly significant can be a very unlikely candidate for finding historical effects, thus supporting the argumentation specifically focus on the mechanisms proven to be of importance. Secondly, I think it is important to keep in mind the difference between a historical interference and present interference and set up the study in a way that allows a separation of these two factors. Finally, another approach to historical factors would be to look deeper into the response time of bacterial communities. This factor will most likely be difficult to observe and prove in a field experiment, since it will be hard to link the effects of perturbation back to one event when other perturbations are occurring simultaneously. If the study is conducted in a laboratory were natural conditions are emulated one might be able to see the effect, time length and frequency of this factor and thereby be able to predict its consequences for bacteria in their natural environment.

Acknowledgements

Silke Langenheder for being a good supervisor and supporting me in all the steps of this project, hopefully a little less so by the end of project!

Mercè Berga for taking time to help me with innumerous issues and questions, everything from clogging water filters to philosophical difference between past and present.

Eva Lindström for here help in the field sampling and support with statistical issues and other bacteria related discussions.

Yinghua Zha for her support in the laboratory and supplementing me with notes and instruction papers that mysteriously got missing.

Jason Ji for his help in the laboratory and aiding me with his vast experience of flagellate counting!

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