Techniques in Molecular Biology 1

Investigating Microbial Ecology Using Molecular Techniques

Does Ocean Acidification Affect Bacterial Diversity?

Abstract

The increasing number of oceanic bacterial species discovered in recent years has inspired many studies into their taxonomy. This study is concerned with how oceanic bacterial species diversity will change in the future. Based upon current research; the assumption is made that increased atmospheric CO_2 partial pressures will cause a significant drop in oceanic pH. The experiment was conducted in Bergen (Norway) where 6 mesocosms each containing 12 metric tonnes of native seawater were subject to different environmental conditions. The data was obtained by sequencing and comparing 16S rRNA genes from bacteria extracted from the ambient and high-CO_2 environments. Species were identified primarily by searching the BLAST (Basic Logical Alignment Search Tool) databases. The results and statistical analysis show a significantly larger number of bacterial species are able to survive in the high CO_2 environment (future conditions) compared to the ambient environment (current). For reasons why this occurs we look more closely at each species and its role in the ocean ecosystem. From the findings it is clear that further study is needed in order to comprehend the complex interactions between different groups of microorganisms and also their interactions with the environment.

Introduction

Present day oceanic pH is approximately 8.1[1]; a recent study by J. C. Orr *et al* at the Laboratoire des Sciences du Climat et de l'Environnement predicted that, by the year 2100, oceanic pH could be as low as 7.8[2] due to increasing levels of atmospheric CO_2 dissolving in the ocean to form carbonic acid[3] (H_2CO_3). This is a significant rise in acidity over a relatively short time, especially when we consider that pH is a logarithmic scale. The aim of this experiment is to use molecular techniques to

explore the effects of this rapid acidification on bacterial diversity by simulating expected oceanic pH in the year 2100.

With the increasing awareness of climate change, many institutions are attempting to model expected future conditions and their effects upon organisms. However; most of the existing studies are concerned with ocean acidification focus upon calcifying organisms such as the coccolithophore *Emiliania huxleyi*[2]. Studies into this type of organism are of great ecological relevance as lowered pH would cause these organisms' exostructures to dissolve, releasing carbon in a positive feedback loop. The impact of ocean acidification upon bacteria, in comparison, is a relatively neglected topic [4]. But why is it necessary to study the effects of climate change on bacteria in the first place?

Bacteria are ubiquitous – there are an estimated 5×10^{30} individuals on the earth[6], they drive the carbon and nitrogen cycles (by photosynthesis and nitrogen fixation respectively) as well as decomposing waste organic matter – without them, life as we know it could simply not exist on this planet.

Why Use Molecular Techniques?

Since the 1600s until fairly recently the only way to classify and conduct experimentation on bacteria relied absolutely upon microscopy. Microscopy has several limitations, the most important being the need to culture the specimen – we know today that the overwhelming majority of bacterial species cannot be cultured in the lab, this places serious limitations on any microbiology experiment, especially one concerned with bacterial diversity. Instead of microscopy; a range of powerful molecular techniques were used to analyse the bacteria, the techniques included: colony PCR, Cloning, Agarose Gel Electrophoresis and Dye Terminator Sequencing.

The Experiment

The aim of the experiment is to collect a library of 16S ribosomal gene sequences for high and ambient CO_2 environments. This library will be used to construct a

phylogenetic tree and the species present in each mesocosm will be counted and compared.

The data comes from experiments conducted in Bergen (Norway) in which 6 mesocosms, each containing 12 metric tonnes of native seawater, were subjected to differing environmental conditions. Inorganic nutrients were added to the mesocosms in order to induce an algal bloom. This study is concerned with mesocosms 4 and 1 – ambient (370ppmV) and elevated (700ppmV) levels of bubbled CO_2 respectively. 2 litres of water from each mesocosm were routinely filtered to first remove detritus and particulate matter then the plankton then the bacteria. Our sample data comes from day 14 of the experiment. The DNA was extracted from the bacteria by physical (bead beating) and chemical techniques – removal of membrane lipids using detergent (Sodium Dodecyl Sulphate), removal of histones by protease enzymes then precipitation of the DNA in isopropanol. The extracted nucleotides were sampled using agarose gel electrophoresis to check for purity. The sequences were amplified with PCR using 16S specific primers then ligated into TOPO Invitrogen plasmids along with an antibiotic resistance marker. The plasmid vectors were taken up by *Escherichia coli* which were then cultured on X-gal and antibiotic medium (to select for transformed individuals). In order to find out which insert belonged to which organism, first, colony PCR was used to amplify the target DNA, a sample was checked for the presence of the target sequence using gel electrophoresis (see later), then the sequence data was acquired through capillary electrophoresis sequencing using Big Dye.

The analysis of the results takes place entirely *in silico* and is described in the next section.

Bioinformatics

At this point in the experiment we have a set of sequence data representing the 16S gene for all the bacterial species from both mesocosms. We know which are from the high and which are from the ambient CO_2 partial pressures but we still don't know what species are present. In order to find this out, we can reference our 16S genes against the millions of 16S sequences deposited in the BLAST (Basic Logical

Alignment Search Tool) database. 16S is useful in this way as its structure is conserved enough to make it universal but with enough sequence dissimilarity to differentiate between organisms[5].

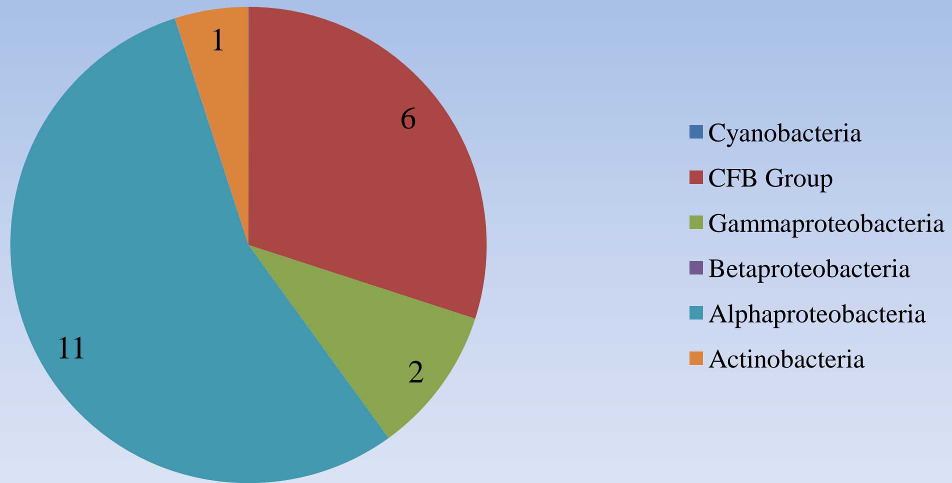
In this system, a new species is defined as one with less than 100% homology with an existing sequence. The sequences are imported into BioEdit™. The most insightful way to visualise the sequence information when observing diversity is to create a phylogenetic tree. Before the tree is produced, the sequences must be aligned, this is achieved using ClustalW. From the tree it is easy to identify which mesocosm the species came from and also its degree of relatedness to the other species. The information gained from the tree will be used to plot pie charts depicting the species in the two different environments. The tree is produced using the BioEdit™ accessory application DNADist ---> Neighbour phylogenetic tree in conjunction with TreeView.

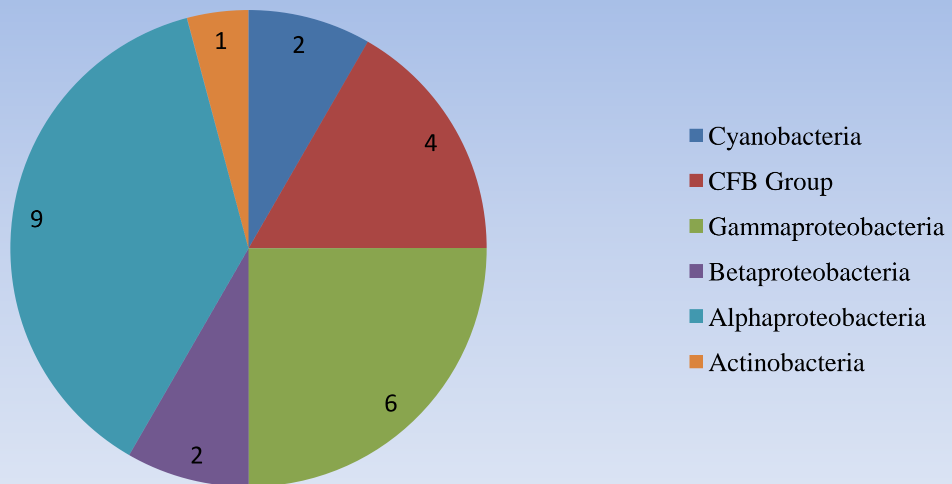
The phylogenetic tree produced is included in Appendix 1

Prediction

Since pH is an important environmental variable, any change is likely to result in a change in bacterial diversity. Every microorganism has an optimum pH range, outside of which its growth is limited or even prohibited

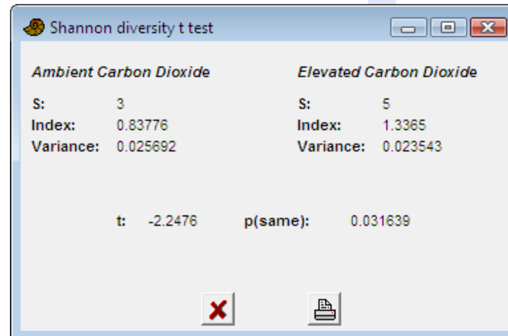
Results


Ambient Carbon Dioxide Partial Pressure
1
6
11
2
Cyanobacteria
CFB Group
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Actinobacteria


Elevated Carbon Dioxide Partial Pressure
1
2
4
9
6
2
Cyanobacteria
CFB Group
Gammaproteobacteria
Betaproteobacteria
Alphaproteobacteria
Actinobacteria

Statistical Analysis

In order to show quantitatively that the difference between the number of species in each environment was significant. A Diversity T Test was performed using the program: PAST, the results are shown below:



A p value of 0.03 was returned, since this is less than 0.05 the difference in the number of species is regarded as statistically significant.

Discussion

The critical aspect to bear in mind when analysing this experiment is that we are looking at *relative diversity* rather than numbers of individuals. A greater number of species does not necessarily mean a more abundant organism, for example, even though there are fewer gammaproteobacter species compared to alphaproteobacteria, it is possible that the gammaproteobacter could be present in far greater numbers. However, for the purposes of this discussion, we will assume a vague correlation between the number of species and the number of individuals for each sample (although this may not always be the case).

From the pie charts we can see that species diversity is greater for the high CO_2 sample than for the ambient sample. This is a somewhat surprising result – a significantly larger number of species can grow at lower pH than at the ambient pH.

In order to understand what is going on, we must look more closely at the species in each microcosm.

The largest number of species belongs to the Phylum: Proteobacteria; the etymology of the word ‘proteobacteria’ is interesting; it is derived from the Greek *Proteus* – meaning ‘the god of the sea’. This is certainly apt as the proteobacteria account for

The Oxbridge Research Group TM

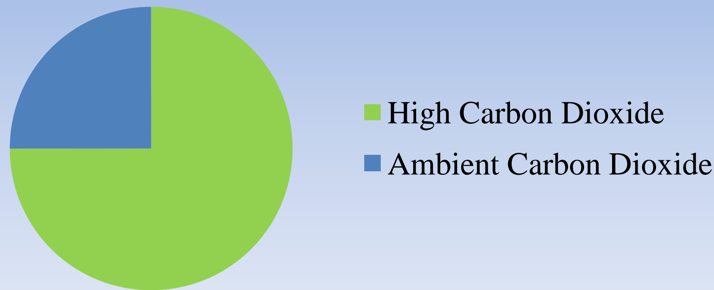
nearly 70% of all the named species in this experiment. Because of this, the proteobacteria are divided into their Orders: Alphaproteobacteria, Betaproteobacteria and Gammaproteobacteria (Delta and Epsilon proteobacteria also exist but were not detected in the samples). Most alphaproteobacteria species are phototrophs – they use light energy to convert CO_2 and H_2O into all of the organic compounds they require for growth, respiration and reproduction. Proteobacterial photosynthesis is not exactly the same process as in plants or even cyanobacteria; it involves the use of bacteriochlorophyll (rather than chlorophyll), as its photosynthetic pigment and electron donor. Apart from this, however, we may regard phototrophic proteobacteria as being similar to cyanobacteria for the purposes of this experiment. Rhodobacter species e.g. *Rhodobacter sphaeroides* is an example of a phototrophic alphaproteobacteria. However most of the alphaproteobacteria were from the SAR11 clade, the most abundant species belongs to this clade; it is *Candidatus Pelagibacter ubique*. *Candidatus Pelagibacter ubique* is generally thought of as being the most abundant organism on earth with a speculated 10^{28} individual cells. They grow by assimilating dissolved organic carbon (DOC) compounds and generate metabolic energy either by a light-driven proteorhodopsin pump or by aerobic respiration[7], in this way they are responsible for recycling thousands of tonnes of DOC every year. The data shows that the number of SAR11 species remains constant throughout the experiment; this indicates that they have a pH tolerance that accommodates the more acidic conditions. Since the SAR11 species are not inhibited by the lower pH, if anything they should fare better because the CO_2 dissolving in water will increase the growth of many species of phytoplankton leading to a higher concentration of dissolved organic carbon as the plankton are killed through lack of light (caused by the increased density of the plankton bloom). This fits with our expectations; the samples were taken on day 14 – it was around this time that the algal bloom peaked.
The order betaproteobacteria consists of several groups of aerobic facultative bacteria which are highly versatile in their degradation capacities. Out of the two betaproteobacteria species present in the high CO_2 environment one is of unknown species but the other is a *Comamonadaceae*. Like many of the betaproteobacteria species, *Comamonadaceae* is a denitrifier – it reduces soluble nitrite (NO_2^-) and

nitrate (NO_3^-) ions and releases gaseous nitrogen gas (N_2). These findings are contrary to what is expected since ocean acidification is accompanied by a reduction in availability of the ammonium ion (NH_3) – one of the key substrates for *Comamonadaceae* respiration. Further study into this area is required – the nitrogen cycle (in which species like *Comamonadaceae* are involved) is of great global significance.

The gammaproteobacteria are present in both mesocosms but there are 300% more species in the high CO_2 concentration. None of the gammaproteobacteria species were identified but it is believed that they all belong to the SAR86 cluster. SAR86 species are phototrophs; they use proteorhodopsin rather than bacteriochlorophyll. Proteorhodopsin works by using light energy to pump protons (H^+) outside the cell and then use the energy associated with the protons return to generate ATP. A possible theory about why the lower pH environment is able to support more species of gammaproteobacteria is that a base environment (as in ambient conditions) will more readily sequester free protons i.e. the protons outside the SAR86 cell therefore taking energy away from the bacteria and attenuating their growth.

The cyanobacteria are another example of a division that is not represented in the ambient CO_2 environment but 2 separate species are present in the high CO_2 environment.

The cyanobacteria are considered capable of ‘full photosynthesis’, more similar to a plant or algae than the other phototrophic bacterial species. They reduce dissolved carbon dioxide to form carbohydrates via the Calvin Cycle. It is not surprising therefore when we observe an increased number of cyanobacterial species in the environment with the increased CO_2. This fits with our common knowledge; we know that carbon dioxide levels are one of the limiting factors in photosynthesis as demonstrated by the increased growth rate of plants in high CO_2 environments. We can go further along this line of thought and compare all the phototrophic bacteria from the two samples:

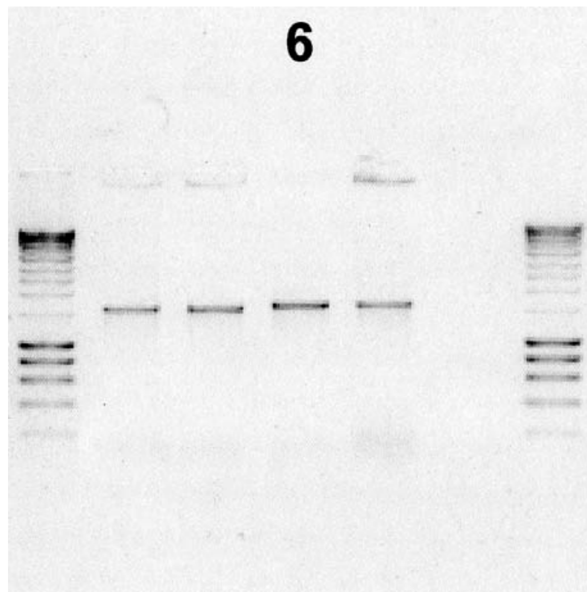


The pie chart shows the number of species in the high CO_2 compared to ambient conditions. Half of the phototrophic species are found only in the high CO_2 environment. An explanation for this could be that the molecular techniques used, powerful and precise as they are, could have failed to spot the phototrophic species in the ambient conditions because they were present in such low numbers; only when supplemented with excess CO_2 could they grow to such numbers as to be obvious to the detection methods employed.

The CFB group lost 33% of its total species number, between the ambient and high CO_2 mesocosms, reducing it from 6 to 4. The group comprises of Cytophaga, Flavobacterium and Bacteroides species grouped together because of their close relatedness. In the ambient CO_2 sample, all the CFB species are *Flavobacteria* but in the high CO_2 sample there are both *Flavobacteria* and *Bacteroidetes*. Both of these species are halophiles (more so than many of the other species) – they can tolerate high salt environments. In 1964, D. J. Kushner *et al* at the Canadian National Research Council proved that halophilic bacteria were particularly susceptible to acidic conditions and a drop in pH proved lethal. This fits with our results – the lower pH environment supports a lesser number of CFB species.

My Clones

I followed the procedure detailed in Appendix 2 and successfully obtained the correct PCR products as confirmed by this gel electrophoresis image:



I then inserted the sequences that roughly corresponded to my clone numbers from the file: MSCStudentsfiles.fas into the full library of sequences (MSCfull.fas). After creating the phylogenetic tree, I identified my sequences and assigned them categories based upon their relative positions in the tree. My clones consisted of: 2 gammaproteobacteria, 1 betaproteobacteria and 1 alphaproteobacteria. In the future, if I wanted to get a more grounded and accurate idea of what species they are, I could BLAST search the sequences.

Experimental Limitations

The fundamental principle of the experiment is to measure bacterial diversity in today's conditions and the simulated conditions of 100 years in the future. An obvious flaw in the experimental design therefore is that the bacteria in the ocean will have 100 years to acclimate to the gradually acidifying conditions whereas the experimental organisms have no time at all – they are simply introduced into the lower pH environment. This throws a serious doubt over the usefulness of the data and conclusions drawn. What is more, there is no way around this problem without waiting 100 years for the bacteria to acclimatise, by which time the data will be irrelevant. One possible solution is to conduct a subsidiary experiment in which the bacteria are cultured for 1 year in an environment where the pH is gradually lowered in order to attenuate the effects of this flaw and investigate its consequences (there are obvious constraints to this approach however).

Another source of potential error comes from the way we define bacterial species – there is such ambiguity surrounding the current method of separating bacterial

species based upon 97% genome homology. If we were to employ this method for mammalian taxonomy humans and chimpanzees would be classed as the same species! (current opinion is beginning to doubt the validity of assumptions like this however). Bacterial taxonomy is grey rather than black and white. Some or many or even all the species groups we have assigned the bacteria in this experiment could turn out to be erroneous. Until we develop a more comprehensive system for classifying bacterial species this problem is likely to persist.

Conclusions

From the information presented in the pie charts and the phylogenetic tree it is possible to answer the question posed in the title. Yes, ocean acidification does affect bacterial diversity – it increases it by a statistically significant amount, though there were no major community changes observed. From the experiment we have learned that movement towards a more neutral pH supports more bacterial species. Because bacterial oceanography is such a fledgling discipline we, as yet, do not have the combined depth of knowledge to know for sure what is causing the shifts in the number of species within each group. There are many complex interactions between the microorganisms of the ocean, not just between bacteria but between algae and plankton as well. More research time must be devoted to studying the oceanic microorganisms because they are so little understood yet so vital to the survival of life on this planet.

References

The Oxbridge Research Group

[1] Key, R. M. *et al* (2004). A global ocean climatology: Results from GLODAP. *Biogeochemical Cycles* **18**, 31-40.

[2] Orr, J. C. *et al* (2005). Anthropogenic ocean acidification over the twenty-first century and its impact on calcifying organisms. *Nature* **437**, 681-686.

[3] Jolly, W. L. (1991), Modern Inorganic Chemistry, 2nd Edition, 11-26, New York, McGraw-Hill

[4] Cox, M. (2006). Fate of Marine Bacteria. *New Scientist* **2568**, 22.

[5] Singleton, D. R. (2001). Quantitative Comparisons of 16S rRNA Gene Sequence Libraries from Environmental Samples. *Applied and Environmental Microbiology* **67**, 4374-4376.

[6] Whitman, W. B. (1998). First-ever estimate of total bacteria on earth. *San Diego Earth Times* **706**. 542-544.

[7] Giovannoni, S. J. *et al* (2005). Genome Streamlining in a Cosmopolitan Oceanic Bacterium. *Science* **309**. 242-262.

BACKGROUND

Giovannoni, S. J. & Stigl, U. (2005). Molecular Diversity and Ecology of Microbial Plankton. *Nature* **437**. 343-348.

Schmidt, T. M. *et al* (1991). Analysis of a Marine Picoplankton Community by 16S rRNA Gene Cloning and Sequencing. *Journal of Bacteriology* **173**. 4371-4378.

Fuhrman, J. A. *et al* (1993). Phylogenetic Diversity of Subsurface Marine Microbial Communities from the Atlantic and Pacific Oceans. *Applied and Environmental Microbiology* **59**. 1294-1302.

Giovannoni, S. J. & Rappé, M. (2000), Microbial Ecology of the Oceans, 47-84, Corvallis, Wiley-Liss

The Oxbridge Research Group TM