



# Handbook of Methods for Microbial Ecology

used to  
Study the Biodiversity and Function of  
Microbial Habitats



<http://www.eu-hermes.net/>

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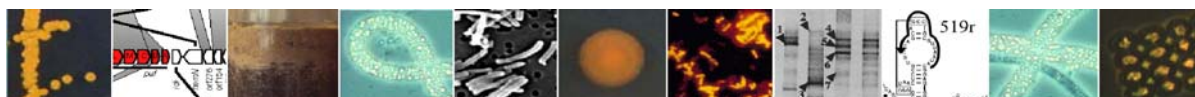


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## 1. Introduction

This handbook has been prepared by the Cardiff University HERMES scientists as fulfilment of a 6 month deliverable in the HERMES research project (funded by the European Commission's Framework Six Programme, under the priority Sustainable Development, Global Change and Ecosystems. Contract No. GOCE-CT-2005-511234-1). It is a handbook of methods for microbial ecology that will be used by the HERMES microbiology partners during their studies of biodiversity and function in a variety of coastal marine habitats that are part of the HERMES multidisciplinary research programme.

All HERMES microbiologists have been asked to contribute methods that they will use or that they think will be useful to others. The resulting collection is comprehensive and representative of the variety of microbiological approaches that will be used by HERMES microbiologists. It is not expected to be an exhaustive collection of all the methods that will be used as microbiological approaches to ecology and biogeochemistry are evolving very rapidly and it would be unwise to restrict investigators to a standard set of approaches. Also it was beyond the scope of this handbook to include the chemical, sampling or statistical methodologies that all of us use to some extent. Providing these methods to all HERMES microbiologists will stimulate interaction, technical developments and the rapid education of PhD students, postdoctoral scientists and others who are new to this branch of science and who are contributing to the HERMES programme.

The methods are written as protocols for use at the bench, during the planning of experiments and during data analysis. All the individual sections start with an introductory paragraph which states the aim of the method and/or a summary of the approach used. All sections also have relevant references which are collated in alphabetical order at the end of the handbook. At the end of each section is a contact name, brief statement of this persons location and their e-mail address to facilitate communication. In most cases the contact person has written the protocol, uses the approaches described routinely, has published studies using the methodology and is available for consultation in cases of difficulty. The protocols do not follow a standard format apart, from the common elements described here, instead in requesting, collating and editing the contributions I have tried to encourage the sections to be written in a way that suits the subject matter of the approach. I apologise if the editorial changes that I have made are not completely endorsed by the authors.

Although this handbook was completed on 30 September 2005 it need not be static, as it will be possible to add material to the body of the work or as appendices as is thought appropriate in the future. No such book is ever complete or fully comprehensive, but rather aims to act as a signboard for us all to use on our journey through scientific discovery.

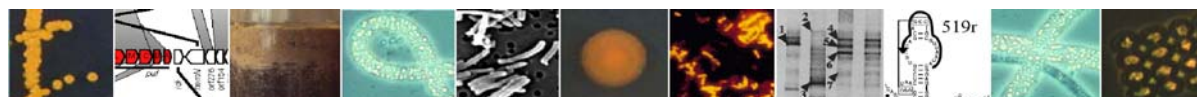
Comments and suggestions for future development and improvement will be welcomed.

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30 September 2005





## 1.1. Acknowledgements

I am very grateful to all those HERMES microbiologists and others who have willingly contributed to this handbook. I hope that we will all find it useful. We of course all thank the European Commission who have funded HERMES. I am especially grateful to Antje Boetius (MPI, Bremen) for her guidance and support during the book's planning and to all my colleagues, HERMES microbiologists and others, who have helped me in various ways. Thanks also go to the scientists working on the Metrol EU programme (EVK2-CT-2002-00080) whose collection of methods formed the basis for some of the protocols presented here.

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## 2. Sample identification & data management for microbiology samples

During HERMES we will collect a diversity of microbiological samples from a number of sites. Some groups will work on samples from pre-HERMES cruises and collaborative projects. One of the main tasks for every investigator in HERMES will be to keep and provide clear sample identification (the so-called metadata) for each data point. The HERMES database and metadata archive is PANGAEA ([www.pangaea.de](http://www.pangaea.de)). To facilitate sample identification and data management, a few basic rules need to be considered.

### Sample storage:

The procedures for sample storage are given in each method description. Most importantly:

- Keep all samples identifiable! They need a clear identification number, which gives a reference to the geographical position of the site where the samples have been obtained. You need to keep sample IDs and a station list for each cruise you have participated in, and for each sample you will receive.
- DNA samples need to be stored frozen (at -20°C or below)
- RNA samples are very sensitive and need to be handled rapidly. They are stored at -80°C.
- Samples for cell counts are stored in 2% formalin, in the cold and dark, and preferably in plastic vials, never frozen
- FISH samples are briefly (1-4 h) fixed in 2% formalin and then washed thoroughly and stored frozen in 50% ethanol/PBS.
- Samples for activity measurements are kept at *in situ* temperature until analysis.
- Samples for cultivation are best kept as bulk sediments in glass vials at *in situ* temperature in the dark. Aerobic samples need aeration, anaerobic samples anaerobic storage.

### Data storage:

Each data point needs a reference to the site and date where and when it was sampled. Usually, this information is provided by the station list of a scientific expedition, which you need to store and use to keep records of the station number and device with which the sample was obtained. It is very important also to keep track of the sediment horizon, which was sampled, and all subsequent handling (storage temperature, fixatives, dilutions etc).

PANGAEA is the data base selected for HERMES. It has already defined parameters for most of the data generated through HERMES microbiologists, including a variety of biomass and activity measures. HERMES is also concerned with biodiversity of microbes. The global solution at the moment for storing information about gene and protein sequences are international databases such as GenBank <http://www.ncbi.nih.gov/Genbank/>. However, unfortunately most available databases provide poor geographical and environmental information. One of the goals in HERMES WP4 is to tackle this problem – and here you need to help by keeping metadata information available!



### Data required as metadata for HERMES:

The basic metadata information will allow proper identification of samples and the data required includes the following.

#### Campaign/cruise

- Project name, institute(s), campaign/cruise name, basis (ship)

#### Site

- Site label (site number), latitude/longitude, elevation (- below sea level, + above sea level), date, time, area

#### Event (core/sample/measurement)

- Core/sample/measurement label (“event label”), latitude/longitude, gear, depth in water/depth in sediment, recovery instrument, date, time

#### Data

- Full name of investigated parameters (method such as cell numbers, thymidine incorporation etc) and parameter units (following SI standard or internationally used/widely accepted format)
- Complete list of abbreviations used in the data table
- Short description of the analytical or calculating methods (laboratory device(s), analytical process, age model, ...); reference(s) for the used method, principle investigator (name, address, email)

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### 3. Rate and Activity Measurements

#### 3.1. Thymidine incorporation

Thymidine is one of the four bases of DNA. By measuring the rates of incorporation of tritiated thymidine into prokaryotes we can obtain a measure of population growth. There are a number of provisos of this method. Methanogenic *Archaea* and many sulphate-reducing bacteria do not incorporate thymidine, relying instead on de novo synthesis. Thus thymidine incorporation is more usefully a measure of growth in the heterotrophic population. Thymidine may be utilized by starving prokaryotes as a carbon source and be metabolized rather than incorporated into DNA. Incubation periods must consequently be short – typically a few hours.

#### Field:

Sediment subcores are sampled in 26 mm ID acrylic tubes with injection ports filled with silicone rubber.

About 10 µl (~200 kBq) radioactively labeled <sup>3</sup>H-thymidine solution is injected into the sediment in 1-cm depth intervals and incubated for 3-12 h at *in situ* temperature.

Activity is terminated by extruding the sediment sample into cold Trichloroacetic acid (TCA) in a 50 ml centrifuge tube and storing at 0-4°C

Alternatively, where individual 5 ml syringe mini-cores are used then 37 µl of tritiated thymidine (~ 750 K bq) is injected along the centre line of the syringe and incubations are terminated as described above or by directly freezing the syringe for long term storage followed by defrosting in TCA when processing begins.

Blank samples are prepared by adding 37 µl of tritiated thymidine to a well mixed slurry of sediment sample (5 ml) and TCA (5 ml) in a centrifuge tube at 0-4°C

#### Laboratory:

Processing methodology is adapted from Wellsbury *et al.* (1996) as originally derived from Karl (1982) and Craven & Karl (1984).

#### DAY 1

1. If not already done (see above), transfer sample (5 ml) to 50 ml centrifuge tube containing 5 ml of 10% TCA at 0-4°C. Mix thoroughly, and store in a fridge or cold room at 0-4°C until extraction.
2. Centrifuge at 2000 g for 15 min at 2°C
3. Decant and collect the supernatant in a Sterilin bottle. Add another 10 ml of 5% TCA at 0-4°C to the centrifuge tube, mix and centrifuge at 2000 g for 15 min at 2°C. Decant and add the supernatant to the Sterilin bottle, repeat rinse for a third time with a further 10 ml of 5% TCA. Thoroughly mix the Sterilin bottle on a vortex mixer and count a 5 ml sub-sample of the combined supernatant. This is the **UNINCORPORATED** fraction. Discard remaining supernatant to sink and soak “Sterilin” bottle in Decon prior to disposal into bin.





4. Rinse (re-suspend, vortex mix and centrifuge at 2000 g and 2°C) sediment twice in 10 ml of 95% ethanol at 0-4°C, collecting both supernatants in a new Sterilin bottle. Re-suspend sediment pellet in 7 ml of 95% ethanol and transfer to a 15 ml centrifuge tube. (This is best done by re-suspending the sediment initially in 5 ml of ethanol, tipping it into the 15 ml centrifuge tube, then re-suspending any residual sediment with a further 2 ml of ethanol before adding it to the 15 ml centrifuge tube). Centrifuge at 2000 g and 2°C and add supernatant to that already acquired. Vortex mix and count a 5 ml sub-sample of the combined supernatant. This is the **LIPID** fraction. Discard remaining supernatant to sink and soak Sterilin bottle and large centrifuge tube in Decon prior to disposal into bin.
5. Leave the samples to dry off overnight under an extractor with the lids off the tubes. Max temp 37°C.

## DAY 2

6. Add 7 ml of 1M NaOH, mix and incubate in a water bath for 1 hr at 37°C. Centrifuge at 2000 g for 15 min at 2°C
7. Transfer 5 ml of supernatant to a new 15 ml centrifuge tube. Discard remaining supernatant to sink. Keep sediment pellet, this is the **PROTEIN** fraction (replace lid, ensure tube is suitably identified and store in freezer). Do not process for protein at this stage. **GO TO STEP 13**
8. To the 5 ml of supernatant add 1.5 ml of 'acidifying solution', 50 µl of cold carrier DNA (0.05 mg) and 50 µl of cold carrier RNA (0.05 mg), and a small amount of Kieselguhr. Mix and cool on ice to 0-4°C.
9. Centrifuge at 3000 g for 15 min at 2°C. Count a 2 ml subsample of the supernatant. This is the **RNA** fraction. Discard remaining supernatant very carefully to sink ensuring that the tiny pellet at the bottom of the tube is not disturbed.
10. Rinse (re-suspend, vortex mix and centrifuge at 3000 g for 15 min at 2°C) remaining pellet twice with ice cold 5% TCA carefully discarding the supernatant to sink and retaining the pellet.
11. Add 5 ml of 5% TCA, vortex mix and incubate at 100°C in a water bath for 30 min. (You may need to loosen the caps to prevent the tubes bursting).
12. Cool on ice rapidly, centrifuge at 3000 g for 15 min at 2°C. Count a 2 ml sample of the supernatant. This is the **DNA** fraction. Discard remaining supernatant to sink. Dispose of extracted pellet and soak centrifuge tube in Decon prior to disposal into bin.

## Protein extraction:

13. Sediment Pellet Rinse (re-suspend, vortex mix and centrifuge at 3000 g for 15 min at 2°C) sediment pellet once with 5% TCA and once with 95% ethanol. Discard rinses to sink.
14. Add 5 ml of 2M NaOH, mix and incubate at 37°C for 18 hrs. Centrifuge at 2000 g for 15 min at 2°C.
15. Count a 2 ml sub-sample of the supernatant. This is the **PROTEIN** fraction. Dispose of sediment to sink and soak tubes in Decon prior to disposal into bin.



### Reagents:

10% (w/v) Trichloroacetic acid (TCA) in Milli-Q water.

5% (w/v) TCA solution.

95% (v/v) ethanol solution

1 M NaOH in Milli-Q water

2 M NaOH in Milli-Q water

'Acidifying solution' 20% (w/v) TCA in 3.6 M HCl

DNA solution 1 mg/ml in Milli-Q water(e.g., Sigma D-6898 or D-1501)

RNA solution 1 mg/ml in Milli-Q water(e.g., Sigma R-7125)

Kieselguhr (Sigma D-5384)

### Centrifugation:

Centrifugations are carried out at 2000 x g and 3000 x g and the RPM required is calculated from:

$$\text{RPM} = \sqrt{\frac{g \times 1,000,000}{11.18 \times R}}$$

Where: RPM = revolutions per minute; g = g-force; R = average sample radius in rotor (cm)

### References:

Karl, D.M., (1982) Selected nucleic acid precursors in studies of aquatic microbial ecology. *Appl. Environ. Microbiol.*, 44:891-902

Craven D.B. and Karl, D.M., (1984). Microbial RNA and DNA synthesis in marine sediments. *Mar. Biol.* 83:129-139.

Wellsbury, P., Herbert, R.A., and Parkes, R.J., (1996). Bacterial activity and production in near-surface estuarine and freshwater sediments. *FEMS Microbiol. Ecol.*, 19:203-214.

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### 3.2. Leucine incorporation

The method aims at measuring bacterial biomass (secondary) production by measuring the rate of incorporation of tritiated Leucine ( $^3\text{H}\text{-Leu}$ ) into bacterial cells. Biomass production is calculated from rates of protein synthesis by using empirical factors. This method, indeed, has become the mostly common method for measuring bacterial production in both water column and sediments (Kirchman *et al.*, 1986; van Duyl & Kop, 1994).

Sediment samples are preferentially collected using multiple corers, which allow recovering undisturbed, intact sediment cores. Immediately after retrieval, sediment sub-samples are gently removed from the corer inserting cut-off 10-ml plastic syringes along the axis of the core. The syringe plunger is held fixed at the sediment surface while the barrel is pushed into the sediment for 1 centimetre, in an overall procedure that is analogous to piston coring. The syringes are then removed from the sediment and the sediment is transferred into a sterile tube and resuspended in sterile seawater to form an homogeneous slurry (dilution 1:1).

Sediment sub-samples (0.2 ml) are then transferred to 2-ml sterile Eppendorf tubes and incubated in the dark for 1 hour at *in situ* temperature with 30  $\mu\text{l}$  of an aqueous solution of L-[4,5- $^3\text{H}$ ] leucine (Amersham). Saturation concentrations of leucine is generally at 0.5  $\mu\text{M}$  (final concentration), but can vary in different areas/samples and should be thus assessed using different concentrations. The measurement of bacterial C production in deep sea samples generally is generally not carried out under *in-situ* pressure condition, so that estimates may be biased (Yayanos, 1995).

After incubation, bacterial incorporation is stopped with 1.7 ml of 80% ethanol. The sediment sub-sample is centrifuged (10000 x g, 5 minutes), the supernatant gently removed and the sediment resuspended in 1.7 ml of 80% ethanol. This centrifugation-resuspension procedure is carried out twice. After the second procedure, the sediment-ethanol slurry is passed through a polycarbonate filter (0.2  $\mu\text{m}$  mesh size). The filters are rinsed four times with 2 ml of 5% TCA each (Trichloroacetic Acid), transferred to sterile pyrex tubes, added with 2ml of NaOH and heated for 2 hours in a water bath at 100°C. After centrifugation of the tubes, 1 ml of supernatant is transferred to scintillation vials containing 10 ml of scintillation liquid (Perkin Elmer or similar). Measurements of radioactivity are carried using a liquid scintillation counter.

For each sediment sample, a total of three replicates and two blanks are analysed. Sediment blanks are run as described for sediment samples but adding 1.7 ml of 80% ethanol immediately before  $^3\text{H}\text{-Leucine}$  addition. Data are normalised to sediment dry weight after desiccation (60°C, 24h).

Data of  $^3\text{H}\text{-leucine}$  incorporation are converted into bacterial Carbon production by using the following formula:

$$\text{Bacterial C Production } \text{g}^{-1} = [\text{nmol incorporated leucine (100/7.3)} \cdot \text{M} \cdot 0.86 \cdot \text{R} \cdot 2] / \text{g}$$

Where:

- the ratio 100/7.3 is the percentage of leucine into the total bacterial aminoacid pool
- M is the molar weight of leucine
- 0.86 is the conversion factor of bacterial protein production to bacterial C production



- R is the correction for radioactive decay of the  $^3\text{H}$ -leucine
- 2 is the intracellular isotope dilution
- g is sediment dry weight (grams).

#### References:

Kirchman DL, Newell SY, & Hodson RE (1986) Incorporation versus biosynthesis of leucine: implications for measuring rates of protein synthesis and biomass production by bacteria in marine systems. *Mar Ecol Prog Ser* **32**:47-59

van Duyl FC, & Kop AJ (1994) Bacterial production in North Sea sediments: clues to seasonal and spatial variations. *Mar Biol* **120**:323-337

Yayanos AA (1995) Microbiology to 10,500 meters in the deep sea. *Ann Rev Microbiol* **49**:777-805

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### 3.3. Sulphate reduction

Sulphate reduction rates are measured experimentally in sediment cores using a whole-core injection method modified from Jørgensen (1978). The distillation procedures are performed either hot (Fossing and Jørgensen, 1989) or cold (Kallmeyer et al., 2004) depending on the expected sulphate reduction rates.

#### Field:

- Sediment subcores are sampled in 26 mm ID acrylic tubes with injection ports filled with silicone rubber.
- About 5  $\mu\text{l}$  (400 kBq) radioactively labelled  $^{34}\text{SO}_4^{2-}$  solution is injected into the sediment in 1-cm depth intervals and incubated for 6-24 h at *in situ* temperature.
- The bacterial sulphate reduction is stopped in 20% (w/v) zinc acetate by mixing sediment with a known amount of 20% zinc acetate (ZnAc) solution (20 g zinc acetate dihydrate in 100 mL water). The ZnAc volume to sediment volume should be 2:1. ZnAc preserves the radiolabelled sulphide as  $\text{Zn}^{35}\text{S}$  and stops bacterial activity. It is important for optimal storage, samples should be frozen.
- Blank samples (i.e. sediment samples with no  $\text{Zn}^{35}\text{S}$  formed) are prepared when 5  $\text{cm}^3$  sediment is preserved in 10 ml 20 %-ZnAc (w/v) prior to  $^{35}\text{SO}_4^{2-}$  addition. About 10  $\mu\text{l}$  (800 kBq)  $^{35}\text{SO}_4^{2-}$  is added to the preserved sample after at least 1 hour (i.e. when sulphate reduction has come to a complete stop). The blank samples are used during distillation to estimate the amount of non-reduced  $^{35}\text{S}$  that are transferred from the labeled sediment to the trap. See Kallmeyer et al.(2004) for a complete discussion of blanks and detection limits.

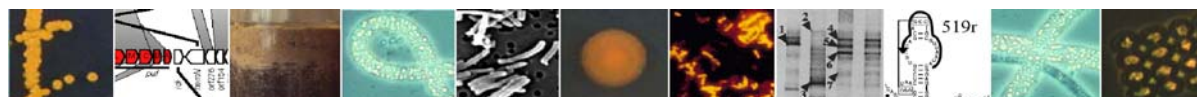
#### Laboratory:

- The weight of each vial + sediment + ZnAc is determined. The empty vial weight (mean of several weighings of empty vials) and the weight of ZnAc solution may be subtracted to give the sediment wet weight.
- The samples are centrifuged (4500 rpm, 5 min; sandy sediments 6000 rpm) and the supernatant carefully removed and kept for further analysis.
- $^{35}\text{SO}_4^{2-}$  radioactivity of the supernatant is determined on a 0.1 ml sample in 1 ml  $\text{H}_2\text{O}$  and 7 ml Lumasafe plus (Lumac BV.) in the scintillation counter.

#### Distillation techniques:

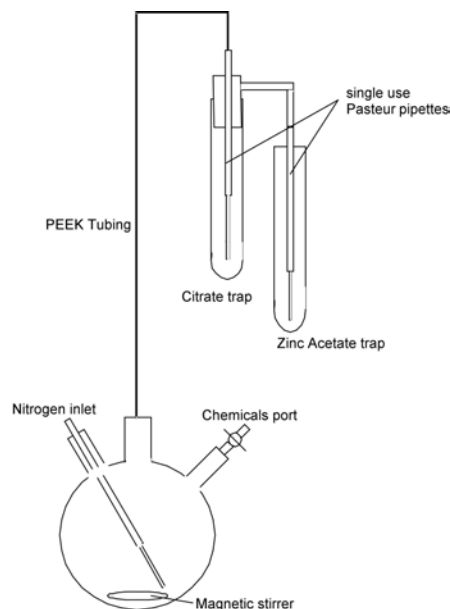
Hot distillations can be performed on samples with an expected sulphate reduction rate  $> 1 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ day}^{-1}$  (see also Fossing and Jørgensen, 1989):

- Approx. 1-2 g sediment (exact weight must be known) is transferred to a flask and mixed with 10 ml 50% ethanol. The flask is attached to the distillation apparatus and connected to a distillation trap through a condenser.
- The flask is flushed with  $\text{N}_2$  for 10 min, after which 8 ml HCl (6 M) and 16 ml CrII-solution (1M) are added to liberate all reduced inorganic sulfur species (mono- and disulphides and elemental sulfur) when boiled for 40 min.
- The released  $\text{H}_2\text{S}$  is trapped in 10 ml 5% ZnAc as ZnS. To reduce foaming in the trap a drop of antifoam is added to the pipet tip above the trap solution.





- The activity of the total reduced inorganic sulfur species (TRIS) is determined by liquid scintillation counting. Half the volume of the ZnAc trap is quantitatively transferred into a counting vial and mixed with 10 ml of Lumasafe plus (Lumac BV.)



Cold distillation is recommended for samples having expected sulphate reduction rates of  $< 1 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ day}^{-1}$  (see also Kallmeyer et al., 2004):

- The sediment is resuspended in 20 ml 1,2 N-N dimethylformamide (technical grade), transferred to a flask with a magnetic stirrer and a drop of antifoam, and the flask is attached to the distillation apparatus.
- In cases where the samples contain only small amount of reduced sulfur carrier is added, usually 0.5 mL of 50 mM ZnS suspension
- The flask is flushed with  $\text{N}_2$  for 10 min, after which 8 ml HCl (6 M) and 16 ml CrII-solution (1M) are added to reduce all reduced inorganic sulfur species (mono- and disulphides and elemental sulfur). The released  $\text{H}_2\text{S}$  is trapped in 7 ml ZnAc (5 %) during 2 h of distillation. To reduce foaming in the trap a drop of antifoam is added to the pipet tip above the trap solution.
- The activity of the total reduced inorganic sulfur species (TRIS) is determined by liquid scintillation counting. The entire volume of the ZnAc trap is quantitatively transferred into a counting vial and mixed with 14 ml of Lumasafe plus (Lumac BV.)

#### Calculation:

The sulphate reduction rate is calculated as

$$SRR = \frac{a}{A+a} \cdot \frac{24}{t} \cdot [\text{SO}_4^{2-}] \cdot 1.06 \text{ nmol SO}_4^{2-} \text{ cm}^{-3} \text{ day}^{-1}$$



where  $a$  is the total radioactivity of ZnS in one volume of fresh sediment,  $A$  is the total radioactivity of  $^{35}\text{SO}_4^{2-}$  in one volume of fresh sediment after incubation,  $t$  is the incubation time in hours,  $[\text{SO}_4^{2-}]$  is the sulphate concentration in  $\text{nmol per cm}^{-3}$  fresh sediment, and 1.06 is the correction factor for the expected isotope fractionation.

#### References:

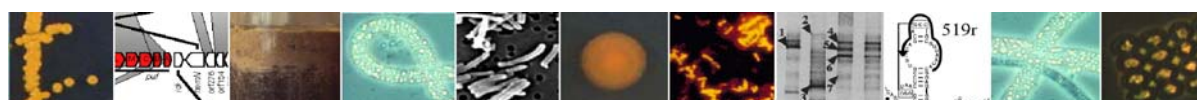
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### 3.4. Methanogenesis from CO<sub>2</sub> + H<sub>2</sub> and from acetate

Methanogenesis from H<sub>2</sub> and CO<sub>2</sub> or from acetate is measured experimentally in subcore samples using <sup>14</sup>C-labeled bicarbonate or acetate as tracers. Methanogenic prokaryotes may reduce CO<sub>2</sub> with hydrogen, or utilize organic compounds (e.g. acetate) to produce methane. This process generally occurs in marine sediments after sulphate-reduction in the upper few metres has depleted, or removed, pore-water sulphate concentrations thus allowing methanogens to successfully compete for hydrogen and organics. Methanogenesis is the dominant prokaryotic process in the deep sub seafloor. After injection and incubation evolved methane is oxidised to CO<sub>2</sub> by flushing through a furnace containing copper oxide and collected with a CO<sub>2</sub> – scrubbing compound (phenethylamine), after the method of Whelan (1985).

#### Field:

- At the core processing station sub-sample mini-cores (2.2 cm diameter) are taken with clean Perspex tubes forced into the cut core surface for 10 cm. During this process a vacuum is applied by sucking a tube attached to a stopper at the top of the tube to ensure that the sediment mini-core is not compressed during sampling. The tubes have been pre-drilled with 1 mm ports at 1 cm intervals along their length and these have been sealed with a silicone based aquarium sealant.
- The tubes are stoppered with a butyl rubber bung and stored temporarily, at the *in situ* temperature before being transported to the isotope station.
- Typically the mini-cores are left to equilibrate for 6 – 12 hours prior to injection. A 10 µl injection micro-syringe (Hamilton) is flushed thoroughly with the isotope (at least 5 times), ensuring that there are no air bubbles. The micro-syringe needle is inserted laterally through the ports in the side of the tube at 2 cm intervals and 2 µl of isotope are injected.
- Amounts of radiotracer injected at each port are approximately;
  - i) <sup>14</sup>C-bicarbonate 2 µl = 50 Kbeq as sodium <sup>14</sup>C-bicarbonate (Amersham, UK), previously diluted 1:4 with de-gassed, filter-sterilized (0.2 µm) distilled water
  - ii) <sup>14</sup>C-acetate 2 µl = 15 Kbeq as sodium [1-(2)<sup>14</sup>C] -acetate (Amersham, UK), used undiluted.

At the conclusion of the injections the micro-syringe is thoroughly rinsed with distilled water (10 times), to remove any residual isotope.

- After injections all mini-cores are incubated at *in situ* temperature for 6 hours (acetate) or 18 hours (bicarbonate).
- Incubations are terminated by piston extrusion of 2 cm sections of mini-core that are sliced off and put immediately into glass jars containing 7 ml of 1.0 M NaOH. The jars are tightly sealed with a butyl rubber bung, shaken to reduce the core section to, a slurry, taped for security and stored upside down at room temperature to await processing.



### Laboratory:

- Samples are processed through a “methane-furnace rig” using a method adapted from that described by Whelan et al (1985). The principle of this apparatus is that the sample vials are connected, using large diameter needles (19G), to a stream of carrier gas (99% N<sub>2</sub>, 1% O<sub>2</sub>) flowing at 70 ml/min. The headspace is blown along copper tubing through a Vost tube of indicating silica-gel desiccant, followed by a CO<sub>2</sub> trap (Supelco, UK) and a second desiccant trap. After this the gas sample passes through a wider (6 mm) steel tube packed with copper oxide in a cylindrical furnace (Carbolite, UK) at 800°C where the <sup>14</sup>CH<sub>4</sub> is oxidized to <sup>14</sup>CO<sub>2</sub>. The CO<sub>2</sub> is collected by bubbling the gas flow through a series of two rubber-stoppered scintillation vials containing 10 ml of scintillant (Opti-Phase 3, Perkin Elmer, UK) mixed (93:7) with β-phenethylamine to capture the <sup>14</sup>CO<sub>2</sub>. The scintillations are then counted on a scintillation counter and rates of methanogenesis are calculated.

### Reference:

Whelan, J.K., Oremland, R., Tarafa, M., Smith, R., Howarth, R., and Lee, C., (1985). Evidence for sulfate-reducing and methane producing organisms in sediments from Sites 618, 619 and 622. In; Bouma, A.H., Coleman, J., Meyer, A.W., et al., Init.Repts. DSDP, 96: Washington (U.S. Govt. Printing Office), 767-775

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### 3.5. Anaerobic oxidation of methane

The oxidation of methane is measured experimentally in sediments, either using a whole core injection technique or small glass tubes sealed with butyl rubber stoppers. Sediments are incubated with  $^{14}\text{CH}_4$  as a tracer. The method is modified from Iversen and Jørgensen (1985).

- The samples are incubated anaerobically with  $^{14}\text{CH}_4$ -tracer, which is oxidized to  $^{14}\text{CO}_2$ . Depending on the expected rate, the incubation time varies between 8 and 24 hours.
- The ongoing AOM process with the tracer is stopped by transferring the sample to 50 ml glass jars containing 25 ml NaOH (2,5 %, w/v) → separation of  $\text{CO}_2$  (dissolved in NaOH) and  $\text{CH}_4$  (gaseous in headspace and is measured first)

a)  $^{12+14}\text{CH}_4$  concentration is measured by gas-chromatography:

- 200  $\mu\text{l}$  of the headspace are subsampled for GC analyses. This amount is negligible in comparison to the total volume of the headspace. Artefacts in the  $^{14}\text{CH}_4$  measurements are therefore not introduced.
- the methane concentration is calculated from the methane peak area of the GC measurement:

$$\text{CH}_4 [\text{nmol ml}^{-1} \text{ sed}^{-1}] = \frac{\text{GC - Area} * C_F * \text{Vol}_{\text{Headspace (ml)}}}{22.4 * \text{Vol}_{\text{Sediment (ml)}}}$$

$C_F$  is the calibration factor (0.0007 at the MPI GC system), which is determined with standard gas mixtures for 200  $\mu\text{l}$  injection volume; 22.4 is the gas constant ( $\text{mol l}^{-1}$ ) at room temperature.

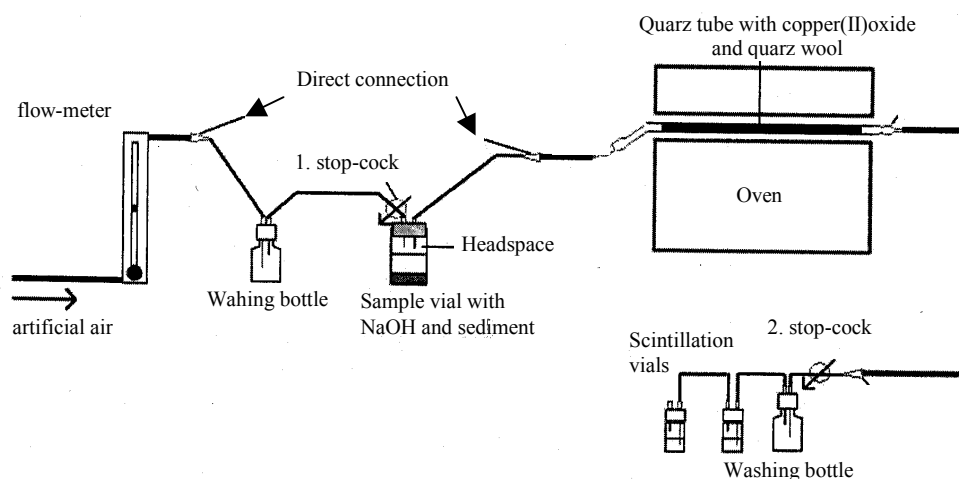
b) Headspace (containing  $^{12+14}\text{CH}_4$ ) is burned and  $^{14}\text{CH}_4$  is measured indirectly as  $^{14}\text{CO}_2$  after burning:

- The sample headspace is connected to the oven by rubber tubing. It is then purged with air and the air/methane mixture is subsequently burned to  $^{14}\text{CO}_2$ . The  $^{14}\text{CO}_2$  is trapped in two succeeding 20 ml scintillation vials containing 1 ml phenylethylamine + 7 ml ethylenglycolmonomethylether.
- Both vials are measured in the wet scintillation counter after the addition of 10 ml Ultima-Gold. The resulting counts (i.e., CPMB) of both vials are summed and the amount of  $^{14}\text{CO}_2$  calculated from the activity and the blank counts determined from a vial containing only the scintillation chemicals:

$$^{14}\text{CH}_4 [\text{KBq}] = \frac{\text{CPMB}_{\text{Sample a+b}} - \text{CPMB}_{\text{Blank}}}{60000}$$







c) CO<sub>2</sub>-diffusion method: <sup>12+14</sup>CO<sub>2</sub> is removed from the NaOH by acidification:

- The lid is removed and the 50 ml jars containing the samples are weighed (if sediment volume is unknown).
- The samples are transferred from the jars into 250 ml Erlenmeyer flasks. The empty glass jar is weighed again to determine sample mass, which can be calculated to volume with porosity values.
- 6 ml scintillation vial containing 1ml phenylethylamine and 1ml 0,5M NaOH is connected with the rubber stopper (rubber-stopper construction) in a “free floating” position.
- 6ml 6M HCl are injected between rubber stopper and flask neck to acidify the sample in order draw out the <sup>14</sup>CH<sub>4</sub>.
- The closed Erlenmeyer flask are shaken for 4 hrs to promote trapping of the <sup>14</sup>CO<sub>2</sub> in the phenylethylamine.
- the radioactivity is measured in the wet scintillation counter after the addition 3ml of Ultima Gold. The amount of <sup>14</sup>CO<sub>2</sub> calculated from the activity of the sample and a blank containing only the scintillation chemicals:

$$^{14}\text{CO}_2 \text{ [KBq]} = \frac{\text{CPMB}_{\text{Sample}} - \text{CPMB}_{\text{Blank}}}{60000}$$

d) Rate calculation:

Two formulae may be applied to calculate AOM:

$$\text{AOM} = \frac{^{14}\text{CO}_2}{^{14}\text{CH}_4 + ^{14}\text{CO}_2} \times \frac{\text{conc. CH}_4}{\text{incubat. Time}} \quad (1)$$



Here,  $^{14}\text{CO}_2$  and  $^{14}\text{CH}_4$  are the activities (Bq) of carbon dioxide and methane, whereas conc.  $\text{CH}_4$  is the concentrations of methane at the beginning of the incubation.

If significant amounts of methane have escaped during the incubation, the following formula can be used as it accounts for this loss.

$$\text{AOM} = \frac{^{14}\text{CO}_2}{^{14}\text{CH}_4} \times \frac{\text{conc. CH}_4}{\text{incubat. Time}} \quad (2)$$

Here,  $^{14}\text{CO}_2$  and  $^{14}\text{CH}_4$  are the activities (Bq) of carbon dioxide and methane, whereas conc.  $\text{CH}_4$  is the concentrations of methane at the end of the incubation.

#### Reference:

Iversen, N. and Jørgensen B.B. (1985) Anaerobic methane oxidation rates at the sulfate-methane transition in marine sediments from Kattegat and Skagerrak (Denmark). *Limnol. Oceanogr.* 30: 944-955.

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### 3.6. In vitro sulphate reduction with methane

#### 3.6.1. In vitro rate determination from substrate concentration changes

The sediment samples taken from the sulfate-methane transition zone are anoxically stored in butyl rubber sealed bottles (250 ml) without headspace (or methane in the headspace) at *in situ* temperature until further processing in the laboratory. All manipulations are done under an anoxic atmosphere of N<sub>2</sub> using either the Hungate technique and its modifications (Widdel and Bak, 1992; Breznak and Costilow, 1994) or an anoxic glove box (Mecaplex). The original sediment is mixed with approximately the same volume of anoxic seawater. In this way, a slurry with 0.2 to 0.3 g sediment dry mass per ml is obtained which can be transferred to tubes by means of plastic tubes (length, 150 mm; inner diameter, 5 mm) connected to a syringe (preflushed with nitrogen). Sulphate reduction with methane at partial pressures of 0.1 MPa (1 atm) or less is measured in culture tubes (volume, approx. 20 ml; length, 135 mm; inner diameter, 14 mm) with a tapered, Viton rubber-sealed orifice (inner diameter, 9 mm). The culture tubes are provided with approx. 3 ml of sediment slurry and 9 ml of anoxic sea water (pre-reduced with 0.5 mM sulphide) or artificial sea water medium with ammonium (4 mM), phosphate (1 mM), trace elements, vitamins, bicarbonate (30 mM) and sulphide (0.5–1 mM) as used for cultivation of SRB (Widdel and Bak, 1992). The pH at the beginning of incubation is 7.5. Pure methane of atmospheric pressure (0.1 MPa) is applied in the headspace. In experiments with artificial seawater medium and methane of atmospheric pressure, an amount of CO<sub>2</sub> corresponding to 1/10 of the headspace volume is in addition injected by means of a syringe. A commercial N<sub>2</sub>-CO<sub>2</sub>-mixture (90/10, [vol/vol]) is applied for controls. Lower methane partial pressures are achieved in tubes with the indicated N<sub>2</sub>-CO<sub>2</sub>-mixture into which defined volumes of methane are injected. Tubes are incubated horizontally to facilitate diffusion of methane into the sediment; they are gently shaken for a few seconds once per day. Tubes are not continuously shaken to avoid possible disintegration of microbial associations. Samples for chemical analyses (100 µl) are withdrawn during incubation via microliter syringes (preflushed with N<sub>2</sub>).

The simultaneous determination of methane utilisation and sulphide production to investigate the stoichiometry of the process can be carried out in an incubation experiment without gas phase. A relatively large gas phase of methane compared to the aqueous phase would not allow reliable measurement of methane consumption, particularly at the beginning of the experiment when the decrease in the total amount of methane is still relatively small. Therefore, a special glass tube is used (modified from Alperin and Reeburgh, 1985) that allows head space-free incubation of sediment samples with dissolved methane concentrations above the saturation limit given at ambient pressure. At one end, the glass tube (length, 180 mm; inner diameter, 17 mm) is tapered towards a regular orifice (inner diameter, 9 mm) with a stopper (Viton rubber, fixed by screw cap with hole) that allows withdrawal of aliquots with a syringe. At the other end, the tube is tapered towards a smaller elongated glass tube (length, 65 mm; inner diameter, 9 mm) that harbours a gas-tight piston (silicon-lubricated Viton rubber); the piston is held in position by a screwing device. If a sample is withdrawn via the opposite stopper, the piston is simultaneously pushed into the tube so as to avoid any underpressure and formation of gas bubbles. High dissolved methane concentrations are achieved via an initial small (5 ml) headspace in which 0.4 MPa (4 atm) of methane is kept for some hours under shaking. The gas phase is finally replaced by anoxic medium, and remaining bubbles are allowed to escape via an inserted hypodermic needle while the piston is slightly moved. This device, which is provided with 6 ml sediment slurry in a total volume of 40 ml, is incubated as the tubes described above.



Analyses:

Sediment dry mass is determined after drying at 80°C for 2 days.

Sulphide is determined colorimetrically using the methylene blue formation reaction in a miniaturised assay (Aeckersberg *et al.*, 1991) or the formation of colloidal copper sulphide (Cord-Ruwisch, 1985).

For the quantification of sulphate, 1.5 ml of a particle-free water sample is mixed with 0.1 ml of 2 M HCl. After heating in a boiling water bath, 0.4 ml of 0.5 M BaCl<sub>2</sub> solution is added. Precipitated BaSO<sub>4</sub> is quantitatively collected on a nitrocellulose filter (25 mm diameter, 0.2 µm pore size), washed with 10 ml distilled water, dried at 60°C and quantified by weighing.

Methane is determined using a GC 14B gas chromatograph (Shimadzu) equipped with a Supel-Q Plot column (30 m x 0.53 mm; Supelco) and a flame ionisation detector. The carrier gas was N<sub>2</sub> at a flow rate of 3 ml min<sup>-1</sup>. The column temperature was 110°C.

Calculations:

The geometry inside the inoculated tubes and their handling (occasional shaking) do not allow application of diffusion models to calculate the actual methane concentration in the sediment during incubation. Only rough estimation of a lower limit appears possible below which the methane concentration in the sediment is unlikely to drop. In the horizontally incubated culture tubes, the settled sediment forms a loose layer nearly over the whole length. The height of the liquid, which can be regarded as the approximate diffusion distance, is  $\Delta x = 0.8$  cm (maximum in the middle). The loose thin sediment layer is not expected to impede diffusion significantly. In the incubation experiment with various methane pressures, application of 0.1 MPa (1 atm) methane results in an increase of the sulphide concentration in the medium of approx.  $0.07 \mu\text{mol cm}^{-3} \text{ d}^{-1}$ , which is  $0.84 \mu\text{mol d}^{-1}$  for the culture volume (12 ml). With a surface area of the settled sediment of roughly  $8 \text{ cm}^2$ , the flux of sulphide into the medium and hence the flux of methane into the sediment would be  $J = 0.105 \mu\text{mol cm}^{-2} \text{ d}^{-1}$ . The diffusion coefficient for methane in seawater at 12°C is  $D = 0.86 \text{ cm}^2 \text{ d}^{-1}$  ( $1 \cdot 10^{-5} \text{ cm}^2 \text{ s}^{-1}$ ; Iversen and Jørgensen, 1985). Hence, the calculated flux would be associated with an approximate concentration difference between the sediment and the medium surface of  $-\Delta C = 0.1 \mu\text{mol cm}^{-3}$  (0.1 mM), according to Fick's first law of diffusion (for linear gradient,  $J = -D \Delta C / \Delta x$ ). The occasional shaking favours the methane supply to the sediment. Hence, if the concentration of methane in the upper medium remains at 1.4 mM (dissolved methane upon addition of 0.1 MPa, 12°C), the concentration in the sediment under the given conditions should not be lower than 1.3 mM. If the rate  $v$  of sulphide production (and hence of methane oxidation) depends on the methane concentration  $C$  according to Michaelis-Menten kinetics, an increase of the concentration from  $C_1$  to  $C_2$  would result in an increase of the rate by a factor of  $v_2/v_1 = [C_2(K_M + C_1)]/[C_1(K_M + C_2)]$ .

Free energy changes ( $\Delta G$  values) under in situ or incubation conditions can be calculated from  $G^\circ_f$  values via  $\Delta G^\circ$  values. For SO<sub>4</sub><sup>2-</sup>, HCO<sub>3</sub><sup>-</sup> and HS<sup>-</sup> in seawater, activity coefficients of 0.1, 0.5 and 0.5, respectively, were estimated (Stumm and Morgan, 1996). The influence of temperature on  $\Delta G^\circ$  can be calculated via the integrated Gibbs-Helmholtz equation including the enthalpy ( $\Delta H^\circ$ ). Redox potentials of half-reactions (viewed as being in equilibrium) are calculated from  $\Delta G^\circ$  values via  $E^\circ$  values using concentrations and activity coefficients as for the calculation of  $\Delta G$ , and assuming a pH of 7.5.



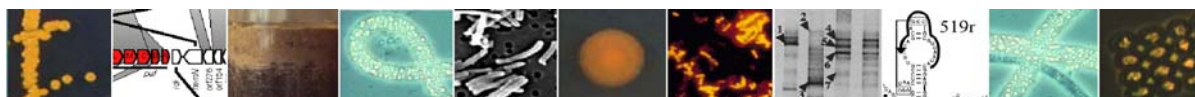
Reference:

Nauhaus K, Boetius A, Krüger M, Widdel F (2002). In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environmental Microbiology* 4, 296-305.

Widdel, F. and Bak F. (1992). The gram negative mesophilic sulfate reducing bacteria, In: *The Prokaryotes* (ed. Dworkin, M.), pp. 3352-3378. Springer Verlag.

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### 3.6.2. In vitro rate determination from radio-labelled tracer turnover

*In vitro* methane and sulphate turn over in sediment slurries can be measured in long term incubations with radio-labelled methane and sulphate according to a modified method of Nauhaus et al. (2002; see section 2.6.1). For this purpose, Hungate tubes (20 ml; n = 5 for AOM and SR, respectively) are provided with 3 ml of sediment slurry (containing ca. 1.5 ml of sediment) and 15 ml of anoxic, artificial seawater medium (Widdel and Bak, 1992) in a glove box under strictly anoxic conditions. The remaining headspace is flushed with a CH<sub>4</sub>/N<sub>2</sub> mixture to adjust methane concentration in the media. The slurry is then pre-incubated in a horizontal position to facilitate diffusion of methane into the sediment for 1 day to 1 week (depending on the expected activity). During this time period, the headspace is flushed several times with the CH<sub>4</sub>/N<sub>2</sub> mixture to maintain constant methane concentrations in the medium. After the pre-incubation period, the methane headspace is replaced with artificial seawater medium containing the same methane concentration as the sediment slurry. 50 µl <sup>14</sup>C-labelled methane and 5 µl <sup>35</sup>S-labelled sulphate (tracer dissolved in water, 10 kBq and 50 kBq, respectively) are injected in the tubes in equilibrium with artificial seawater medium. The Hungate tubes are then incubated in a horizontal position to facilitate diffusion of methane into the sediment for 1 day to 1 week (depending on the expected activity). The incubations are stopped by fixing the sediment slurries in glass jars containing 25 ml NaOH (2.5 %, w/v) and in falcon tubes containing 20 ml of Zn-Acetate solution (20%, w/v) for AOM and SR rate measurements, respectively. Further processing and rate calculations are according to the ex situ AOM and SR rate measurements (sections 2.5 and 2.3, respectively).

#### Reference:

Nauhaus K, Boetius A, Krüger M, Widdel F (2002). In vitro demonstration of anaerobic oxidation of methane coupled to sulphate reduction in sediment from a marine gas hydrate area. *Environmental Microbiology* 4, 296-305.

Widdel, F. and Bak F. (1992). The gram negative mesophilic sulfate reducing bacteria, In: *The Prokaryotes* (ed. Dworkin, M.), pp. 3352-3378. Springer Verlag.

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### 3.7. Assessment of extracellular enzymatic activities of benthic assemblages

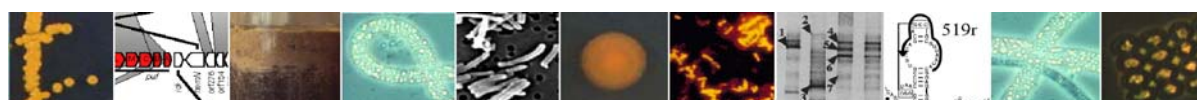
This method allows measuring the degradation rates of high-molecular-weight (HMW) organic compounds by extracellular microbial enzymes. These enzymatic activities, which are recognised as the key step in the degradation and utilisation of organic polymers by bacteria (Hoppe 1991; Meyer-Reil 1991), are measured by using fluorogenic model substrates (Hendel and Marxen 1997). The *in vitro* degradation of these fluorogenic analogues provides a reliable estimation of the rates of enzymatic activity and, in the deep sea, has been primarily focused on leucine aminopeptidase,  $\beta$ -D-glucosidase and alkaline phosphatase.

Extracellular enzymatic activity is measured immediately after sediment retrieval (Meyer-Reil, 1987; Meyer-Reil and Koster, 1992). Activities of L-aminopeptidase,  $\beta$ -D-glucosidase and alkaline-phosphatase are quantified fluorometrically by the cleavage of artificial fluorogenic substrates (Hoppe, 1993), using L-Leucine-4-methylcoumarinyl-7-amide (Leu-MCA), 4-methylumbelliferone  $\beta$ -D-glucoopyranoside (MUF-Glu), 4-methylumbelliferone phosphate (MUF-P) as substrates, respectively.

Marine sediment collection for the assessment of extracellular enzymatic activities must be carried out avoiding any contamination of the sample that could affect estimates. Sediment samples from deep localities for enzymatic assays are preferentially collected using multiple corers. Immediately after retrieval, sediment sub-samples are gently removed from the corer inserting 10-ml plastic syringes along the axis of the core. The syringe plunger is held fixed at the sediment surface while the barrel is pushed into the sediment, in an overall procedure that is analogous to piston coring. The syringes are then removed from the sediment and the sediment is transferred in a sterile tube and resuspended with sterile seawater to produce a sediment slurry (1:1 ratio).

Substrate incubations are performed in the dark at *in situ* temperature for 1 hour (enzymatic activities generally increase linearly with time up to 3 hours), in a final volume of 5 ml containing sterile seawater, an aliquot of the sediment slurry (500  $\mu$ l) and the fluorogenic substrate. The substrate is added at saturating concentrations (which are generally at 100-200  $\mu$ M, final concentrations), but saturating conditions must be estimated with caution, after kinetic runs, using the Michaelis-Menten equation. The measurement of enzymatic activities in deep sea samples is generally not carried out under *in-situ* pressure condition. However, previous studies reported that aminopeptidase activities did not change significantly between decompressed and recompressed abyssal sediment samples (Poremba 1995), but Deming and Baross (2000) reported a 5-fold increase of aminopeptidase activity after abyssal sediment recompression. These contrasting results indicate that the extracellular enzymatic activities measured in the deep sea must be considered with caution.

After incubation, the slurries are centrifuged (3000 x g, 5 minutes) and supernatants analysed fluorometrically (at 380 nm excitation, 440 nm emission for Leu-MCA and 365 nm excitation, 455 nm emission for Glu-MUF and MUF-P). Immediately after substrate inoculation (at t = 0), the fluorescence of each sample is measured (blank) and then subtracted from fluorescence after 1 hour of incubation. Data are normalised to sediment dry weight (60°C,



24 h) and reported as nmol of MUF or MCA released per g of sediment dry weight  $h^{-1}$ . The replicates must be generally run per each sediment samples.

Solutions of 7-amino-4-methylcoumarin and 4-methylumbelliferone (0.1 to 1.0  $\mu$ M) are used as standards for Leu-MCA, and for MUF-Glu and MUF-P respectively. Standard solutions should be freshly prepared using pre-filtered and autoclaved seawater.

Aminopeptidase and  $\beta$ -glucosidase activities can be transformed into equivalents of C mobilised assuming that 1 nmol of substrate hydrolysed enzymatically corresponds to 72 ng of mobilised C.

### References:

Deming JW, Baross JA (1993) The early diagenesis of organic matter: bacterial activity. P. 119-144. In M.H.Engel and S.A. Macko (ed.), organic geochemistry: principles and applications. Plenum Press, New York, NY

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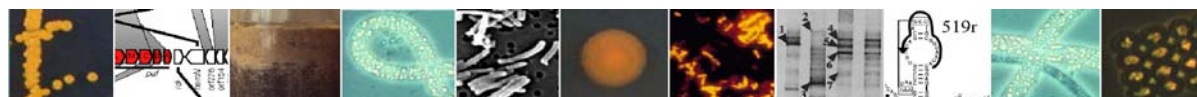
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### Contact:

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## 4. Counting Methods

### 4.1. Acridine orange direct counting (AODC) of prokaryotic cells in sediment

This method aims to count the total number of prokaryotes present in sediment and the proportion that are growing, as indicated by cells that are dividing or have just divided. Acridine orange is used to differentially stain prokaryotes green when viewed under blue light by epifluorescence microscopy. Samples (mini-cores) are taken from the core using a sterile (autoclaved) 5-ml plastic syringe from which the luer end has been removed. A 1 cm<sup>3</sup> plug is ejected directly into a serum vial (previously furnace at 450°C) containing 9 ml of filter sterilized (0.2-µm) 2% formaldehyde in artificial seawater, crimp sealed, and shaken vigorously to disperse the sediment plug.

In the laboratory the samples are processed using acridine orange staining and epifluorescence microscopy based on the general recommendations of Fry (1988). Generally, between 5 µl and 25 µl of formaldehyde-preserved sub sample are stained with acridine orange (50 µl of 1 g/l solution) in 10 ml of filter sterilized (0.1 µm pore size) 2% formaldehyde for three minutes and then vacuum filtered through a polycarbonate (0.2 µm pore size) membrane. The membrane is then rinsed with a further 10 ml of 2% filter sterilized formaldehyde and mounted in a minimum of paraffin oil under a cover slip.

The mounted membrane filters are viewed under incident illumination with a Zeiss Axioskop microscope fitted with a 50-W mercury vapour lamp, a wide-band interference filter set for blue excitation, a 100 X (numerical aperture = 1.3) Plan Neofluar objective lens, and 10 X eyepieces. The volume of sample stained and filtered should be adjusted to optimize filter coverage by particles at around 50-70%. This adjustment is very important as too much sediment on the slide will obscure too many cells, as particles will overlap, and so underestimate numbers grossly, whilst too little sediment makes counting very tedious. Sediment particles will appear orange/red and prokaryotic cells will glow with a bright green/blue light. Sometimes prokaryotes that are not on particles will appear as orange/red prokaryote shaped cells, these should be counted.

Three replicate filters are prepared from each sample to minimize count variance (Kirchman et al., 1982). A minimum of 200 fields of view, or 200 bacterial cells are counted. The total number of bacteria and the numbers of dividing and divided cells are separately counted. The number of cells counted on opaque particles is doubled to account for cells hidden from view (Goulder, 1977). Blank membranes are regularly counted and bacterial population size is calculated after subtraction of the appropriate blank.

Total bacterial numbers are calculated from:

$$\left( \left( \left( \frac{2C_{ON} + C_{OFF} + C_{DG} + 2C_{DD} + \left( \frac{C_{ON}}{2C_{ON} + C_{OFF}} \times (C_{DG} + 2C_{DD}) \right)}{\text{VIEW}} \right) \times A \right) - B_T \right) \times \frac{1000}{V_{CT}}$$

D



- $C_{ON}$  and  $C_{OFF}$ : Number of cells counted ON and OFF particles. For the purposes of this calculation transparent particles e.g., diatom frustules, are not particles.
- $C_{DG}$  and  $C_{DD}$ : Numbers of cells observed DIVIDING (a cell with an invagination) and DIVIDED (two adjacent cells of identical morphology with a distinct space between them). Cells counted in these two categories are not also tallied under ON and OFF particle categories.
- VIEW: The total number of fields of view observed during a cell count on a filter.
- A: Filter area ratio. Total countable area of the filter divided by the area of filter observed for one field of view.
- $B_T$  and  $B_D$ : Blank correction terms for the total cell number ( $B_T$ ) and the dividing and divided cell numbers ( $B_D$ ). Calculated from counts of blank membranes and using the same equation with the omission of the correction term. In this instance  $V_{CT}$  will equal 10050 (10 mL of formaldehyde +50  $\mu$ L of acridine orange) and D will equal 1.
- VCT: Volume of formaldehyde-preserved sample that is stained ( $\mu$ L)
- D: Dilution factor of original sample expressed as a proportion, e.g., 1 cm<sup>3</sup> of sediment in 9 ml of formaldehyde will give  $D = 0.1$
- Numbers of dividing and divided cells are calculated from the same equation with the omission of the terms “ $2C_{ON} + C_{OFF}$ ” at the start of the equation, and the substitution of  $B_D$  for  $B_T$ .
  - The percentage of dividing and divided cells is calculated from the numbers of dividing and divided cells expressed as a percentage of the total bacterial numbers. Where total bacterial numbers approach the calculated detection limit, or numbers of cells counted approach the number of cells observed in the blanks, then this calculation becomes unreliable.

#### References:

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## 4.2. Fluorescent In Situ hybridization (FISH)

The aim of FISH is to stain prokaryotic cells with a fluorescently tagged molecular probe so that different groups of organisms can be counted directly with epifluorescence microscopy with suitable filter sets to visualise the bacteria that have hybridized with the probe. Multiple probes can sometimes be used with fluorescent tags that can be seen with different filter sets.

### Field:

- 15 ml vials are prepared with 3 ml formaldehyde (4% formaldehyde in 0.2 µm sterile filtered seawater)
- The sediment is sampled with a 5 ml capped syringe of which 1 ml is transferred to the formaldehyde and vortexed.
- Vials are left for 3-4 h for fixation at 4°C and mixed well before 2 ml of the suspension is transferred to a 2 ml Eppendorf vial with a cut-off pipette tip.
- The Eppendorf vials are centrifuged (max rpm for 2 min) and the supernatant is discharged
- The pellet is resuspended in 1.5 ml 1xPBS (10 mM sodium phosphate pH 7.2; 130 mM NaCl) and centrifuged again. This washing step is repeated and the pellet resuspended in 1.5 ml 1xPBS/EtOH (1:1) and stored at -20°C

### Laboratory:

Sonication (optional but highly recommended for marine sediments)

- Dilute sample 1:10 (75 µl fixed sample + 675 µl 1xPBS:EtOH (1:1) for sediments
- Apply sonication at a setting of 20 s, amplitude 42 µm, and <10 W while keeping the sample on ice.  
(MS73 probe, Sonopuls HD70, Bandelin, Berlin, Germany).

### Filtration and Hybridisation

- Mix 10-20 µl (sediments) of the sonicated sample with 5-10 ml 1xPBS.
- Put a cellulose nitrate filter (0,45 µm, Sartorius ) on a filter-tower and place a GTTP polycarbonate filter (0,2 µm, Millipore, Germany) on top of it, shining side up.
- Add sample and apply vacuum; let the filter dry on paper tissue.
- Cut the filter in quarters and label them with a pencil (do not use edding). For each probe use only one of the quarters.
- For each probe prepare 2 ml hybridisation buffer:
 

360 µl	NaCl (5 M)
40 µl	Tris-HCl (1 M) pH = 7,5
x µl	formamide
	(amount depends on the probe; the higher the stringency; optimum has to be tested in advance)
ad 2 ml	Milli-Q H <sub>2</sub> O
2 µl	SDS (10 %)
- Put filter on a glass slide
- Mix 13,5 µl of the hybridisation buffer + 1,5 µl probe (50 ng/µl) per quarter-filter and carefully pipette it on the filter. With the rest of the 2 ml hybridisation buffer moisten a



piece of paper tissue and put it into a 50 ml falcon tube. The filter tissue protects the filters from drying during hybridization (“humid chamber”). Place the glass slide on the filter tissue. Incubate for 2 h at 46 °C.

It is highly recommended to use separate slides for different probes to avoid mixing of the probes during hybridization!

- Prepare washing buffer in a 50 ml falcon tube and preheat in water bath to 48 °C.

50 ml washing buffer per sample contains:

x µl	NaCl (5 M) depending on amount of formamide used during hybridization (see table)
1 ml	Tris-HCl (1 M) pH = 7.5
500 µl	EDTA (0.5 M) pH = 8,0 (≥20% FA used)
add 50 ml	Milli-Q H <sub>2</sub> O
50 µl	SDS (10%)

Formamide concentrations in washing buffer:

Formamide [%] (used for hybridisation)	NaCl [µl] in 50 ml washing buffer	NaCl in mol
0	9000	0,900
5	6300	0,636
10	4500	0,450
15	3180	0,318
20	2150	0,225
25	1490	0,159
30	1020	0,112
35	700	0,080
40	460	0,056
45	300	0,040
50	180	0,028
55	100	0,020
60	40	0,014
65	---	0,010
70	--- + 350 µl EDTA only	0,007
75	--- + 250 µl EDTA only	0,005
80	--- + 175 µl EDTA only	0.0035

- After incubation transfer glass slide with the filters into the washing buffer and incubate for 15-20 min in the water bath at 48 °C. Try to keep samples warm, i.e. work quickly!
- Wash filters sections in 50 ml of deionized water, and then dehydrate with absolute ethanol. Let sections air dry.
- The filter sections can now be counterstained (e.g. with the DNA stain 4',6'-diamidino-2-phenylindol, DAPI): Prepare glass slide with 10 µl DAPI (1 µg/ml) per filter. Dip the filter into it upside down and incubate for 3-10 min. Shield the filters from light during and after dyeing with DAPI.
- Rinse filter first in H<sub>2</sub>O and afterwards in EtOH (80 %) and dry on paper tissue. Make sure that it is completely dry before proceeding.
- Microscopy is performed after embedding the filters in Citifluor. Sections could also be stored at -20°C until further processing. For sediments it is recommended to incubate the embedded filters at 4°C overnight before microscopy.

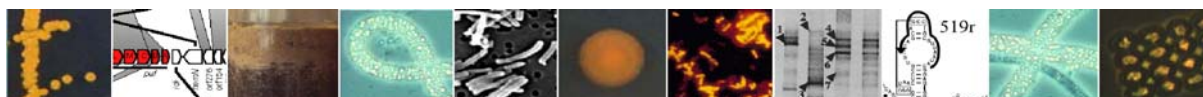


Reference:

Perntaler, J., Glöckner, F.-O., Schönhuber, W. & Amann, R. (2001). Fluorescence *in situ* hybridization (FISH) with rRNA-targeted oligonucleotide probes. *Methods in Microbiology* 30, 207-226.

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### 4.3. CARD-FISH

This protocol for catalyzed reporter deposition fluorescence in situ hybridization (CARD-FISH) is based on the method of Pernthaler et al. (2002) with slight modifications.

#### I) Preparation of filters

1. Boil 0.2% (w/v) low-gelling point agarose in a microwave oven.
2. Drop the agarose on a clean glass plate and let it cool down to 40 to 35°C.
3. Put the filters with both sides into one drop of agarose and put the filters face-up onto the glass plate (using a separate drop for each filter avoids cell loss)
4. Let the filters dry at 20 to 40°C for ca. 10 to 30 min.
5. To remove the filters from the glass plate, pipette ethanol (96% to 80% [v/v]) onto the filters and carefully peel them off.
6. Let the filters air dry on paper tissue.

#### II) Inactivation of endogenous peroxidases

1. Incubate filters in 0.01 M HCl at RT for 10 min.
2. Wash in MilliQ water.

**Very important:** Test, if endogenous peroxidases have been bleached completely (follow the protocol without using any probe). If peroxidase activity is still present, the bleaching protocol needs to be optimized.

#### III) Permeabilization

**Permeabilization procedure varies and needs to be optimized for each probe and sample!**

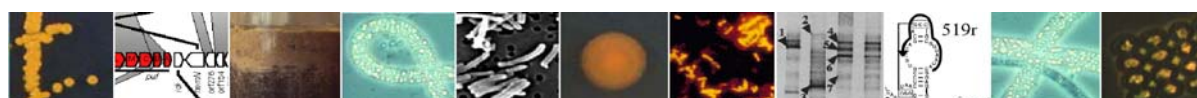
After inactivation of endogenous peroxidases, try the treatments described below to permeabilize cell walls. *Archaea* are more difficult to permeabilize than *Bacteria*. A proper permeabilization of target cells is crucial for bright hybridization signals. Optimizing this step is highly recommended, e.g. by comparing each single treatment, varying single treatments (incubation time, concentrations) or the combinations of different treatments.

Permeabilization of bacterial cell walls:

Incubate filters with lysozym (10mg/ml in 0.05 M EDTA, 0.1 M Tris/HCL pH-8,0) at 37°C for 60 min

Permeabilization of archaeal cell walls:

1. Incubate filter sections for 1 minute in 0.1 M HCl  
Rinse filters with excess water and dehydrate with Ethanol abs.  
Let filters air dry
2. Incubate filter sections for 10 minutes in 1xPBS containing 1% Triton X-100  
Rinse filters with excess water and dehydrate with Ethanol abs.  
Let filters air dry
3. Incubate filter sections for 10 minutes in 1xPBS containing 0.5% SDS  
Rinse filters with excess water and dehydrate with Ethanol abs.



- Let filters air dry
4. Incubate filter sections in TE buffer containing Proteinase K (~1 mg/ml – 1 µg/ml)  
Rinse filters with excess water and dehydrate with Ethanol abs.  
Let filters air dry  
Filter sections can be stored at -20°C until hybridization.

#### IV) Hybridization

Preparation of hybridization buffer: pipette in a 50 ml tube:

3.6 ml	5 M NaCl
0.4 ml	1 M Tris-HCl, pH 8.0
20 µl	20% SDS
x ml	MilliQ water (See Table 1)
x ml	formamide (See Table 1)
2.0 ml	10x Blocking Reagent

Add 2.0 g dextran sulfate. Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. Aliquots of the buffer can then be stored at -20°C for several months.

**Table 1.** Volumes of formamide and water for **20 ml of hybridization buffer**

% formamide in hybridization buffer	ml formamide	ml water
20	4	10
25	5	9
30	6	8
35	7	7
40	8	6
45	9	5
50	10	4
55	11	3
60	12	2
65	13	1
70	14	0

To obtain formamide concentrations of 75% reduce the amount of blocking reagent in the hybridization buffer to 1 ml.

Preparation of washing buffer: pipette in a 50 ml tube:

0.5 ml	0.5 M EDTA, pH 8.0
1.0 ml	1 M Tris-HCl
x ml	5 M NaCl (volume see Table 2)
add 50 ml	MilliQ
25 µl	20% SDS



1. Mix hybridization buffer and HRP-labelled probe (50 ng/ $\mu$ l) 300:1. Place filter sections in a reaction vial (0.5 ml to 2 ml, depending on the number of sections) and pipette the hybridization mix onto the sections. At least  $2/3^{\text{rd}}$  the total volume of the reaction vial should be filled with buffer. Hybridize on a rotation shaker (app. 10 rpm) for 4 hours at 35°C (over night hybridizations result in brighter signals!).
2. For stringent washing, prepare washing buffer and preheat at 37°C. Wash sections after hybridization for 5 minutes (when hybridized over night extend washing step) in 50 ml of washing buffer.  
Do not let the filter sections run dry after washing; this will reduce the activity of the HRP.

## V) CARD

For the preparation of amplification buffer pipette into a 50 ml tube:

4 ml	10× PBS
0.4 ml	10x Blocking Reagent
16 ml	5 M NaCl
ad 40 ml	sterile MilliQ water

Add 4 g dextran sulfate. Heat (40 to 60°C) and shake until the dextran sulfate has dissolved completely. The amplification buffer can be stored at 4°C for several weeks.

1. To equilibrate the probe delivered HRP, remove the sections from the washing buffer and incubate them in 50 ml 1x PBS for 15 minutes at room temperature.
2. Prepare fresh 100 x H<sub>2</sub>O<sub>2</sub> stock solution by mixing 1000  $\mu$ l of 1 x PBS with 5  $\mu$ l of 30% H<sub>2</sub>O<sub>2</sub>.
3. Mix 1000  $\mu$ l of amplification buffer with 10  $\mu$ l of the 100 x H<sub>2</sub>O<sub>2</sub> stock (30% H<sub>2</sub>O<sub>2</sub> 1:200 diluted) and 1 to 2  $\mu$ l of fluorescently labelled tyramide.
4. Put filter section in a reaction vial and pipette the amplification buffer with the tyramide onto the sections. Incubate at 37°C for 10 to 15 minutes in the dark.
5. Remove excess liquid by dabbing filters onto blotting paper. Wash sections in 50 ml of 1x PBS for 15 minutes at room temperature in the dark.
6. Wash sections in 50 ml of deionized water, and then dehydrate with absolute ethanol. Let sections air dry. The filter section can now be counterstained (e.g. with the DNA stain 4',6'-diamidino-2-phenylindol, DAPI). Microscopy is performed after embedding the filters in Citifluor. Sections could also be stored at -20°C until further processing.

It might be necessary to optimize probe concentrations, tyramide concentration, incubation temperature and time for each sample and probe to reduce background autofluorescence and/or increase probe signal intensity!

## VI) Multicolor CARD-FISH

1. Inactivate the probe delivered peroxidase from the first hybridization by incubating the filter sections in 0.01 M HCl for 10 min at room temperature.
2. Wash sections twice with 50 ml of MilliQ water.





3. A second hybridization followed by a second CARD using another fluorescently labelled tyramide can now be performed as described above.

Reference:

Pernthaler, A., J. Pernthaler, and R. Amann (2002). Fluorescence in situ hybridization and catalyzed reporter deposition for the identification of marine bacteria. *Appl. Environ Microbiol.* 68, 3094-3101.

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## 5. Biomass and Biomarker Methods

### 5.1. Phospholipids for biomass estimation

Phospholipids are a major component of all prokaryotic cells and so can be used to estimate prokaryotic biomass in sediments. The method described here is based on that of Findlay *et al.* (1989).

#### Chemicals:

chloroform (pure)

methanol (pure)

phosphate buffer:

8.7 g  $K_2HPO_4$  filled up with distilled water to a final volume of 1 liter; pH 7.4

potassium-peroxodisulfate-solution:

add 5 g  $K_2S_2O_8$  to 100 ml 0.36N  $H_2SO_4$

ammonium-molybdate-solution:

add 2.5 g  $(NH_4)_6 Mo_7 O_{24} \cdot 4 H_2O$  to 97.5 ml 5.97N  $H_2SO_4$

malachite-green-solution:

add 0.113 g Polyvinylalcohol (98%) to 100 ml of warm (80°C) distilled water;

let it cool down and add 0.011 g malachite-green

#### Accessories:

50 ml Oak Ridge Teflon centrifugation vials (solvent resistant) with screw-caps;

centrifuge;

multi-varipettes (50, 12.5, 2.5 ml);

Eppendorf-varipettes (10-100  $\mu$ l, 100-1000  $\mu$ l);

vacuum pump;

glass funnels and Whatman-filters (2 V, 12.5 cm);

Wheaton vials (2 ml);

welding torch for glass vials;

nitrogen gas cylinder.

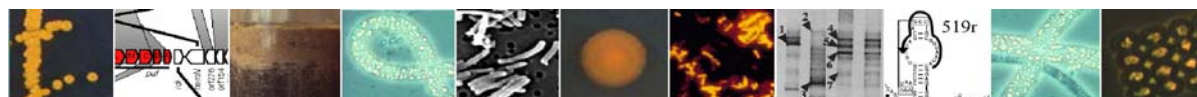
#### Method:

##### *First day:*

- 1.) add 2 ml of wet sediments to the centrifuge test tubes
- 2.) add 1 ml phosphate buffer, 4 ml chloroform and 8 ml methanol
- 3.) block up the tubes and give it a good shake (homogenized mixture)
- 4.) leave it for the next day

##### *Second day:*

- 1.) add 4 ml distilled water and 4 ml chloroform (separation into two phases)



- 2.) block up the tubes and give it a good shake (homogenized mixture)
- 3.) leave it for the next day

*Third day:*

- 1.) centrifugation: 10 min. at 6000 rpm, 400 x g
- 2.) suck of the water phase quantitatively
- 3.) filter the chlorophyll phase into glass test tubes
- 4.) pipette 2 ml of the chloroform extract into a glass vial
- 5.) evaporate the chloroform with nitrogen in a water bath at 40°C
- 6.) when vials are totally dry inside add 0.5 ml potassium-peroxodisulfat-solution
- 7.) heat seal the glass vials
- 8.) incubate vials at 95°C till next day

*Fourth day:*

- 1.) open vials and
- 2.) add 0.1 ml ammonium-molybdate-solution and leave it for 10 min.
- 3.) add 0.5 ml malachite-green-solution and leave it for 30 min.
- 4.) measurement of the final solution at 610 nm with a photometer

Standards:

0.6805 g  $K_2HPO_4$  filled up with distilled water to a final volume of 100 ml;  
 1 ml of this solution filled up with distilled water to a final volume of 100 ml  
 = standard solution of 0.5  $\mu\text{mol/ml}$ .

ml distilled water.	ml standard solution	nmol/ml end concentration
1.00	0.00	0.0
0.95	0.05	0.5
0.90	0.10	1.0
0.80	0.20	2.0
0.60	0.40	4.0
0.40	0.60	6.0
0.00	1.00	10.0

Add 20  $\mu\text{l}$  of each end conc. solution to 0.5 ml potassium-peroxodisulphate for measurements.

Reference:

Findlay, R.H., G.M. King & L. Watling (1989). Efficiency of phospholipid analysis in determining microbial biomass in sediments. - *Appl. Environ. Microbiol.*, **55**: 2888-2893.

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## 5.2. Protein for biomass estimation

Proteins are a major component of all prokaryotic cells, are quickly degraded out side of cells by exoenzymes and so can be used to estimate prokaryotic biomass in sediments.

### Principle:

The proteins are extracted from the cells and divided by NaOH and temperature into reactive fragments. The temperature may not be higher than 60°C, since otherwise the fragments become too small and are not detectable any longer. Measurements are done photometrically, using a colour reaction of the protein fragments with Coomassie blue.

### Accessories:

Photometer  
Plastic cuvettes (half micro)

### Execution:

Immediately after sampling the sediment (5 ml syringe) is shock-frozen with -80°C, then with stored at -18°C. For the first hydrolysis the sediment is cut in centimetre horizons and put in numbered test tubes.

Now the sediment is dried with 60°C at least for 48 h (it must be completely dry).

Then 3 ml 0.5N NaOH is added, the tubes are vortex mixed and finally hydrolyzed for 2 hours in the water bath with 60°C.

Afterwards, once again vortex mixed and centrifuged at 3500 U/min for 10 minutes. The supernatant (sample) is decanted and the sediment is washed with 2ml 0.5N NaOH (vortex mixed and centrifuged for 10 minutes at 3500 U/min).

Both extracts are combined and measured (1st hydrolysis).

If further extractions should be desired, the sediment is dried again etc..

The individual hydrolyses are carried out as described above. After the 6th hydrolysis all extracts are combined, the volume is again determined and the protein content is measured.

### Measurement:

#### Preparation:

Test tubes (some more than necessary for the measurements) are filled with 2 ml Coomassie blue (keep cool as for a long time as possible to); prepare plastic cuvettes (half micro), stop clock and Whirlmix. The thawed out hydrolysate should not stand on the bench for a long time, since concentration-reduction will take place. One should try to thaw only as much as as can be measured in the next few hours. Controls should be carried out to check the standing time that is safe to avoid degradation.



### Measuring procedure:

One measures with a wavelength of 595 nm and with a gap width of 2.

At first the blank values of the cuvettes are determined using 0.5N NaOH (subtract afterwards from the sample values or use auto-zero).

Pipette 0.35 ml hydrolysate into a test tube already filled up with coomassie blue, whirlmix for 10 seconds. The mixture is poured into the appropriate cuvette and measured after 2 minutes. During this response time further mixtures can be prepared. Two mixtures per sample should always be prepared and measured! If the deviation is larger than 20 fluorescence units, further parallel analyses must be made. The response time must be kept absolutely exactly constant (use a stop clock).

After the measurement the cuvettes are soaked immediately in soapy water, so that the dyestuff can be completely removed (then rinse the cuvettes carefully, otherwise a blue film remains, and the blank value is much too high and can't be adjusted any longer). The cuvettes can be used if rinsed for up to five times.

Since the dyestuff is unstable, one calibration must be measured per measuring day. The calibration solutions can be kept frozen, but repeated thawing out and freezing harms them! Therefore divide the 100 ml master solution in 10 ml portions and freeze only in such a way. The calibration curve is linear between 0 and 150  $\mu\text{g}$  Globulin/ml. If the protein concentration of the samples exceeds this range, the mixture must be diluted.

### Computation:

$\mu\text{g -Gl.}\ddot{\text{A}}\text{qu.}/5 \text{ cm}^3 \text{ Sed. after 6 hydrolyses} = 100\%$

$\mu\text{g -Gl.}\ddot{\text{A}}\text{qu.}/5 \text{ cm}^3 \text{ Sed. after 1 hydrolysis} = x\%$

$x\% =$  relative protein concentration of the first hydrolysis

- multiply by the dilution, with the first hydrolysis  $\times 6.13$ , with six hydrolyses  $\times 6 \times 6.13$
- divide by the sediment volume :  $1.13 \text{ cm}^3$
- convert with water content, because 1 ml is not equal with  $1 \text{ cm}^3$

### Reagents:

0.5N NaOH:

20 g NaOH is solved in a litre of distilled water.

Coomassie blue:

40 mg Serva blue are solved in approx. a half litre of distilled water. In addition comes 100ml phosphoric acid and 50 ml ethanol. Fill up with distilled water to 1 litre. The final solution must be mixed before use for at least 24 h.



Calibration master solution:

10 mg  $\gamma$ -Globulin is dissolved in 50 ml 0.5N NaOH.

$\gamma$ -Globulin as powder has to be stored with +4°C, and as master solution at -18°C.

Standards:

Protein conc. [ $\mu$ g $\gamma$ -Globulin/ml]	Master solution [ $\mu$ l]	0.5N NaOH [ $\mu$ l]
0	0	3000
4	50	2950
10	150	2850
20	300	2700
30	450	2550
40	600	2400
50	750	2250
70	1050	1950
100	1500	1500

Chemical list:

NaOH (Merck No. 6498)

Serva blue (Serva No. 35050)

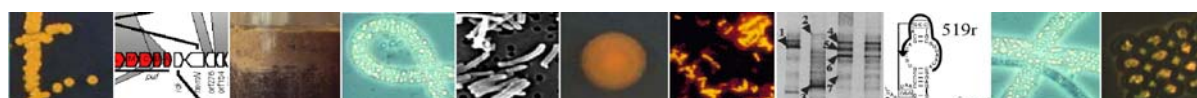
$\gamma$ -Globulin (Serva No. 22550)

85% ortho phosphoric acid (Merck No. 573)

Ethanol, p.A. (Merck No. 983)

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### 5.3. Phospholipid/biomarker analysis

The aims of the biomarker analyses are to determine the abundance and, where possible, carbon isotopic compositions of compounds derived from *Archaea*, sulfate-reducing bacteria and other *Bacteria*. These will serve alongside other microbiological analyses as tracers for microbial biomass and species composition. By linking such analyses to geochemical data via their carbon isotopic compositions, we will be able to link taxonomic groups to specific biogeochemical processes.

#### Sample Collection:

At least 50 g (dry weight) of sediment – preferably 100 g – will be collected from cores in a manner consistent with shipboard decisions regarding intervals of interest. Of critical importance is that all samples be collected in conjunction with samples collected for geochemical and microbiological analysis, insuring comparability among data sets. Samples, must be excised from the core in a manner to minimize cross-contamination and contamination from handling; specific sources of contamination include plastics (phthalates), improper handling with hands (cholesterol), and introduction of any petroleum-type products. To address both external and cross-contamination, samples will be removed with a metal spatula and/or knife that will be washed thoroughly with hot water and then rinsed with methanol between samples. After collection, samples will be wrapped in ashed aluminium foil and then placed in a sealed bag. Samples will be stored at  $-20^{\circ}\text{C}$  as soon as possible and maintained at that temperature until analysis.

#### Analytical Scheme:

In general, sediments will be freeze-dried (in the case of carbonate crusts, dried and then ground) and then extracted. The extracts will then be separated into neutral, acid and phospholipid fractions which can then be analysed by either GC or LC-based techniques. In *some* cases, samples will be further degraded into more GC-amenable components (i.e. saponification of phospholipid fraction) or further fractionated (i.e. neutral fraction into apolar and polar sub-fractions) to facilitate further analyses.

#### Lipid extraction:

Two extraction techniques will be used, depending on the type and quantity of sample; a bligh dyer extraction will be used when attempting to characterise intact phospholipids, and a soxhlet extraction will be used for general screening for bacterial and archaeal biomarkers. Note: in all cases, at least 25 g dried sediment will be archived.

*Soxhlet extraction:* Sediment and carbonate powder is weighed (40 to 80 g) into pre-extracted cellulose thimbles. The samples are then extracted using the soxhlet apparatus using 2:1 DCM/MeOH as the carrier solvent for 24 hours. After extraction, solvents are evaporated using a rotary evaporator and the total lipid extracts are transferred to a vial and stored.

*Bligh dyer extraction:* The bligh-dyer extraction mixture (4 parts by volume of acidified/ buffered water: 3 parts chloroform:10 parts methanol) is added to the sample and placed in an sonication bath for 13 minutes. After sonication, the mixture is centrifuged and the supernatant decanted into a separating funnel. This process is repeated 3 times. To the total supernatant extraction, 2ml of buffered water and 2ml of chloroform is added. The organic



layer is removed and the aqueous layer was extracted with 3×2 ml chloroform. The aqueous extracts are combined in a round-bottom flask, rotary evaporated to 1ml, transferred to a vial and finally evaporated under N<sub>2</sub>.

#### Standard and archives:

The quantity of total lipid extracts of all samples is determined by weighing dried samples in pre-weighted vials; subsequently, a standard consisting of, for example,

- Androstane-1840 µg
- 2-hexadecanol-2020 µg
- Hexadecyl-octadecanoate-7390 µg

is added. The extracts will be split into two 50:50 fractions (half archived).

#### Bond-Elut and Flash-Column Chromatography:

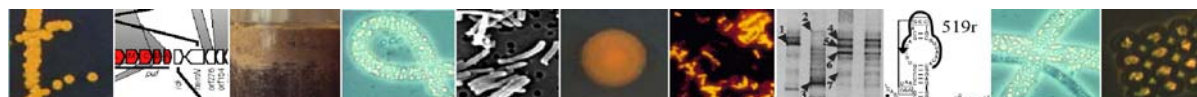
The total lipid extracts (2/3 – 1/3 to be archived) are loaded onto an NH<sub>2</sub> column (an amino bond-elut column) that has been pre-washed with methanol and 2:1 DCM:isopropanol. Using 12ml DCM, 12 ml 2% acetic acid ethyl acetate and 12 ml methanol the total lipid extract is fractionated into neutral lipid, acid and phospholipid fractions, respectively, and eluted into 100ml round bottom flasks. The neutral lipids are then loaded onto a column consisting of extracted and deactivated (by storing at 60°C) alumina and are further split into apolar and polar neutral fractions by eluting with 9:1 hexane:DCM and 1:2 DCM:methanol, respectively.

#### Saponification and Methylation of Glycolipids and Phospholipids:

One-half of the phospholipid and glycolipid (the latter eluting in the acid fraction) fractions are heated with 1 ml 0.5M 95% methanolic NaOH at 70 °C for 1 hour in a boiling tube placed in a heating block, subsequently acidified to pH 1-2 with 1M HCL and extracted with 3×2 ml hexane. The combined extracts are evaporated under N<sub>2</sub> in a 12 ml pyrex culture tube, after which 100µl BF<sub>3</sub>/methanol is added and the fractions heated again at 70 °C for a further hour. After cooling, 1 ml of double-distilled water is added and the methyl esters are extracted by 3×2ml of DCM; extracts are combined into a 100 ml round-bottom flask and rotary evaporated to ~1 ml. To remove any residual water, the fatty acid methyl extracts are eluted through a pre-cleaned (3 ml DCM) anhydrous sodium sulphate column into a 3.5 ml sample vial. The dry FAMES are then evaporated under N<sub>2</sub>, and ca. 200 µg of a *n*-C<sub>19</sub> standard is added to both fractions.

#### Preparation of Tetraethers by Cleavage of Ether bonds by HI/LiAlH<sub>4</sub> reduction:

For particularly active samples of interest, the remaining half aliquots of the phospholipid fraction and glycolipid fraction are subjected to chemical cleavage with hydriodic acid (HI) to release archaeal biphytanes from tetraether lipids and to release phytanes from diether lipids. The polar/phospholipid and glycolipid fractions are dissolved in 2:1 DCM:isopropanol and then transferred into a 12ml Quickfit tube. The solvent is then evaporated under a stream of N<sub>2</sub>; a stir bar and 2ml of HI is added to the sample and then refluxed at 120°C for 4 hrs.



Following reflux, *n*-hexane (3-4 ml) is washed through the HI three times to extract the alkyl iodides. In addition sodium thiosulphate (5 % Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> in H<sub>2</sub>O) is used to remove any HI from the sample. 3ml of Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> is subsequently added to the sample and washed through until the pink colour (caused by excess free iodine ions) disappears completely. The *n*-hexane (upper) layer is then transferred to a fresh tube and remaining Na<sub>2</sub>S<sub>2</sub>O<sub>3</sub> is washed through (x2) with 3ml *n*-hexane to ensure recovery of sample. The *n*-hexane is evaporated under a stream of N<sub>2</sub>, until ~1ml remains. The sample, in 1 ml *n*-hexane, was eluted through a magnesium sulphate (MgSO<sub>4</sub>) column with 3 ml *n*-hexane, to remove any residual water from the sample. The alkyl iodides are isolated by column chromatography using an Al<sub>2</sub>O<sub>3</sub> stationary phase and hexane/DCM (9:1, v/v) as the eluent.

The alkyl iodides are then reduced to hydrocarbons using lithium aluminium hydride (LiAlH<sub>4</sub>). The alkyl iodides fraction is transferred to a reflux tube and evaporated to dryness under a stream of N<sub>2</sub>. A stir bar and 3 ml 1,4-dioxane were added followed by 2 scoops of LiAlH<sub>4</sub>. The mixture is then refluxed at 120 °C for 1 hour. Following reflux, the LiAlH<sub>4</sub> is quenched with 2ml of ethyl acetate and 5ml of double-distilled water. The mixture is then centrifuged for 2min at 3000rpm and the supernatant is decanted to a fresh tube. 2ml DCM is then added and the supernatant decanted after centrifugation, and repeated, and all solvent extracts are combined. In order to facilitate separation of solvent and aqueous phases, 250µl 2M HCl is added to acidify the water. The organic (lower) phase is removed to a round-bottomed flask, and the water phase extracted twice more with 2 ml DCM. The solvent is then evaporated with a rotary evaporator. Any water remaining in the sample was removed by elution of the sample through an MgSO<sub>4</sub> column.

If sodium methane thiol (NaSCH<sub>3</sub>) is used in place of LiAlH<sub>4</sub>, methylthioethers are formed. These can be run on a GC-MS to confirm the position of the original ether bonds in the lipid (Schouten *et al.*, 1998).

#### Gas chromatography – Mass spectrometry:

##### Derivatisation of alcohols:

Polar fractions need to be derivatised prior to GC and GC-MS analyses as alcohols are not well resolved on most capillary columns. Similarly, the FAME fractions generated by saponification and methylation of the acid and phospholipid fractions could contain acidic compounds that also bear hydroxyl groups (e.g. hydroxy fatty acids), also requiring derivatisation. Thus, prior to GC or GC-MS analyses, the above fractions are reacted with BSTFA to form trimethylsilyl ethers from the alcohols. To do this, 30 µl of pyridine and 30 µl of BSTFA (N,O-bis(trimethylsilyl)trifluoroacetamide) is added to the sample vials, which are then heated at 70°C for about 35 minutes. After heating, samples are brought up to appropriate volumes for GC and GC-MS analyses with ethyl acetate as the solvent.

Gas Chromatography and Gas chromatography- Mass spectrometry (GCMS) will be performed on all generated apolar lipid, neutral polar lipid and saponified/methylated acid and phospholipid fractions. GC analyses are performed on a Carlo Erba GC equipped with a Flame Ionisation Detector. Samples are injected (in either hexane or ethyl acetate as described above) at 40°C using an on-column injector, and the oven is initially heated to 130 °C at 20 °C per minute after which the heating rate is 4 °C per minute to 300°C and held at this temperature for 20 minutes. The GC and GC-MS is equipped with a Chrompack fused



silica capillary column (50m length, 0.32mm internal diameter) with a CP-Sil 5 CB stationary phase (dimethylpolysiloxane equivalent, film thickness 0.12 $\mu$ m). GC-MS will be performed using a Thermoquest Finnigan Trace GC interfaced to a Thermoquest Finnigan Trace MS operating with electron ionisation at 70eV and scanning an  $m/z$  range of 50 to 850. GC conditions for GC-MS analyses are the same as for GC analyses as described above.

#### High Temperature Gas Chromatography:

High temperature gas chromatography (HTGC) will be performed on the tetraether lipids from the polar neutral fractions and generated from the HI cleavage and LiAlH<sub>4</sub> reduction of the phospholipid fraction and glycolipid fraction on the same model GC as described above. Prior to analysis the samples are derivitised with BSTFA and pyridine as above. The samples are injected in ethyl acetate at 40°C using an on column injector, with the oven initially heated up to 140°C at a rate of 20°C per minute, after which the temperature is increased at a rate of 6°C per minute up to 400°C and held at this temperature for 10 minutes. The HTGC is equipped with a SGE bonded phase aluminium clad column (6m length, 0.53mm internal diameter) with a HT5 non-polar stationary phase (5% phenyl equivalent polycarbane siloxane, film thickness 0.1 $\mu$ m).

#### High Pressure-Liquid Chromatography-Atmospheric Pressure Ionisation-Mass Spectrometry:

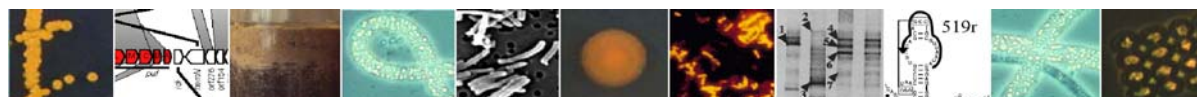
Polar head groups are removed from the tetraether lipids before analysis by LC-MS. This is achieved by a two-step process. Firstly by acetolysis: 100  $\mu$ l 9:1 acetic anhydride:pyridine is added to the dry sample and heated for 1 hr at 60°C and dried under a stream of N<sub>2</sub>. Secondly by acid methanolysis: 100  $\mu$ l HCl-methanol is added to the dry sample and heated for 1 hour at 60°C, then dried under a stream of N<sub>2</sub>. The sample is then ready to be run through the LC-MS in *n*-hexane or DCM. LC-MS will be performed using a Walters 600MS LC instrument coupled to a Finnigan MAT TSQ 700 triple quadrupole mass spectrometer. Separation of tetraether lipids is achieved on a Spherisorb NH<sub>2</sub> column (4.6m x 250 mm, 5  $\mu$ m: Phenomenex) maintained at 30 °C. 10 $\mu$ l is injected each time, into a 20  $\mu$ l loop. Tetraethers were eluted with 99% *n*-hexane and 1% iso-propanol, for 30 minutes with a flow rate of 1 ml/min<sup>-1</sup>. After each analysis 95:5 v/v *n*-hexane:iso-propanol is used to backflush the column, at 1 ml min<sup>-1</sup> for 5 minutes. Detection is achieved using atmospheric pressure chemical ionisation mass spectrometry (APCI-MS), under the following conditions: nebulizer pressure 60 psi, vaporizer temperature 400 °C, capillary voltage -3 kV, capillary temperature 200 °C corona 7  $\mu$ A (4.5 kV), drying gas (N<sub>2</sub>) flow 6 litres min<sup>-1</sup> at 250 °C. Positive ion spectra are generated by scanning the range  $m/z$  1000 to 1400 in 1 s. Data will be analysed with the ICIS II data system

#### Reference:

Schouten S., Hoefs M. J. L., Koopmans M. P., Bosch H. -J., & Sinninghe Damsté J. S. (1998). Structural characterization, occurrence, and fate of archaeal ether-bound acyclic and cyclic biphytanes and corresponding diols in sediments. *Org. Geochem.* 29, 1305-1319

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## 6. Molecular Methods for Investigating Diversity: Clone Libraries

### 6.1. DNA extraction from sediments

#### 6.1.1. Modified Zhou et al (1996) method

For the extraction of genomic DNA from environmental samples, we use a protocol modified after Zhou et al. (1996).

- Be careful **not to shear DNA** at any step!
- **Never vortex DNA solutions**, mix gently by inversion
- Use **pipette tips with a wide opening!!!** (“cell saver” tips)

All equipment and solutions have to be autoclaved or if this is not possible “sterilised” with Ethanol to get rid of any DNases!

#### Solutions:

- Extraction buffer
 

TrisHCl (pH 8,0)	100 mM
EDTA (pH 8,0)	100 mM
NaPhosphate (pH 8,0)	100 mM (see below)
NaCl	1.5 M
CTAB	1%

 (CTAB = Hexadecyltrimethylammoniumbromide,  
→ removal of polysaccharides)
- SDS (20%)
- Proteinase K (10 mg/ml) dissolved in autoclaved Milli-Q-water and 0.2 µm sterile filtered  
Do not autoclave Proteinase K! Prepare aliquots, storage at -20°C
- Chloroform/Isoamylalkohol 24:1 (v/v)
- Isopropanol, storage at room temperature!
- Ethanol 80%, dilute Ethanol (96%) in autoclaved Milli-Q water, storage at -20°C!
- Milli-Q water, autoclaved

How to prepare 1 M NaPhosphate-buffer:

1. Prepare 1 M Na<sub>2</sub>HPO<sub>4</sub> stock solution
2. Prepare 1 M NaH<sub>2</sub>PO<sub>4</sub> stock solution
3. Mix stock solutions in an appropriate ratio (see Sambrook et al.: Molecular cloning: a laboratory manual)) and adjust the pH with Na<sub>2</sub>HPO<sub>4</sub> and NaH<sub>2</sub>PO<sub>4</sub>, respectively.

#### Extraction:

- To 5 g frozen sediment add 13.5 ml extraction buffer (thaw sediment in extraction buffer) use 15 ml or 50 ml polypropylene tubes
- Freeze in liquid N<sub>2</sub> and thaw at 65°C three times, let it cool down to 37°C
- Add 100 µl Proteinase K, shake horizontally at 37°C for 30 min
- Add 1.5 ml 20% SDS, incubate at 65°C for 2 h with gentle end over end inversion every 25 min





- Centrifuge at ca. 4000 x g for 10 min  
collect supernatant and extract pellet again with 4.5 ml extraction buffer and 0.5 ml SDS.  
Try to resuspend the sediment without vortexing, if not possible: vortex 10 sec.  
Incubate at 65°C for 10 min and centrifuge again at 4000 x g for 10 min.  
**Store supernatant on ice!!!**  
(if possible: centrifuge at 6000 x g!!!)
- Combine supernatants and add an equal volume of chloroform/isoamylalcohol- fumehood!  
Mix thoroughly but carefully and centrifuge at 4000 x g for 10 min  
(if possible: centrifuge at 6000 x g!!!)
- Collect aqueous phase and if necessary extract aqueous phase again.
- Precipitate DNA by addition of 0.6 vol of isopropanol by incubating for 1 h at room temperature. Do not use cold isopropanol because it would result in enhanced simultaneous precipitation of salt
- Incubate at room temperature for 1 h
- Centrifuge at >14000 x g for 25 min at RT, use max. speed (if possible 20000 x g)
- Decant supernatant and wash pellet by addition of 10-20 ml cold 80% (v/v) ethanol
- Centrifuge at >14000 x g for 10 min at 4°C
- Decant supernatant, dry pellet
- Resuspend in 100-400 µl Milli-Q water  
(depending on pellet size; in general: ca. 400 µl/5 g sediment)

#### Controls for quality and quantity of extracted DNA:

The quality and quantity of the extracted genomic DNA can be controlled on an agarose gel (~1%). For length determination of the DNA we use the  $\lambda$ -Hind III standard marker (Invitrogen, Karlsruhe, Germany); for quantification we use the “Low Mass DNA Ladder” (Gibco, Eggenstein, Germany).

#### DNA purification:

Co-extracted substances, such as e.g. humic acids, can inhibit PCR reactions and must be removed. This can be done with commercially available DNA purification kits, such as the “Wizard Clean-up System” (Promega, Madison, USA) (for specific procedures see the protocol of the manufacturer).

Alternatively, the DNA can be purified via dialysis (a desalting method based on osmosis: due to osmotic pressure, low-molecular substances diffuse through a semi-permeable membrane into a salt-free dialysis reservoir, while high-molecular substances (DNA) are retained by the membrane).

Fill an 8-well plate with autoclaved Milli-Q water and cover it with a 0.025 µm nitrocellulose-membrane (type VSWP, Ø 13 mm, Millipore, Bedford, USA). Pipette an aliquot of the genomic DNA raw extract onto the membrane. Incubate at room temperature until the brownish staining of the DNA solution has vanished (~3 h). Transfer the purified





DNA-solution to a clean reaction tube. Quality and quantity controls of the purified DNA as described above. Store at  $-20^{\circ}\text{C}$ .

### Agarose gel electrophoresis:

Reagents and solutions:

- 50 x TAE buffer
  - 2 M Tris-HCl
  - 0.95 M Acetic acid
  - 50 mM EDTA (pH 8.0)
- Loading buffer
  - 0.5 % (w/v) SDS
  - 1-2% (w/v) Bromophenol Blue
  - 40% (w/v) Saccharose
  - 0.1 M EDTA (pH 8.0)
- DNA sizers
  - $\lambda$ -DNA Hind III (Invitrogen, Karlsruhe)
  - “1 kb Ladder” (Gibco, Eggenstein)
  - “Low Mass DNA Ladder” (Gibco, Egenstein)
- Ethidium bromide (EtBr) staining solution
  - 0.4  $\mu\text{g/ml}$  EtBr in Milli-Q water

Mix 4  $\mu\text{l}$  of DNA solution with  $>0.2$  vol. loading buffer. Load the mixture on an agarose gel (0.8% w/v in 1 x TAE) submersed in 1x TAE buffer in an electrophoresis chamber. Run electrophoresis according to size of the gel for 20-40 min at  $\sim 100$  W. Stain gel in EtBr solution for 15-60 min (gentle shaking). Detect DNA with UV trans-illumination and document the stained agarose gel with a photo.

### Reference:

Zhou J., Bruns M. A., and Tiedje J. M. (1996). DNA Recovery from soils of diverse composition. *Appl. Environ. Microbiol.* 62, 316-322.

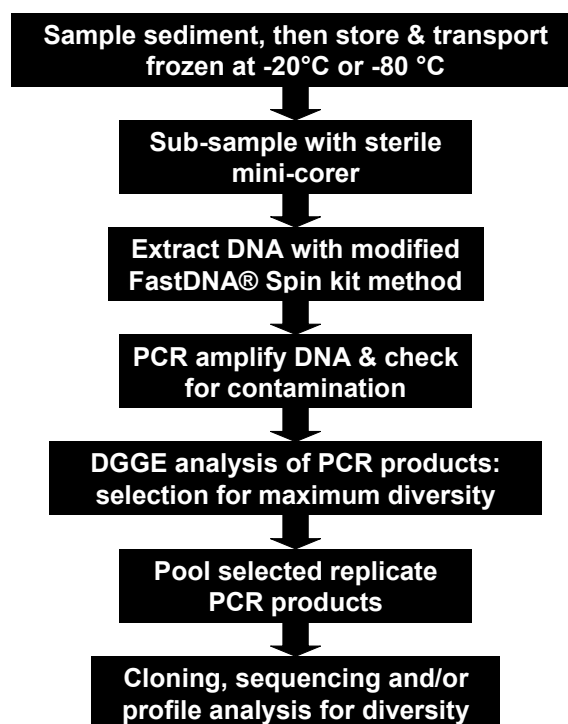
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### 6.1.2. Modified FastDNA<sup>®</sup> Spin kit (Q-BIO gene) DNA extraction method and tips for subsequent molecular diversity studies in low DNA sediments

In many subsurface sediments it can be very hard to extract DNA that is visible on electrophoresis gels, especially from sediments that are very deep or from low productivity areas. The method of DNA extraction described here works especially well for these types of sediment. This method, its development, comparisons with other methods and the history of the problems with DNA extraction from similar sediments are described in Webster *et al.* (2003). If the DNA extracted with this method is not clearly visible on agarose gels it is also prudent to check for contamination and that preferential amplification of just a few types of prokaryotes is not occurring in subsequent PCR reactions used to study diversity further by cloning or profiling methods. Hence, Webster *et al.* (2003) also recommends screening the extracted DNA with replicate PCR reactions followed by denaturing gradient electrophoresis (DGGE). Then only PCR reactions producing maximum band diversity on the DGGE gels should be used. An experimental protocol similar to that recommended by Webster *et al.* (2003) is shown in the accompanying flow chart. This approach has worked well in recent prokaryotic diversity studies on marine sediments (Newberry *et al.*, 2004; Parkes *et al.*, 2005).



#### Sample handling:

Sediment samples, preferably as whole round cores (WRC), for use in diversity studies should be frozen as soon as possible after sampling at -20°C or -80°C and transported frozen back to the laboratory. Sub-sampling in the laboratory should then be carried out in a laminar flow cabinet with a sterile 2-cm diameter stainless steel corer. WRC can be softened by partial thawing if required and the corer can be hammered into the sediment if necessary. Only sediment from the centre of the cores should be used, as studies have shown that the outer layers are more likely to be contaminated than the central section (House *et al.*, 2003). Sub-samples should then be stored frozen before DNA extraction.

#### Method:

Use the FastDNA<sup>®</sup> Spin kit for soil (Q-BIO gene). **Note it is important to use the kit for soil as there are other FastDNA kits.**

1. Add 6 × 0.8 g of sediment to Lysing Matrix E Tube.
2. Add 122 μl MT Buffer, 20 μl Polyadenylic acid (10 mg ml<sup>-1</sup>) and 780 μl Sodium Phosphate Buffer.



3. Secure tubes in FastPrep<sup>®</sup> Instrument and process for 30 sec at speed 5.5.
4. Centrifuge Lysing Matrix E Tubes at  $13,200 \times g$  for 8 min.
5. Transfer supernatant to a clean tube (High-yield Nucleic Acid Recovery Tube). Add 250  $\mu$ l PPS reagent and mix by carefully inverting the tube by hand 10 times.
6. Centrifuge at  $13,200 \times g$  for 5 min to pellet precipitate. Transfer all supernatant to a sterile universal tube. Add 1ml Binding Matrix Suspension (resuspend before use) to the supernatant.
7. Invert by hand for 2 min to allow binding of DNA to matrix and place tube in a rack for 30 min to allow continued binding and settling of silica matrix.
8. Remove 500  $\mu$ l of supernatant being careful to avoid settled Binding Matrix and discard. Resuspend Binding Matrix in the remaining amount of supernatant. Transfer 750  $\mu$ l of the mixture to a SPIN<sup>™</sup> Filter and centrifuge at  $14,000 \times g$  for 1 min. Empty the catch tube and add the remaining supernatant to SPIN<sup>™</sup> Filter and spin again.
9. Add 500  $\mu$ l SEWS-M to the SPIN<sup>™</sup> Filter and centrifuge at  $14,000 \times g$  for 1 min. Decant flow-through and replace SPIN<sup>™</sup> Filter in Catch tube. Centrifuge at  $14,000 \times g$  for 2 min to dry the matrix of residual SEWS-M wash solution.
10. Remove SPIN<sup>™</sup> Filter and place in fresh Catch Tube. Air dry the SPIN<sup>™</sup> Filter for 5 min at room temperature.
11. Add 100  $\mu$ l DNase/RNase free water and gently flick matrix on filter membrane to resuspend the silica for efficient elution of the DNA. Place in a rack for 20-30 min to allow efficient DNA elution. Centrifuge at  $14,000 \times g$  for 2 min to transfer eluted DNA to catch tube.
12. Transfer  $6 \times 100 \mu$ l eluted DNA into one Microcon filter (YM-100; Millipore) and centrifuge at  $7,000 \times g$  for 10 min. Decant flow-through, wash with 500  $\mu$ l DNase/RNase free water and centrifuge at  $7,000 \times g$  for 10 min. Repeat washing with step with 500  $\mu$ l DNase/RNase free water.
13. Take out the membrane filter, invert and place in fresh Microcon tube. Add 40  $\mu$ l DNase/RNase free water and centrifuge at  $3,000 \times g$  for 4 min and discard the filter.
14. DNA yield is examined by agarose (1.2% w/v) gel electrophoresis with 10  $\mu$ l DNA sample and molecular weight markers.

#### References:

House, C.H., Cragg, B.A., Teske, A. *et al.* (2003). Drilling contamination tests during ODP Leg 201 using chemical and particulate tracers. In: Proceedings of the Ocean Drilling Program, Initial Reports, 201 (D'Hondt, S.L., Jørgensen, B.B., Miller, D.J., *et al.*, Eds.), pp. 1-19 [CD-ROM]. Ocean Drilling Program, College Station, Texas, USA.

Newberry, C.J., Webster, G., Cragg, B.A., Parkes, R.J., Weightman, A.J. & Fry, J.C. (2004). Diversity of prokaryotes and methanogenesis in deep subsurface sediments from the Nankai Trough, Ocean Drilling Programme Leg 190. *Environmental Microbiology* 6:274-287.

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Webster, G., Newberry, C.J., Fry, J.C. & Weightman, A.J. (2003). Assessment of bacterial community structure in the deep sub-seafloor biosphere by 16S rDNA-based techniques: a cautionary tale. *J Microbiol Methods* 55:155-164.

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## 6.2. PCR

Specific genes are amplified from genomic DNA with the polymerase chain reaction (PCR) performed in a thermocycler. Since the PCR conditions for gene amplification can strongly vary according to the nature of the template DNA and the targeted genes, a specific protocol must be checked and modified if necessary. In principle, all parameters can be modified (e.g., primers, amounts of the various reagents, type of the Taq DNA polymerase, numbers of reaction cycles, annealing temperature, etc.) and the best result is open to tests. The following protocol was successfully used as a standard protocol for the amplification of 16S rDNA:

### Reagents and solutions:

- 10 x Taq-Reaction buffer (TaKaRa, Shiga, Japan)
- 10 x dNTP solution (Roche, Mannheim, Germany)
  - 2.5 mM dATP
  - 2.5 mM dCTP
  - 2.5 mM dGTP
  - 2.5 mM dTTP
- 10 x BSA solution (bovine serum albumine): 3 mg/ml in PCR water
- 5 U/ $\mu$ l Taq DNA polymerase (TaKaRa, Shiga, Japan)
- 50 pmol/ $\mu$ l primers (Interactiva, Ulm, Germany)

Standard PCR primers for the amplification of 16S rDNA from environmental samples:

Primer	Target	<i>E. coli</i> binding site	Sequence 5'→3'	Reference
GM3 F	Bacteria	8-24	AGA GTT TGA TCM TGG C	Muyzer et al. 1996
GM4 R	Bacteria	1492-1507	TAC CTTT GTT ACG ACT T	Kane et al. 1993
Arch20 F	Archaea	20-38	TTC CGG TTG ATC CYG CCR G	Massana et al. 1997
Uni1392 R	Archaea Bacteria Eucaria	1392-1407	ACG GGC GGT GTG TRC	Pace et al. 1986

F = forward primer, R = reverse primer

M = A/C, R = A/G, Y = C/T

Standard reaction volume: 20  $\mu$ l:

- 1 x Taq buffer
- 1 x BSA
- 10 pmol forward primer
- 10 pmol reverse primer
- 0.2 U Taq DNA polymerase
- ad 19  $\mu$ l PCR water

Use 1  $\mu$ l of extracted DNA (try various dilutions) as DNA template for the PCR reaction. Add the template DNA at 70°C to the reaction mix in order to avoid unspecific amplifications ("hot start").

PCR conditions: First cycle with heating to 70°C and addition of template DNA as quick as possible ("hot start"), 1 min at 94°C (DNA denaturation), 1 min at x°C (annealing, see



below) and 3 min at 72°C (extension), followed by 30-40 cycles (the less the better!) with 92°C for 1 min, 40°C for 1 min, and 72°C for 3 min, and a final extension step consisting of 10 min at 72°C. Store the PCR products at 4°C or -20°C.

The annealing temperature varies according to the used primers:

GM3 F, GM4 R: 42-46°C

Arch 20 F, Uni1392 R: 58°C

Check for correct length of the amplified DNA fragments (for 16S rRNA genes ~1500 bp) by electrophoresing an aliquot (2-4 µl) of each amplification product on a 1% agarose gel stained with ethidium bromide (for DNA standards use e.g., "1 kb ladder" or "Low Mass DNA Ladder", see above).

Purify the PCR products, e.g., by using the purification protocol from the QIAquick purification Kit (QIAGEN, Hilden, Germany). Check the efficiency of the purification and the DNA concentration by electrophoresis of an aliquot of the purified solution (we use "Low Mass DNA Ladder" as a DNA standard).

#### Preparation of DNA from agarose gels:

If the PCR, even after optimising, produces unspecific amplifications (i.e., more than one band produced by the electrophoresis), it might be necessary to isolate the specific band by cutting the band with the correct length from the agarose gel. We use the QIAquick gel extraction protocol (QIAGEN, Hilden, Germany): Investigate the EtBr-stained agarose gel with UV trans-illumination, find the band with the correct length (~1500 bp) and cut an agarose block containing the correct band with a sharp and clean scalpel. Follow instructions of the QIAquick extraction protocol to extract the DNA from the agarose block. Store the extracted DNA at -20°C.

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### 6.3. Selecting and confirming efficacy of 16S rRNA gene primers

Most microbiologists who study diversity of prokaryotes by molecular means apply PCR amplification technology to 16S rRNA genes as part of their armoury of methodologies to obtain clone libraries or as the basis for profiling techniques such as DGGE or T-RFLP. However, many 16S rRNA gene primers have been designed over a decade ago when knowledge of prokaryotic diversity was much less than it is today. This means that many PCR primer pairs for 16S rRNA genes are not as effective as researchers suppose (e.g. Baker *et al.*, 2003).

The aim of this protocol is to describe the use of a computer program, called OligoCheck, written and developed by Kevin Ashelford at Cardiff that enables researchers to screen primer pairs for their effectiveness at amplifying specific prokaryotes that they are interested in. Use of OligoCheck helps the selection of primers, the assessment of old favourites and the design of new, more effective primer pairs. This programme has been widely used in Cardiff over about the last 5 years and has been found to be invaluable. An example of the utility of the program for studying methanogen diversity can be found in Banning *et al.* (2005).

#### Background:

A phylogenetically 'robust' oligonucleotide primer is one that retains high levels of specificity even when conditions result in numerous mismatches. Software such as ARB (Ludwig *et al.*, 2004) and PRIMROSE (Ashelford *et al.*, 2002) are already available to identify potentially useful oligonucleotides. It is also possible to assess the robustness of individual 16S rDNA oligonucleotides using the Ribosomal Database Project (RDP) tool PROBE\_MATCH (Maidak *et al.*, 2001). What PROBE\_MATCH or any other current tool cannot do is assess the robustness of pairs of primers (i.e. primer sets) simultaneously. Assessing forward and reverse primers separately is unsatisfactory because it is difficult to determine how their target-ranges overlap, especially when an increasing number of base-pair mismatches is allowed for. OligoCheck was written to make this assessment possible.

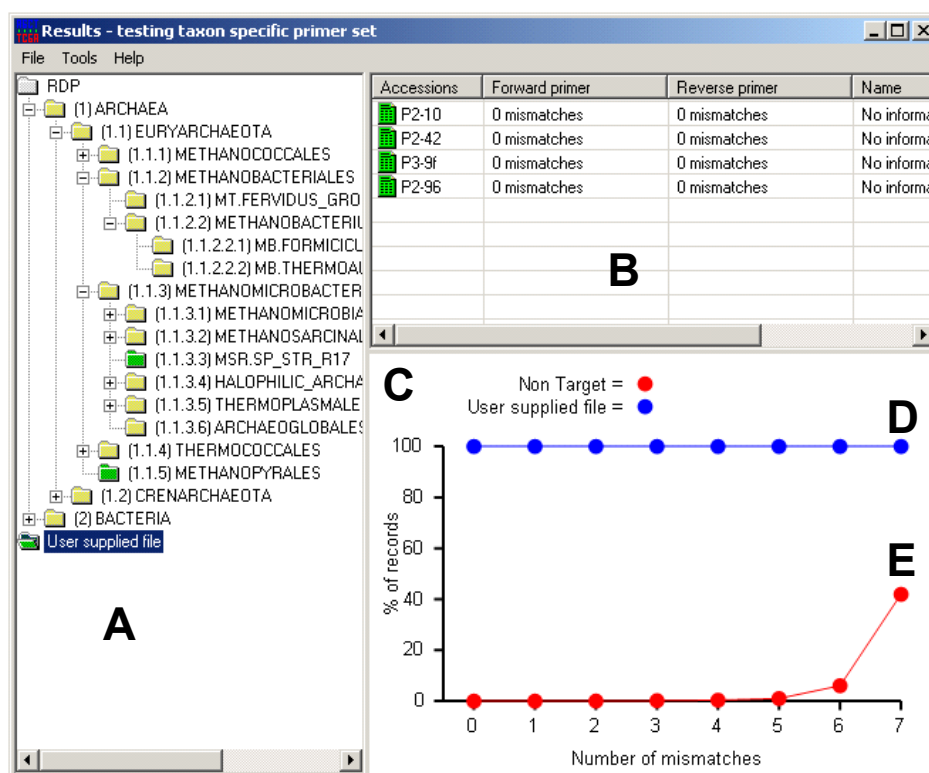
#### Using Oligocheck:

The program proceeds as follows. The user enters forward and reverse primers as the enquiry sequences and loads one or more GenBank or Fasta formatted files containing the subject sequences to be searched. The user may also use the RDP aligned database (release 8.1) supplied with the program if the 16S rRNA gene is being targeted. The user also has the option of specifying the maximum number of mismatches allowed between each primer and subject sequence. The user can also specify what length of each primer at the 3' end must match perfectly with target sequence, with the program accepting any value from 0 upwards. The program considers each primer separately against each subject sequence in the database. Successful matches between primer and subject sequence are recorded as the number of base-pair mismatches between enquiry and subject is increased (to a maximum of seven base-pair mismatches for each primer). The resulting output is then summarised, as illustrated in Fig. 1. Taxa loaded into the program are displayed in the left-hand panel of the result window (A). If the RDP aligned database has been selected, this is presented as an expandable hierarchy browser. Records within the currently selected taxon are presented in the top-right-hand panel (B). Selecting an individual taxon also reveals the results for that particular taxon as a plot in the bottom-right-hand panel (C). A blue line (D) illustrates the percentage of records



in that taxon that are successfully targeted by the primer set, as the number of base-pair mismatches are increased. Conversely, a red line (E) shows what percentage of the rest of the database is targeted with increasing base-pair mismatches. Thus, the ability of the primer set to describe the taxon identified, to the exclusion of all other taxa, can be rapidly assessed. Colour coding of the taxon icons, aids in the identification of the records that are targeted. For example, the plot in Fig. 1 shows the profile of a phylogenetically specific primer set, from the perspective of the taxon it was designed to target. The ‘target’ taxon in this case is represented by a novel group of 16S rDNA sequences loaded into the program (in this example named ‘user supplied file’). Line D shows that all records within this taxon are successfully targeted. Line E, in contrast, shows that at least six mismatches are required in either primer before around 6% of the rest of the database (12,661 in this example) are targeted. Through further exploration of the RDP aligned database (not shown), the identity of non-target-taxon records targeted by the primer set can be identified.

**Fig. 1.** A typical results window for OligoCheck. Panel A displays the RDP release 8.1 aligned database (partially expanded), along with a dataset supplied by the user. Panel B lists the contents of the currently highlighted taxon (in this case, the user-supplied file). Panel C summarises the effectiveness of the primer set in targeting this highlighted taxon (D) in contrast to the rest of the database (E).



Besides assessing 16S rRNA gene primer sets, OligoCheck can also assess individual oligonucleotides (e.g. hybridisation probes) in the same way and can be used for non 16S genes when appropriate user databases are supplied.



### Downloading the program and requirements:

OligoCheck is released under the terms of the GNU General Public Licence and is freely available for downloading from <http://www.cf.ac.uk/biosi/research/biosoft/>. Further information can be found at <http://www.cf.ac.uk/biosi/research/biosoft/OligoCheck/>. A comprehensive help file is included with the program.

OligoCheck is written in the Java computer language and will run on any computer with the Java Runtime Environment installed. Java is available for all major operating systems including Microsoft Windows 98, ME, NT, 2000 and XP and Apple Mac OS X, and can be downloaded for free from [www.java.com](http://www.java.com).

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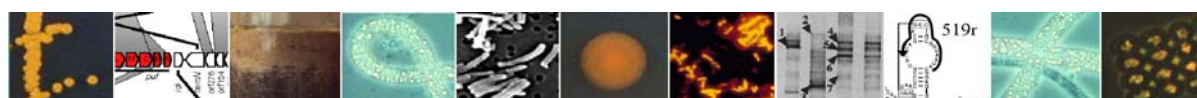
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## 6.4. Selecting and using primers for functional genes

### 6.4.1. Methyl coenzyme M reductase (*mcrA*) genes for methanogens

Methyl coenzyme M reductase (MCR) catalyses the terminal step in methanogenesis and therefore a key target enzyme. Specific primers have been developed to target the methyl coenzyme M reductase (*mcrA*) gene (Hales et al., 1996; Luton et al., 2002). The development of this molecular marker for methanogens has improved the characterisation of methanogenic populations within a number of environments to date, including deep marine sediments, gas hydrate sediments, Antarctic sediments, brackish sediments, peat bogs and wetland soils.

DNA samples for PCR should be stored at  $-80^{\circ}\text{C}$  until required to prevent degradation of sample over time by coextracted DNAses and other contaminating substances.

#### Reagents and disposables:

PCR reaction tubes  
 Taq DNA polymerase (5 units  $\mu\text{l}^{-1}$ )  
 dNTP solution (25 mM each dNTP)  
 10x PCR buffer  
 50 mM  $\text{MgCl}_2$  solution  
 Sterile DNase-free molecular grade water (Sigma)  
 Bovine serum albumin (BSA) 10 mg  $\text{ml}^{-1}$   
 PCR primers (20 pmol  $\mu\text{l}^{-1}$ )

The *mcrA* PCR primers sets that have been used routinely to detect methanogens in environmental samples are listed in Table 1:

#### PCR:

Make a 10-fold serial dilution of extracted DNA and test several dilutions by PCR to find the best concentration of template DNA that gives a good specific product. Prepare a master mix for the total number of PCR reactions to be undertaken as by adding for each reaction the following reagents:

10x PCR reaction buffer	10 $\mu\text{l}$
dNTP mix	1 $\mu\text{l}$
Forward primer	1 $\mu\text{l}$
Reverse primer	1 $\mu\text{l}$
$\text{MgCl}_2$	3 $\mu\text{l}$
BSA	1 $\mu\text{l}$
Taq DNA polymerase	0.25 $\mu\text{l}$
Molecular grade water	up to 49 $\mu\text{l}$

Gently mix the master mix by careful inversion and gently tap with finger to collect contents in bottom of tube. Dispense 49  $\mu\text{l}$  to each PCR tube and add 1  $\mu\text{l}$  of DNA template to each tube and close. Insert each tube into a thermocycler and start appropriate PCR program.



When the run has completed store reactions at 4 or -20°C until required. Analyse 5 µl of the PCR product by 1-2% (w/v) agarose gel electrophoresis with appropriate DNA marker. Note, always perform PCR with the following controls (1) without addition of DNA template (negative control) and (2) with addition of known DNA template (positive control).

**Table 1. *mcrA* PCR primers used for study of methanogens.**

Primer pair	Primer sequence (5' to 3')	Target sequence length (bp)	Reference
*ME1f	GCM ATG CAR ATH GGW ATG TC	760	Hales <i>et al.</i> , 1996
*ME2r	TCA TKG CRT AGT TDG GRT AGT		
*MLf	GGT GGT GTM GGA TTC ACA CAR TAY GCW ACA GC	464 to 491	Luton <i>et al.</i> , 2002
*MLr	TTC ATT GCR TAG TTW GGR TAG TT		
MCRf	TAY GAY CAR ATH TGG YT	490	Springer <i>et al.</i> , 1995
MCRr	ACR TTC ATN GCR TAR TT		
F	GCN ATG CAR ATH GGN ATG	700	Ohkuma <i>et al.</i> , 1995
R	GCN CCR CAY TGR TCY TG		

\* have been used on marine sediments and PCR protocols listed below (Table 2). It should be noted that the *mcrA* primers of Hales *et al.* (1996) provide reasonable sequence information (sequence length) with good methanogen coverage whereas the *mcrA* primers of Luton *et al.* (2002) target more known methanogenic groups but provide smaller amount of sequence information.

**Table 2. PCR cycling conditions**

Primer pair	PCR program
ME1f/ME2r	5 min at 94°C, followed by 35 cycles of 45 s at 94 °C, 45 s at 50 °C, and 1 min 45 s at 72 °C, with a final extension at 72 °C for 7 min.
MLf/MLr	5 min at 94°C, followed by 10 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min 30 s at 72 °C (ramped to 72 °C at 0.1 °C/s), followed by 25 cycles of 1 min at 94 °C, 1 min at 52 °C, and 1 min 30 s at 72 °C, with a final extension at 72 °C for 7 min.

## References:

Hales, B., Edwards, C., Ritchie, D., Hall, G., Pickup, R. & Saunders, J. (1996) Isolation and identification of methanogen-specific DNA from blanket bog peat by PCR amplification and sequence analysis. *Appl. Environ. Microbiol.* 62, 668-675.



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### 6.4.2. Quantitative Real-time PCR of the *dsrAB* genes from sulphate-reducers

This method allows quantifying the sulphate-reducing microorganisms present in environmental samples, by using the *dsrAB* genes (dissimilatory sulphite reductase) as a target sequence, assuming that one copy of this gene is present per cell (Wagner *et al.*, 1998). The principle of Real-time PCR is a combination of the exponential amplification of specific regions of the genomic DNA and the quantification of these PCR products by highly sensitive and automated fluorescence detection (for more details, see review Sharkey *et al.*, 2004). Real-time PCR monitors the amount of amplicon in the reaction as it is produced during each PCR cycle. The cycle at which the product is first detectable (the threshold cycle or  $C_t$ ) is inversely proportional to the amount of starting material. We use a SyBrGreen based Real time PCR detection technique. This dye specifically binds double-stranded DNA and emits fluorescence only upon binding. The fluorescent signal increases proportionally with the amount of amplified PCR product. During each PCR cycle, DNA is only double stranded after the annealing step and during the extension step. Therefore, the fluorescent SYBR Green signal is usually detected immediately after the extension step of each cycle. This method is highly sensitive to humics acids or other chemicals contaminants known as PCR inhibitors. Thus, DNA has to be purified prior quantification. To confirm the expected size of the product and the absence of non-specific amplicons, a melting curve analysis has to be performed after PCR. By slowly increasing temperature, PCR product will be denaturated and a rapid loss of fluorescence will be observed. Then by plotting fluorescence as a function of temperature, a melting curve can be obtained. As the melting curve is dependent of GC content, length and sequence, different PCR products can be distinguished.

All materials for the PCR mix are stored at  $-20^{\circ}\text{C}$ , and thawed on ice before use. The reaction mix ( $V_r = 25 \mu\text{l}$ ) is composed of: 12,5  $\mu\text{l}$  of MasterMix SyBrGreen 2X (Eurogentec, Seraing, Belgium), 1  $\mu\text{l}$  of Bovine Serum Albumin (BSA) ( $3 \text{ mg}\cdot\text{ml}^{-1}$ ), 1.25  $\mu\text{l}$  of *dsr1F+* and *dsrR* primers ( $1 \mu\text{M}$ ), and 2  $\mu\text{l}$  of template DNA. It might be necessary to add 1  $\mu\text{l}$  of  $\text{MgCl}_2$  (25 mM) depending on the sediment nature and purity of DNA extracts.

Primers used:

<i>dsr1F+</i> :	5'-ACS CAC TGG AAG CAC GGC GG-3', Kondo <i>et al.</i> (2004)
<i>dsrR</i> :	5'-G TGG MRC CGT GCA KRT TGG-3', Kondo <i>et al.</i> (2004)

Quantitative PCR is carried out in a ABI Prism 7700 (Applied Biosystems), as follows: 2 minutes at  $50^{\circ}\text{C}$ , 10 minutes at  $94^{\circ}\text{C}$  for initial denaturation; 40 cycles of 15 seconds at  $94^{\circ}\text{C}$ , 1 minute at  $59^{\circ}\text{C}$ , 45 seconds at  $72^{\circ}\text{C}$ . Results are analysed using the ABI Prism 7700 sequence detection system (Applied Biosystems). Melting curves are analysed using the software "dissociation curve" (Applied Biosystems). All measurements have to be done in triplicates.

To obtain final quantitative data, a calibration curve is constructed by performing a serial dilution of known amount of *dsrAB* genes, carried by pKSAII (plasmid constructed by Leloup *et al.*, 2004). Thus, the copy number of target is plotted as a function of  $C_t$  values (Fig. 1).

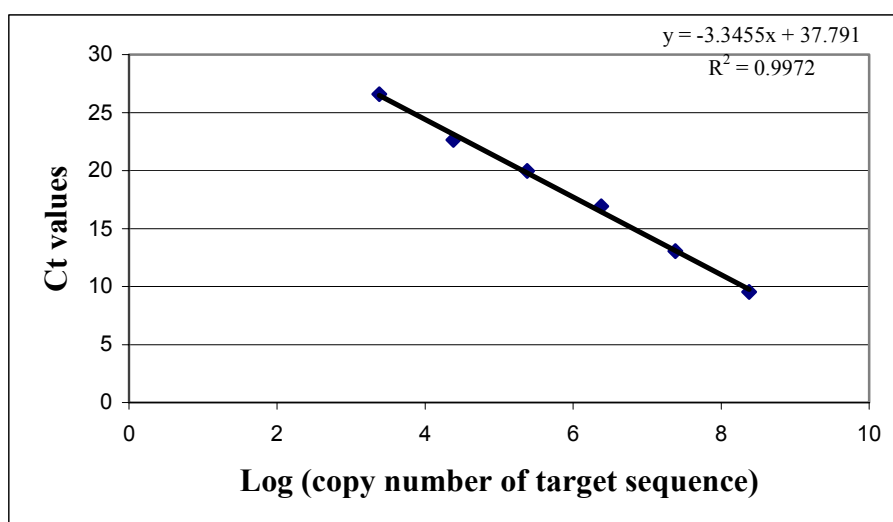


To estimate that your calibration is accurate, the efficiency of the amplification has to be equal to 1:

$$E \text{ (Efficiency)} = [10^{(-1/\alpha)}] - 1$$

Thus, the slope  $\alpha$  has to be near -3.32 and the  $R^2$  value near to 1.

**Fig. 1. Plot of copy number against  $C_t$  values**



#### References:

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### 6.4.3. Other functional genes and small subunit rRNA groups

#### DNA isolation from sediments:

The MoBio UltraClean Soil DNA kit (MoBio, USA) will be used according to the manufacturer's recommendations.

#### PCR amplification, cloning and sequencing:

The extracted DNA will be amplified by PCR for the investigation of the Bacteria, Archaea and Eukarya domains using specific primers for the 16S rRNA gene. Also, the diversity of the certain functional genes, specific for methanogens (methyl coenzyme M reductase), methylotrophs (particulate and soluble methane monooxygenase, and methanol dehydrogenase), sulfate reducers (dissimilatory sulfite reductase) and sulfide oxidizers (sulfide monooxygenase) will be investigated. The primers for each specific PCR are shown in Table 1.

In order to eliminate some of the PCR's innate limitations (e.g. Witzingerode *et al* 1997), all PCRs will be performed using the optimum number of thermal cycles, i.e. the minimum number of cycles that produce a visible PCR product in a 1.2% agarose gel.

PCR products will be purified using the Microcon purification kit (Millipore, USA) and then will be cloning by using the TOPO XL cloning kit (Invitrogen, USA). Approximately 100 clones from every sample will be checked for having the right-sized insert by PCR. All true-positive clones will be screened for unique phylotypes by restriction fragment length polymorphism (RFLP) by using 4 or 5 different restriction enzymes (*AluI*, *RsaI*, *HinfI*, *HaeIII*, *HhaI*). Unique phylotypes will be sequenced by capillary electrophoresis in an ABI 3700 sequencer at Macrogen Ltd (Seoul, Korea).

#### Phylogenetic analyses:

The resulting sequences will be checked for chimeras using the CHIMERA\_CHECK function of the Ribosomal Database Project (Maidak *et al* 2001) and the Bellerophon server (Huber *et al* 2004). All chimeric sequences will be discarded from further analyses and the remaining ones will be submitted to GenBank (NCBI). Phylogenetic analyses will be performed using the ARB ([www.arb-home.de](http://www.arb-home.de)) and PAUP\* (Swofford 2000) softwares.



**Table 1. Primers for specific groups of organisms: 16S rRNA, 18S rRNA and functional genes**

Gene	Primer		Reference
16S rRNA - Bacteria	EUB338f-mix	5'-ACTCCTACGGGAAGGCAG C-3'	Amann <i>et al</i>
		5'-ACTCCTACGGGAAGGCTG C-3'	(1990)
	Univ1492r-mix	5'-ACACCTACGGGTAGGCTG C-3'	Dammis <i>et al</i>
		5'-ACACCTACGGGTAGGCAG C-3'	(1999)
	5'-TACCGTTACCTTGTTACGACT T-3'	Lane (1991)	
		5'-TACCGCTACCTTGTTACGACT T-3'	
16S rRNA - Archaea	Arc109f-mix	5'-ACGGCTCAGTAACACGT-3'	Grosskopf <i>et al</i>
		5'-ACTGCTCAGTAACACGT-3'	(1998), Y.
		5'-AAGGCTCAGTAACACGT-3'	Sekiguchi
			(unpubl.)
	Univ1492r-mix	5'-TACCGTTACCTTGTTACGACTT-3'	Lane (1991)
		5'-TACCGCTACCTTGTTACGACTT-3'	
18S rRNA - Eukarya	1f(EUK)	5'-ACCTGGTTGATCCTGCCAG-3'	Moon-van der
			Staay <i>et al</i>
			(2000)
	1775r(EUK)	5'-TGATCCTTCYGCAGGTTAC-3'	Moon-van der
			Staay <i>et al</i>
			(2000)
Methyl coenzyme M reductase ( <i>mcrA</i> )	ME1f 5	5'-CGMATGCARATHGGWATGTC-3'	Hales <i>et al</i>
			(1996)
	ME2r	5'-TCATKGCRTAGTTDGGRTAGT-3'	Hales <i>et al</i>
			(1996)
Particulate methane monooxygenase ( <i>pmoA</i> )	A189f	5'-GGNGACTGGGACTTCTGG-3'	Holmes <i>et al</i>
			(1995)
	A682r	5'-GAASGCNGAGAAGAASGC-3'	Holmes <i>et al</i>
			(1995)
Soluble methane monooxygenase ( <i>mmoX</i> )	mmo882f	5'-GGCTCCAAGTTCAAGGTTCGAGC-3'	McDonald <i>et al</i>
			(1995)
	mmo1403r	5'-TGGCACTCGTAGCGCTCCGGCTCG-3'	McDonald <i>et al</i>
			(1995)
Methanol dehydrogenase ( <i>mxhF</i> )	mxh1003f	5'-GCGGCACCAACTGGGGCTGGT-3'	McDonald <i>et al</i>
			(1997)
	mxh1561r	5'-GGGCAGCATGAAGGGCTCCC-3'	McDonald <i>et al</i>
			(1997)
Dissimilatory sulfite reductase ( <i>dsrAB</i> )	1FI	5'-CAGGAYGARCTKCACCG-3'	Dhillon <i>et al</i>
			(2003)
	1RI	5'-CCCTGGGTRTGGRAYRAT-3'	Dhillon <i>et al</i>
			(2003)
Sulfide monooxygenase ( <i>soxA</i> )	soxF	5'-TCAAGGCCTGGATTGGCTGGTGG-3'	Lei & Tu
			(1996)
	soxR	5'-CGATCAGCGCCTCGGATCCTCAGG-3	Lei & Tu
			(1996)

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Amann RI, Binder BJ, Olson RJ, Chisholm SW, Devereux R, & Stahl DA (1990) Combination of 16s ribosomal-rna-targeted oligonucleotide probes with flow-cytometry for analyzing mixed microbial-populations. *Appl. Environ. Microbiol.* 56:1919-1925

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## 6.5. Cloning, clone selection and sequencing

### 6.5.1. Contribution 1

Cloning is a technique for recombination of DNA, with which a DNA fragment is inserted into a circular DNA molecule (cloning vector) by a ligase enzyme (ligation). The ligation products are brought into competent bacterial strains (transformation). The competent cells duplicate the recombined cloning vectors (e.g. plasmids) during growth and pass the copies on to their daughter generations. Plated on an agar medium the transformed cells build up colonies of genetically identical clones, which contain the recombined DNA.

#### Ligation:

Cloning systems that have been successfully used are the pGEM<sup>®</sup>-T easy Vector System (Promega, Madison, USA) and the TOPO TA Cloning<sup>®</sup> System (Invitrogen, Groningen, The Netherlands). Ligations with the vectors pGEM<sup>®</sup>-T easy and the pCR<sup>®</sup>4-TOPO<sup>®</sup> were performed according to the protocols of the manufacturers. The ligation products can be directly used for transformations or are stored at -20°C.

#### Heat shock transformation:

Materials and solutions:

- Strain *Escherichia coli* TOP10
  - 0.5 M IPTG stock solution:
    - IPTG solution (Isopropyl  $\beta$ -D-thiogalactoside, AppliChem Darmstadt)
    - 0.5 M IPTG in Milli-Q water
    - sterile filtered solution (0.2  $\mu$ m pore size) and store aliquots at -20°C.
- X-Gal solution:
  - X-Gal (5-Bromo-4-chloro-3-indolyl  $\beta$ -D-galactopyranoside, Roth, Karlsruhe, Germany)
  - 80 mg/ml X-Gal in N-N'-Dimethylformamide
  - store aliquoted solution at -20°C.
- Luria Bartani (LB) medium:
  - 10 g/l Bacto<sup>®</sup>-trypton (Difco, Detroit, USA)
  - 10 g/l Bacto<sup>®</sup>-yeast extract (Difco, Detroit, USA)
  - 5 g/l NaCl
  - adjust to pH 7.0 with NaOH, autoclave and store at 4°C.
- Ampicillin stock solution
  - Ampicillin (Biomol Feinchemikalien, Hamburg, Germany)
  - 80 mg/ml Ampicillin in Milli-Q water
  - aliquote and store at -20°C.
- LB-agar pates (Amp<sup>+</sup>, IPTG<sup>+</sup>, X-Gal<sup>+</sup>)
  - Add 15 g Bacto<sup>®</sup>-agar (Difco, Detroit, USA) to 1 l LB medium, autoclave and let cool to 50°C. Add 1 ml each of the IPTG, X-Gal and ampicillin stock solutions (final concentrations 0.5 mM, 80  $\mu$ g/ml, and 80  $\mu$ g/ml, respectively). Mix gently, fill Petri dishes, and store the cooled agar plates at 4°C.
- SOB medium (pH 7.2-7.4)





- 0.5% Yeast extract
- 2% Tryptone
- 10 mM NaCl
- 2.5 mM KCl
- autoclave
- SOC medium
  - 10 mM MgSO<sub>4</sub>
  - 10 mM MgCl<sub>2</sub>
  - 20 mM Glucose
  - sterile MgSO<sub>4</sub>, MgCl<sub>2</sub>, and glucose solutions in autoclaved SOB.

#### Transformation:

Transformation of the competent *E. coli* cells (strain TOP10) according to the manufacturer's protocol: Thaw the competent cells (storage temperature -80°C) on ice. Add 2 µl of the ligation product solution to a TOP10 containing vial and mix gently. Incubate on ice for 20 min, apply heat shock (42°C) for 30 s and transfer vial to ice again. Apply 250 µl SOC medium (room temperature) and incubate gently shaking (~150 rpm) for 1 h at 37°C. Plate various volumes on selection plates and incubate over night at 37°C.

#### Selection of transformed cells:

pGEM®-T easy vector: selection of transformed cells by blue-white-screening. Cells that contain the recombinant plasmid are colorless, while those containing the vector without recombination insert are stained blue.

*Principle:* The pGEM®-T easy vector contains the gene sequence coding for the *LacZα* fragment of β-galactosidase, an enzyme involved in the lactose metabolism of *E. coli*. The expression of this enzyme is induced by the presence of the lactose analogue IPTG. The galactosidase enzyme uses X-Gal (colorless) as a substrate, which it splits to galactose and an inoxyl derivate. The latter oxidizes with atmospheric oxygen to a blue dibrome derivate. Ligation of DNA in the multiple cloning site of the vector results in a fragmented β-galactosidase gene. The expression of this gene results now in a complete inactive protein or a protein of highly reduced activity.

pCR®4-TOPO vector: direct selection of transformed cells; cells without the recombinant vector die.

*Principle:* The DNA fragment is inserted in the *E. coli* lethal gene *ccdB*, which is merged on the vector with the C- terminus of the *LacZα* fragment. Ligation of an insert results in an inactive merged protein. Transformed TOP10 cells can grow, while those containing an un-recombined vector die. Additionally, the ampicillin resistance coded on the vector allows the selection of cells that were not transformed with the plasmid because these cannot grow in the presence of the antibiotic ampicillin.

#### Selection of clones:

After incubation on the LB media (see above), the white bacterial colonies are individually labeled and systematically transferred (sterile pipette tips) to other LB selection plates.



Incubate over night at 37°C; store at 4°C. The entire yield of white colonies is termed a gene bank.

#### Control for correct length of the inserts:

The correct lengths of the inserts in the clones are controlled with PCR using the primers M13 uni and M 13 R which are specific to both vectors (pGEM<sup>®</sup>-T easy, pCR<sup>®</sup>4-TOPO).

Primer	Target	Sequence 5' → 3'	Reference
M13 uni	Vector	ACG ACG TTG AAA ACG ACG GCC AG	Yanish-Perron et al. 1985
M13 R	Vector	TTC ACA CAG GAA ACA GCT ATG ACC	Yanish-Perron et al. 1985

Transfer the clone colonies from LB plates to 150 µl liquid LB medium (100 µl/ml ampicillin) in 96 well-format and incubate shaking for 3-5 h at 37°C. Use 1-µl aliquots of the bacteria suspension for PCR (96-well PCR plates allow high efficiency). Use standard PCR program as described above for the amplification of 16S rDNA, with the following modifications: (i) do not perform a “hot start”; (ii) insert a heat step of 95°C for 5 min before the first temperature cycle in order to lyse cells and inactivate nucleases. Electrophoresis of the PCR products with 1% agarose gel.

#### Sequencing:

*Taq* cycle sequencing of plasmid DNAs from selected clones with vector primers and universal rRNA gene specific primers was performed using a ABI capillary sequencer according to manufacturer's recommendations. Partial sequences are assembled to a single full length sequence using "Sequencher" software (Gene Codes Corporation, Ann Arbor, USA). Sequence data are phylogenetically analyzed with the ARB software package (Ludwig et al., 2004). Phylogenetic trees are calculated for new sequences together with sequences, which are available in the databases EMBL, GenBank and DDJB, by performing parsimony, neighbor-joining and maximum-likelihood analysis on a subset of up to 200 nearly full-length sequences (>1300 bp). Filters are used which exclude highly variable positions. Partial sequences (<1300 bp) are inserted into the reconstructed tree by parsimony criteria with global/local optimization, without allowing changes in the overall tree topology.

#### Reference:

Ludwig, W., O. Strunk, R. Westram, L. Richter, H. Meier, Yadhukumar, A. Buchner, T. Lai, S. Steppi, G. Jobb, W. Förster, I. Brettske, S. Gerber, A. W. Ginhart, O. Gross, S. Grumann, S. Hermann, R. Jost, A. König, T. Liss, R. Lüßmann, M. May, B. Nonhoff, B. Reichel, R. Strehlow, A. Stamatakis, N. Stuckmann, A. Vilbig, M. Lenke, T. Ludwig, A. Bode, and K.-H. Schleifer. (2004). ARB: a software environment for sequence data. *Nucleic Acid Research* 32:1363-1371.

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### 6.5.2. Contribution 2

Sequence analysis of PCR-amplified and cloned 16S ribosomal RNA genes (16S rDNA), is a widely used approach to assess microbial diversity and community composition in environmental samples (Bowman & McCuaig, 2003; von Wintzingerode *et al.*, 1997). The present method aims to investigate the community composition and the general diversity of sediment bacterial communities using conditions to minimize PCR artefacts.

Similar to other methods, based on the use of PCR to enrich multiple alleles of specific genes from complex mixtures of genomes, 16S rDNA clone libraries suffer from methodological constraints that may skew the distribution of phylotypes in the library relative to the community it was derived from (von Wintzingerode *et al.*, 1997). PCR-induced formation of chimeric molecules, heteroduplexes and mutations resulting from Taq-error may also bias the library and introduce 'artificial' diversity. This interference can be largely avoided by lowering the number of amplification cycles and carrying out 'reconditioning PCR' (Thompson *et al.*, 2002). The present protocol was successfully applied in sediment samples obtained from the Mediterranean Sea (Polymenakou *et al.*, 2005) and PCR conditions described here were designed to minimize bias (Polz and Cavanaugh, 1998; von Wintzingerode *et al.*, 1997).

#### 16S rRNA gene amplification:

Genomic DNA must be extracted from sediments using a highly efficient DNA extraction kit. Bacterial 16S rRNA genes are amplified from mixed genomic samples using the polymerase chain reaction (PCR) with the universal bacterial primers 27f modified to match also *Planctomycetales* (5'- AGRGTTTGATCMTGGCTCAG-3') (Vergin *et al.*, 1998) and 1492r (5'- GGYTACCTTGTTACGACTT-3') (Lane, 1991).

For each sample, eight replicate PCR reactions of 30 µl are amplified in a Thermal Cycler with the following program:

1. Initial denaturation at 94°C for 3 min
2. 25 cycles of:
  - 1 min at 94°C
  - 1 min annealing at 55°C
  - 3 min primer extension at 72°C
3. Final extension at 72°C for 7 min.

Each tube contained the following mixture

1. 1-4 ng of target DNA
2. PCR buffer
  - 10 mM Tris-HCl pH 9
  - 50 mM KCl
  - 0.1 % Triton X-100
  - 2 mM MgCl<sub>2</sub>)
3. 100 nM of each primer
4. 200 µM of each dNTP
5. 0.25 U Taq DNA Polymerase

Products from each of the 8 PCR reactions are used as templates 1:10 (vol.) in duplicate 3-cycle reconditioning PCR-reactions (16 reactions total) to eliminate heteroduplex formation



that may introduce artificial diversity in clone libraries (Thompson, 2002). All PCR products are pooled and precipitated with ethanol and sodium acetate (Sambrook & Russel, 2001) followed by gel purification using a PCR purification kit. The concentration of PCR products generated from the different sediment samples are determined by direct comparison to a Low DNA Mass ladder using 2% agarose gel electrophoresis, ethidium bromide staining and UV-transillumination.

#### Cloning:

For each sampling site, 5-10 ng of PCR product are cloned into a vector and transformed into chemically competent cells of *E. coli* using the appropriate cloning kit.

At least 200 positive clones from each clone library (selected by blue and white screening) are transferred to 96-well plates and incubated overnight at 37°C in Luria-Bertani (LB) medium containing 50 µg kanamycin ml<sup>-1</sup>.

Aliquots of the individual clones are

- (i) archived at -80°C in 7% Dimethyl sulfoxide or
- (ii) washed by pelletizing cells in a 30 min centrifugation at 10,000 x g followed by supernatant removal by low-speed centrifugation (<500 rpm) of inverted plates.

Pelletized cells are resuspended in 30 µl sterile and UV-irradiated MQ-grade water. Cells are lysed by heating at 98°C for 10 minutes (using the Thermal Cycler) followed by agitation. The lysates are used (1:10 vol.) as templates in a PCR amplification of the insert using external (vector) primers (e.g. M13f-20: 5'-GTAAAACGACGGCCAG-3' and M13r: 5'-CAGGAAACAGCTATGAC-3' when using the TOPO TA Cloning kit) to avoid co-amplification of *E. coli* host-cell DNA. PCR amplification is carried out for 25 cycles as described before. Positive transformants (clones carrying an insert of correct size) are identified by agarose gel electrophoresis as described above.

#### Clones screening and sequencing:

Aliquots (5 µl) of individual PCR products are digested with two four-cutting restriction enzymes (*HhaI* and *HaeIII*) for 16 h according to instructions supplied by the manufacturer. After inactivation of the enzymes (20 min at 85°C), fragments are sized by electrophoresis on a 2% agarose gel (2.5 hours, 80V, 10°C). Fragments are recorded using ethidium bromide staining and UV-transillumination. A 100 bp DNA ladder is used for determination of fragment size. The resulting restriction fragment length polymorphism (RFLP) patterns are then used to classify clones into operational taxonomic units (OTUs).

All different clones are sequenced on a capillary sequencer using primer 27f (Vergin et al., 1998) and the appropriate kit.

#### Species Richness:

For each clone library, the RFLP-based distribution of clones in different OTUs is used to estimate species richness using the web based Rarefaction calculator software (<http://www2.biology.ualberta.ca/jbrzusto/rarefact.php>).



Species richness is estimated using the nonparametric Chao estimator (Chao, 1984):

$$S^*_1 = S_{\text{obs}} + [a^2/(2 \times b)]$$

The standard deviation (SD) is estimated using the equation:

$$SD = b [ ( a / ( 4 \times b ) ) ^ 4 + ( a / b ) ^ 3 + ( a / 2 \times b ) ) ^ 2 ]$$

Where:

$S_{\text{obs}}$ : the number of 16S rDNA clones observed

$a$ : the number of clones observed just once

$b$ : the number of clones observed twice

### References:

Bowman, J.P. & McCuaig, R.D. (2003). Biodiversity, community structural shifts, and biogeography of prokaryotes within Antarctic continental shelf sediment. *Appl. Environ. Microbiol.* **69**:2463-2483.

Chao, A. (1984). Nonparametric estimation of the number of classes in a population. *Scand. J. Stat.* **11**:265-270.

Lane, D.J. (1991). 16S/23S rRNA sequencing. In Stackebrandt, Goodfellow, M., (Eds.), *Nucleic Acid Techniques in Bacterial Systematics*: Chichester (Wiley and Sons), 115-175.

Polymenakou, P.N., Bertilsson, S., Tselepides, A. & Stephanou, E.G. (2005). Bacterial community composition in sediments from the Eastern Mediterranean Sea: a comparison of four 16S rDNA clone libraries. *Microb. Ecol.*, in press.

Polz, M.F. & Cavanaugh, C.M. (1998). Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.*, **64**:3724-3730.

Sambrook, J., & Russel, D.W. (2001). *Molecular Cloning: a Laboratory Manual*, 3<sup>rd</sup> ed., Cold Spring Harbour, New York.

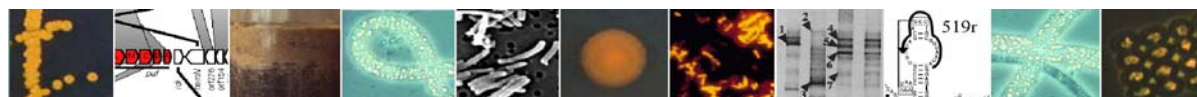
Thompson, J.R., Marcelino, L.A., & Polz, M.F. (2002). Heteroduplexes in mixed-template amplifications: formation, consequences and elimination by 'reconditioning PCR'. *Nucleic Acids Res.*, **30**:2083-2088.

Vergin, K.L., Urbach, E., Stein, J.L., DeLong, E.F., Lanoil, B.D., & Giovannoni, S.J. (1998). Screening of a fosmid library of marine environmental genomic DNA fragments reveals four clones related to members of the order *Planctomycetales*. *Appl. Environ. Microbiol.* **64**:3075-3078.

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## 6.6. Screening clone libraries for artefacts, chimeras and coverage

Once a clone library has been obtained it is sensible to check it for artefacts and to ensure that enough clones have been sequenced for the purposes for which the library was made. The aim of this section of the handbook is to help microbiologists with this process. Most of these methods and the protocols described will be for 16S rRNA clone libraries.

### Outline of protocols:

The first step is to check the sequences in the library for artefacts. Common artefacts are poor sequencing resulting in too many ambiguous base positions (i.e. too many N's or other degenerate base characters), poorly assembled sequences (e.g. inversions or missing sequence) and chimeras. Chimeras are sequences resulting from amplification of more than one gene template during a PCR reaction from environmental DNA, so for example, one half of the gene might originate from one organism and the other half from another (Hugenholtz & Huber, 2003).

Once erroneous sequences are removed, all the sequences are put into a file in FASTA format. I will use one such file as the example throughout. This is available for downloading, at <http://www.cardiff.ac.uk/biosi/research/biosoft/Data/> so that HERMES microbiologists can use it as a test file. This file represents the clone library described in Parkes *et al.* (2005); it is the *Archaea* 16S rRNA gene library from the 42 mbsf depth in the Peru margin sediment at Ocean Drilling program site 1229 in the Pacific Ocean close to the coast of Peru.

The next step is to compare the percentage similarity of all the sequences and put them into groups that are similar. For example, sequences showing 97-98% similarity for the 16S rRNA gene are sometimes referred to as "within a species", whilst 95% can be used as "within genus" similarity value. However, note that there is no precise relationship between 16S rRNA gene similarity and taxonomic closeness and so these values are very rough indeed and are only used as convenient indicators. This process is called "binning" here. Once the numbers of sequences at particular similarity level within each bin is known calculations can be done to estimate coverage, rarefaction curves and perhaps species richness estimators. Rarefaction curves, or collector's curves, enable investigators to estimate if they have collected enough clones to sample a population adequately at a particular similarity level.

### Checking for chimeras and other artefacts:

Care whilst assembling final sequences from multiple reads and carefully checking and re-sequencing if necessary will prevent some of the artefacts described above. However, other methods are needed to detect chimeric sequences. CHIMERA\_CHECK version 2.7 is a program available on the Ribosomal Database Project (RDP) that can be used at the RDP website ([http://rdp8.cme.msu.edu/docs/chimera\\_doc.html](http://rdp8.cme.msu.edu/docs/chimera_doc.html)) but its results are sometimes difficult to interpret. Some other programs are also available (e.g. Bellerophon; Huber *et al.*, 2004). However, here will be briefly described another program called Pintail (Ashelford *et al.*, in press), which checks for chimeras and will also detect other artefacts as well.

Pintail can be downloaded along with full documentation from the Cardiff Bioinformatics Toolkit website (<http://www.cardiff.ac.uk/biosi/research/biosoft/>). Although the use of the program is fully explained on the website, and a tutorial is also available, below is a brief explanation of how it is used.





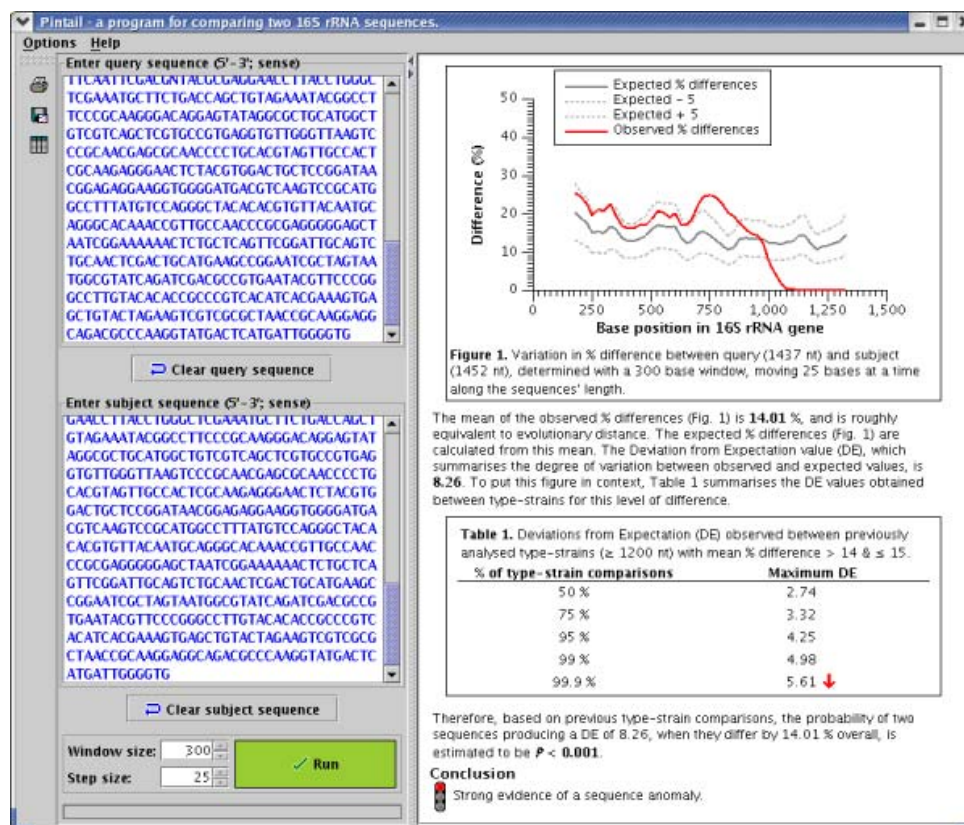
## How to use Pintail - a brief guide to analysing a chimera

Basically what you have to do is to compare your clone library sequences individually with a phylogenetically close and reliable sequence (e.g. from a pure culture or well researched environmental sequence). Look at the screen shot in Fig. 1 below and then read the following and you will see that testing a small clone library will be very quick once you have learnt to use Pintail.

- Enter the 16S rRNA gene sequence to be checked into the text box marked 'query sequence'.
- Enter a reliable 16S rRNA gene sequence in the text box labelled 'subject sequence'.
- Click 'Run' to carry out the analysis.

A chimera is demonstrated if the observed percentage differences line, plotted in red by the program, deviates significantly from the expected percentage difference line plotted in dark grey. This deviation is quantified with the Deviation from Expectation (DE) statistic. The program determines whether the calculated DE value is likely to be indicative of a chimera (or other sequence anomaly) and provides an assessment accordingly. Fig. 1 shows results from one comparison. The left data entry boxes show the test (upper) and reliable (lower) sequences. The graph on the right shows that the red line deviates from between the light grey lines most strongly after about 950 base positions on the 16S rRNA gene and so is almost certainly a chimera. The associated text gives more explanation. If such a sequence was found in a clone library of yours it should probably be removed.

**Fig. 1. Pintail screenshot showing the outcome of analysis of the chimera AY326570.**



### Binning at defined similarity levels:

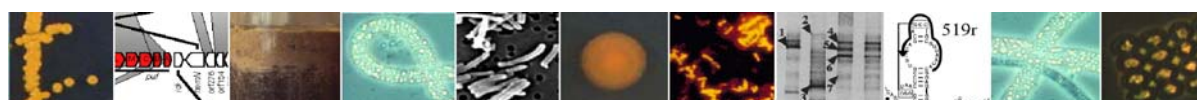
Firstly it is necessary to define the similarity levels at which the groups of sequences or bins should be placed. Schloss & Handelsman (2004) use the following, which they consider widely used for 16S rRNA gene sequences, and it seems reasonable to recommend their use here. Similarity at 97% = species level, 95% = genus level, 90% family/class level and 80% = phylum level. There are several ways to group sequences by similarity and here we describe a simple method using Blastclust, which is a command line driven program, available free within the Blast package of programs at the NCBI website. Schloss & Handelsman (2005) describe another method for those that prefer to use DNADIST in the PHYLIP package.

#### 1. Downloading Blastclust

- Go to the NCBI website at <http://www.ncbi.nlm.nih.gov/>.
- Click FTP site in the left hand menu.
- Click BLAST.... to download the software.
- Double click the folder/directory “executables” and then “release”.
- Double click the most recent folder/directory.
- View the list of files for the whole BLAST package which is available for various operating systems.
- Choose the one for your operating system. For Windows XP, which will be illustrated here, click blast-2.2.12-ia32-win32.exe and save it at your chosen location. Note that it will be easier to run Blastclust if you save it with a small easy to type and remember path, so for example choose C:\Blast which will be used in this illustration
- Double click on the download file name to download the whole BLAST package to your chosen location. The procedure for this varies slightly with your computer set-up but I did it as follows.
  - Click Save in the pop-up box.
  - Choose where to save the downloaded file via the Save As dialog box that appears after clicking Save in the previous step. Go to My Computer and add a folder called Blast in the root of drive C: with the New folder icon above the folder screen. Double click the Blast folder icon to open the folder. Then click Open (bottom right) to Download BLAST, then click Open Folder to view downloaded file.
- Note that all the files are now in folders in the C:\Blast directory. All the executable program files are in the subdirectory called BIN = binary and detailed HTML text help files are in DOC = documentation.

#### 2. Using Blastclust in Windows

Blastclust is a command line driven program that operates in Windows from the Command Prompt window. It needs a FASTA file as input that contains the sequences for the clone library being analysed. It is easiest to use if the input and output files are in the same directory as the program and if the path name to this directory is short. So in this example I have copied the Blastclust program into C:\Blast\clust. It is also easiest to use if very short names are used for the input and output files. You can then experiment with the program and copy the output files you want to keep elsewhere for further analysis. Instructions for using the program in this way follow.



- Copy the file blastclust from C:\blast\bin to a new folder within C:\Blast called C:\Blast\clust.
- Download the example file 42mbsf\_Arch.fas (see Outline of protocols for details) from <http://www.cardiff.ac.uk/biosi/research/biosoft/Data/> into C:\Blast\clust and rename a copy of this file to in.fas.
- Open the Command Prompt via Start/All Programs/Accessories/Command Prompt
- Type cd C:\Blast\clust, followed by a carriage return [ret], to point the command line to the directory that you will use.
- Type dir [ret] to check that you have the expected files in this directory (N.B. you can do this at any time).
- The command line that you must type has the structure indicated in Fig. 2 (N.B. the full details are much more complicated than this [see HTML help file] but the command line in Fig. 1 is all you need).

**Fig. 2. A simple command line to run the Blastclust program from the Windows Command Prompt**

```
C:\Blast\clust>blastclust -i in.fas -o out95.txt -p F -L 0.9 -S 95
```

1
2
3
4
5

**Key to command line:**

C:\Blast\clust> this is the command line prompt

blastclust is the name of the program being executed

Fields 1-5 provide essential information the program needs, note that they are case sensitive. They mean the following:

1. -i indicates the input file name in.fas in this case, always a FASTA file.
2. -o indicates the output file name out95.txt in this case.
3. -p indicates that it is true or false that the input file contains protein sequences. In this case F is used as the file contains DNA sequences.
4. -L indicates the proportion of the sequence that will be used in the comparison of sequence similarity. The default value of 0.9 is used here. This can be adjusted to allow for errors in sequencing or ambiguous base positions. 1.0 means that all the sequence is used, 0.95 means 95% is used.
5. -S indicates the percentage similarity used to bin the sequences, here we use 95 indicative of the genus level (95% similarity).

- So type the command in Fig. 2 and you will find the output in file out95.txt.
- You can run the Windows notepad program to view the output file from the command line by typing the command:

```
C:\Blast\clust>notepad out95.txt
```

- This output is given in Fig. 3, suitably annotated to show how it is interpreted.
  - Note that there is one bin per line in the output and the sequence names are taken from the FASTA file, so use short names in this file.
  - Note that you should turn Word Wrap off in Notepad or else the number of bins and their contents will not be clear. An alternative is to open the file in Word and ensure that there is a clear gap separating each paragraph (selecting the entire text and toggling with Ctrl-0 does this).



- The output in Fig. 3 shows that the 29 sequences in the example library are binned into 17 groups of sequences, with 7 sequences in bin 1, 4 in bin 2, two sequences in the next three bins and 1 sequence in each of the other bins.

**Fig. 3. Annotated output file from the Blastclust command given in Fig. 2 from the example library used here. Note that the raw output is in the left of the panel and the bin numbers and their contents in the two columns on the right.**

Raw Output	Bin Number	Number of sequences in each bin
42-A7_109F 42-B1_109F 42-A3_109F 42-A8_109F 42-C1_109F 42-A10_109F 42-A9_109F	1	7
42-B4_109F 42-A6_109F 42-C4_109F 42-A4_109F	2	4
42-B6_109F 42-C6_109F	3	2
42-B3_109F 42-B10_109F	4	2
42-A1_109F 42-C8_109F	5	2
42-B8_109F	6	1
42-C3_109F	7	1
42-C5_109F	8	1
42-B2_109F	9	1
42-C9_109F	10	1
42-A5_109F	11	1
42-C7_109F	12	1
42-B7_109F	13	1
42-A2_109F	14	1
42-C10_109F	15	1
42-C2_109F	16	1
42-B9_109F	17	1

Calculating coverage:

Statistics calculated using simple formulae have been used to estimate how well a library covers the sampled population and the estimated number of species, genera and other taxonomic units in the sample (e.g. Wagner & Loy, 2002). These formulae use operational taxonomic units (OTU), which can be defined conveniently using the similarities for species, genus, family/class and phylum given earlier in this section.

Good’s coverage statistic (C) is often used; this is defined by the following formula.

$$C = [1-(n_1/N_{max})]*100$$

Where  $n_1$  = Number of unique OTUs (OTUs that contain only one clone) and  $N_{max}$  = Number of clones in library. Furthermore, the following formula can be used to estimate the number of OTUs ( $N_{eOTU}$ ) in the sample from which the library was derived.

$$N_{eOTU} = (N_{OTU}*100)/C$$

Where  $N_{OTU}$  = the number of OTUs observed in the sample and C is Good’s coverage statistic as defined above.

So from the results in Fig. 3 values for C and  $N_{eOTU}$  can be calculated as follows.

$$C = [1-(12/29)]*100 = 58.6\%$$

$$N_{eOTU} = (17*100)/58.6 = 29$$





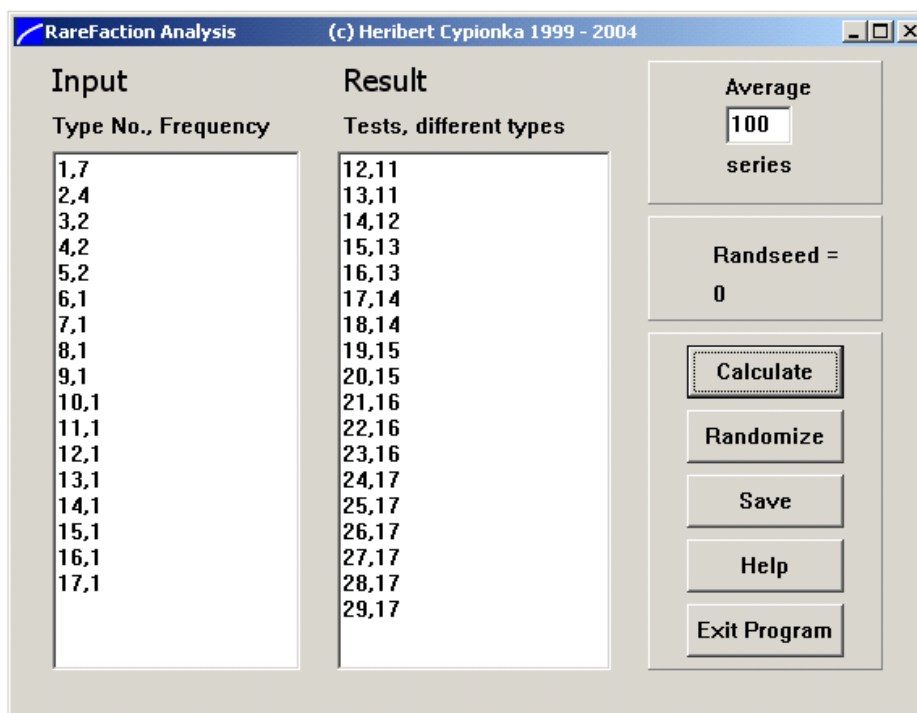
Note however that these statistics and the shape of the rarefaction and species richness curves described below are very sensitive to the value of L used in the Blastclust command. Values of L near to 1.0 give the smallest number of clones in the bins and hence the lowest values of C and the highest values of  $N_{eOTU}$ .

#### Rarefaction curves:

Here we describe the use of a simple program for rarefaction analysis from the University of Oldenberg, Paleomicrobiology Group website. Other programs are also available (e.g. Schloss & Handelsman, 2004). These programs resample the data to give estimated collectors curves for a clone library. The protocol for this is as follows.

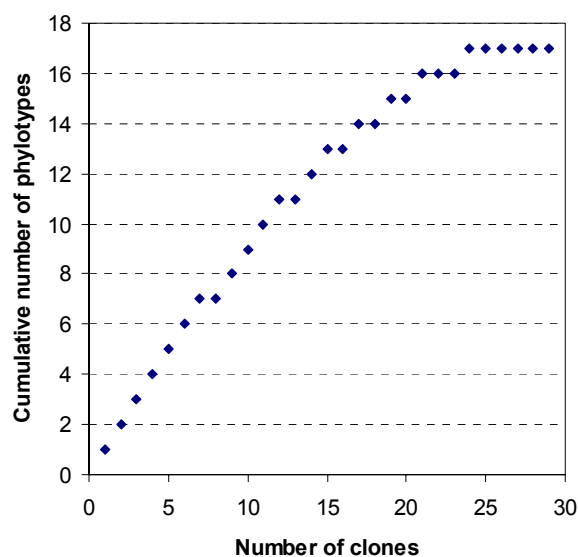
- Visit the website at <http://www.icbm.de/pmbio/> and click Download in the left hand menu bar. Alternatively go direct at <http://www.icbm.de/pmbio/downlist.htm>.
- Download the free rarefaction analysis program into a convenient folder by clicking on RarFac.
- Double click on the icon to run the program, click help in the program dialog box for extra information.
- Type 100 into the Average series box in the top right of the dialog box.
- Put the data into the left hand panel of the dialog box. You already have 10 bins numbered when the program starts followed by commas, you just have to add the numbers of clones in each bin after the comma. Fig. 4 shows this for the test data at 95% similarity (L = 90%; data from the right hand columns of Fig. 3). If there are more than 10 bins then carry on with a similar data input structure until all the data is added.

**Fig. 4. The program window of the RarFac program after rarefaction analysis of the binned data (see Fig. 3) from the example clone library used.**



- Click Calculate to perform the rarefaction analysis and the results are displayed in the right hand panel of the program dialog box (Fig. 4).
- Use the Save command on the right to store the results in a text file.
- You can then modify the input data with the next set of values and save each new set of results in a different text file.
- You can then plot a graph of the data using any graphing package. Below is a brief guide to doing this in MS Excel.
  - Open a blank Excel workbook.
  - Use the File/Open command to open the results text file you have saved from the RarFac program.
  - This will take you into the Text Import Wizard. In step 1 choose delimited as original data type and start importing from row 1. In step 2 choose comma as the delimiter. In step 3 choose general as the format and click finish.
  - Insert a new row above the first line and label the first column “Number of clones” and the second column “Cumulative number of phylotypes”.
  - Highlight the two columns and start the Chart Wizard by clicking the chart icon or with the Insert/Chart menu commands.
  - Go through the 4 steps of the chart wizard choosing X-Y Scatter (without lines) as chart type (step 1), in step 3 remove the chart title and add “Number of clones” in the X-axis box and “Cumulative number of phylotypes” in the Y-axis box, click Finish in step 4 and a graph will be put in the same worksheet as the data.
  - You can then format the chart to your liking by clicking and right clicking on different parts of the chart and adjust things in the dialog boxes that appear. Experimentation is the best way to learn this, one example of the formatted chart is given in Fig. 5.
- The plot of the data shows that, using the Blastclust L value of 90%, the number of clones sequenced are a fair reflection of the number of genus level OTUs in the sample analysed.

**Fig. 5. Example of a rarefaction plot at 95% similarity of the data from Fig. 4 transferred to and plotted in MS Excel, with limited formatting.**





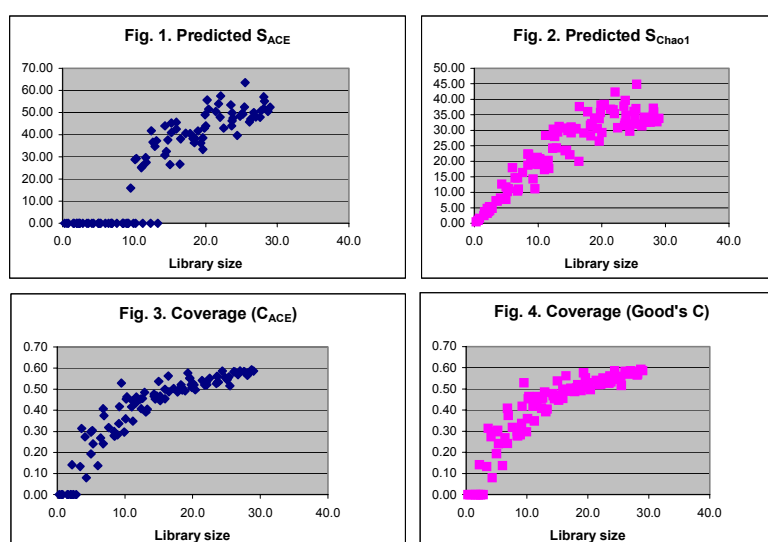
### Species richness curves:

Species richness curves have also been recommended as a way of determining whether the number of phylotypes in a library is large enough. Species richness estimators aim to calculate what the total number of species (or number of other taxonomic groupings) is likely to be. Again for these methods the sequence data must be binned first. Kemp & Aller (2003, 2004) discuss these methods and their relative merits with a variety of clone libraries from many habitats. An inference from their work is that the  $S_{\text{Chao1}}$  species richness estimator is well suited to estimates of phylotype richness from prokaryotic 16S rRNA gene libraries (Kemp & Aller, 2004). Species richness curves often take longer to stabilize with increasing clone number than rarefaction curves. For those who prefer to use a species richness approach Kemp & Aller (2004) provide a web interface for plotting richness estimates against sub-sample size. This interface, form processor, spreadsheet and instructions are found at <http://www.aslo.org/lomethods/free/2004/0114a.html>. The data summary for the test data from this web interface and accompanying spreadsheet is given in Fig. 6.

**Fig. 6. Data summary table and graphs from the species richness analysis provided by the Kemp & Aller (2004) web interface for the test data used here.**

#### DATA SUMMARY

Number of clones in library	29
Number of phylotypes observed	17
Predicted value of $S_{\text{ACE}}$	53.39174
Predicted value of $S_{\text{Chao1}}$	34.86576
Observed phylotypes / predicted $S_{\text{ACE}}$	0.371044
Observed phylotypes / predicted $S_{\text{Chao1}}$	0.53196



### References:

Ashelford, K.E., Chuzhanova N., Fry J.C., Jones A.J., & Weightman A.J. (2005). At least one in twenty 16S rRNA sequence records currently held in public repositories estimated to contain substantial anomalies. *Applied and Environmental Microbiology*, in press.

Huber, T., Faulkner, G., and Hugenholtz, P. (2004). Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. *Bioinformatics* 20: 2317-2319.

Hugenholtz, P. & Huber, T. (2003). Chimeric 16S rDNA sequences of diverse origin are accumulating in the public databases. *International Journal of Systematic and Evolutionary Microbiology* 53:289-293.



Kemp, P.F. & Aller, J.Y. (2003). Bacterial diversity in aquatic and other environments: what 16S rDNA libraries can tell us. *FEMS Microbiology Ecology* 47:161-177.

Kemp, P.F. & Aller, J.Y. (2004). Estimating prokaryotic diversity: When are 16S rDNA libraries large enough? *Limnology and Oceanography: Methods* 2:114-115.

Parkes, R.J., Webster, G., Cragg, B.A., Weightman, A.J., Newberry, C.J., Ferdelman, T.G., Kallmeyer, J., Jorgensen, B.B., Aiello, I.W. & Fry, J.C. (2005). Deep sub-seafloor prokaryotes stimulated at interfaces over geological time. *Nature* 436:390-394.

Schloss, P.D. & Handelsman, J. (2004). Status of the microbial census. *Microbiology and Molecular Biology Reviews* 68:686-691.

Wagner, M & Loy, A. (2002). Bacterial community composition and function in sewage treatment systems. *Current Opinion in Biotechnology* 13:218-227

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## 6.7. Phylogenetic analyses

The aim of the analyses described in this section is to produce phylogenetic trees of unknown sequences generated during your research. By comparing sequences from your unknown prokaryotes and with from known sequences, their phylogenetic relationships can be found and the probable source of the unknown sequences confirmed. Beginners using the software tools described below will find it useful to test their analyses on published trees from reliable sources before working with their own data.

Phylogenetic analyses of molecular sequence data can be broken down into several steps:

- a. Identify a DNA or protein sequence of interest.
- b. Identify other sequences that are related to the sequence of interest.
- c. Align sequences.
- d. Using the subsequent alignment to generate a phylogenetic tree

### Obtaining related sequences by BLAST search:

The **Basic Local Alignment Search Tool (BLAST)** finds regions of local similarity between sequences. The program compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. BLAST can be used to infer functional and evolutionary relationships between sequences as well as help identify members of gene families.

Search the international nucleic acid and protein databases for similar sequences at the following National Center for Biotechnology Information (NCBI) webpage:  
<http://www.ncbi.nlm.nih.gov/BLAST/>.

Paste sequence in Search box in FASTA format. Note, a sequence in FASTA format begins with a single-line description, followed by lines of sequence data. The description line is distinguished from the sequence data by a greater-than (">") symbol in the first column. An example sequence in FASTA format is:

```
>AJ605559 Uncultured candidate division JS1 bacterium partial 16S rRNA gene, clone ChCM-1
TGCAAGTCGAACGAGAAGCTAACTTCTGATTCCCTTCGGGATGATGAGGTTGGCAGAAAGTGGCGA
ACGGGTGAGTAACGCGTGGGTAATCTACCCTGTAAAGTGGGGGATAACCCTCCGAAAGGAGGGCT
AATACCGCATAATATCTTCTCCTCTGAAAGAGGTAAAGATTAAGATGGCCTCCATATTATGCTAT
CGCTTCAGGATGAGCCCGCGTCTTATTAGTTAGTTGGTGGGGTAATGGCCTACCAAGGCGACAATG
GGTAGCCCGTCTGAGAGGATGTACGGCCACACTGGGACTGAGATACGGCCAGACTCCTACGGGA
GGCAGCAGTGGGGAATATTGCGCAATGGGGGAAACCCTGACGCAGCGACGCCGCGTGGATGATG
AAGGCCCTTGGGTTGTAAAATCCTGTTCTGGGGGAAGAAAGCTTAAAGGTCCAATAAACCTTAA
GCCTGACGGTACCCCAAGAGAAAGCTCCGGCTAATTATGTGCCAGCAGCCGCGGTAATACATAAG
GAGCAAGCGTTATCCGGAATTATGGGCGTAAAGAGCTCGTAGGCGGTCTTAAAAGTCAGTTGTG
AAATTATCAGGCTCAACCTGATAAGGTCACCTGAAACTCTAAGAC
```

Choose database to be searched and click BLAST to submit your sequence. After sometime you will get back a response with a list of related sequences (see Fig. 1 and 2). These related sequences can be downloaded and added to sequences within your personal database of sequences which you would like to analyse.



Fig. 1. Top section of a BLAST search results window

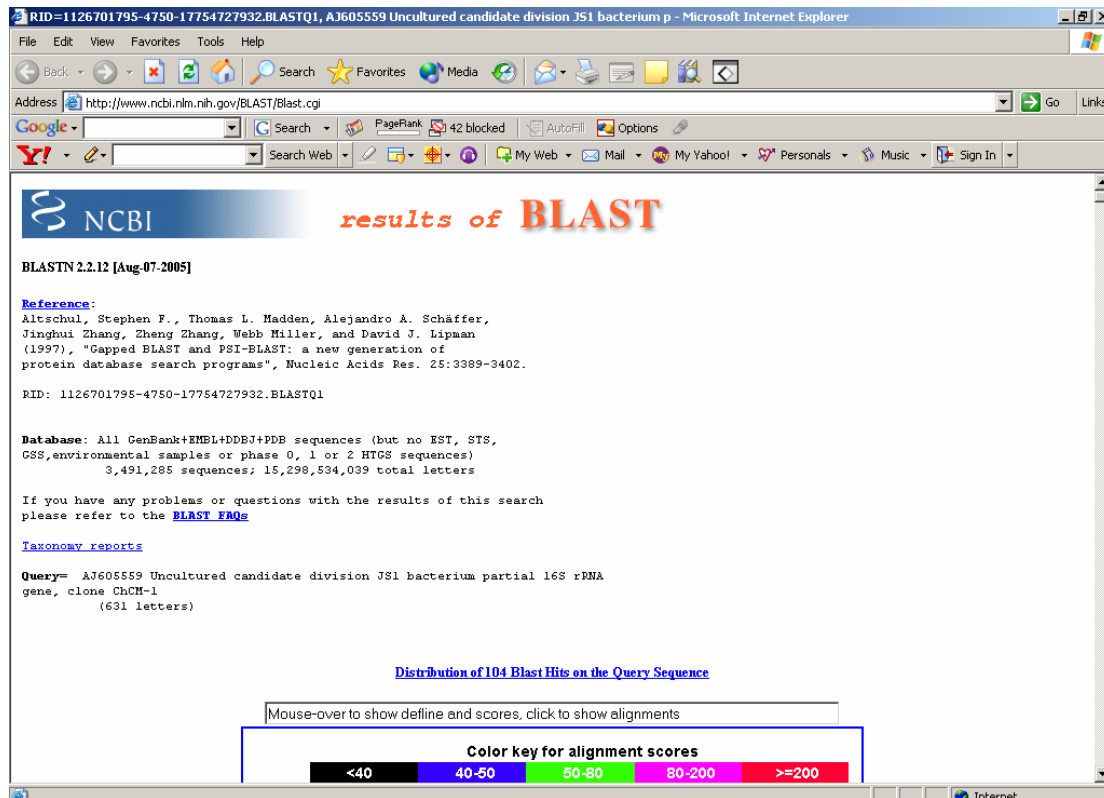
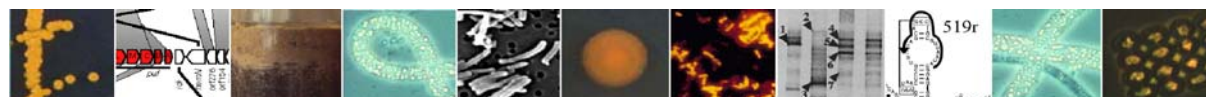
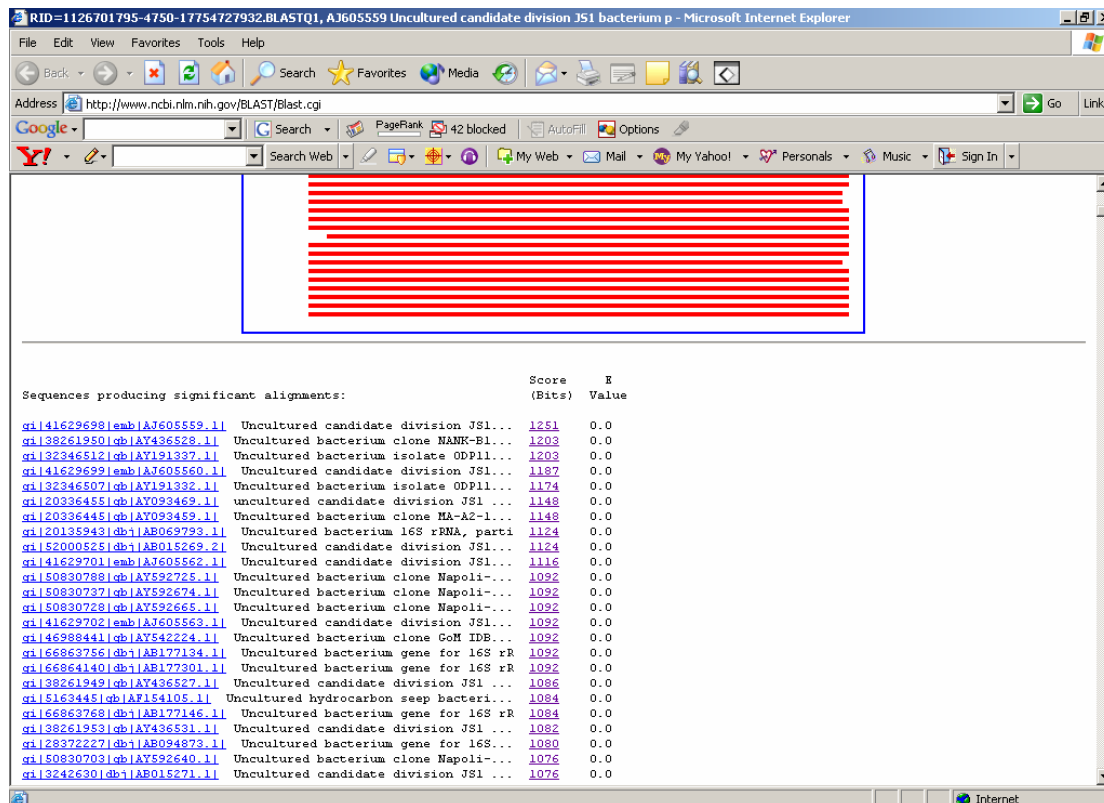


Fig. 2. Lower section of a BLAST search results window

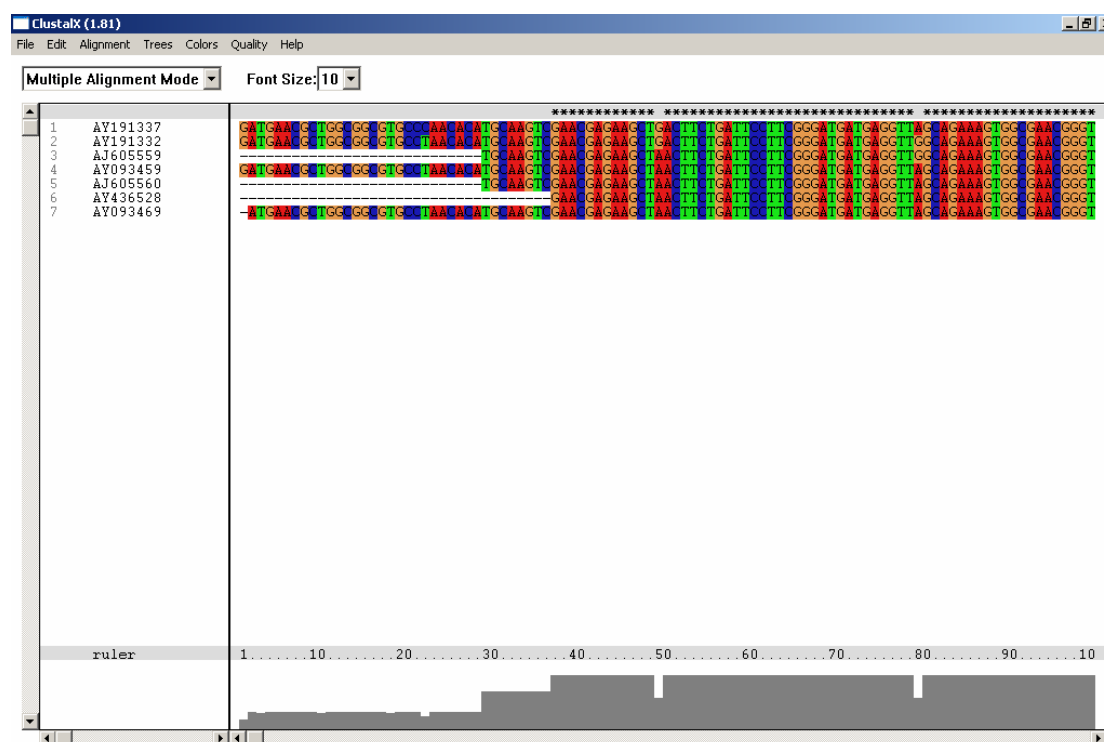


## Alignment:

ClustalX (Thompson *et al.*, 1997) is a multiple alignment program providing alignment files that can be used by other programs. It also displays alignments in ways that allows recognising regions of high similarity easy. Available to download for free at <http://bips.u-strasbg.fr/fr/Documentation/ClustalX/>.

Files can be imported easily in several formats (e.g. FASTA format) and aligned (see Hall, 2001) by pairwise alignments to create a guide tree which it uses to create a multiple alignment (Fig. 3).

**Fig. 3. Alignment of the first part of seven aligned sequences displayed in ClustalX**



The above multiple alignment (Fig. 3) can then be manually edited and aligned using other alignments programs such as BioEdit (Hall, 1999; see Fig. 4). BioEdit can be downloaded from <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>.

## Phylogenetic analysis:

This section is a brief introduction to methods of phylogenetic analysis with emphasis on implementation of some computer software to construct phylogenies. It should be noted that no one method is the best for all circumstances. The method of choice depends both on the user and what they want to learn and the size and complexity of the data set. It is highly recommended that the beginner should read further molecular evolution and systematic texts (e.g. Li, 1997; Graur and Li, 2000) before they attempt phylogenetic analysis.



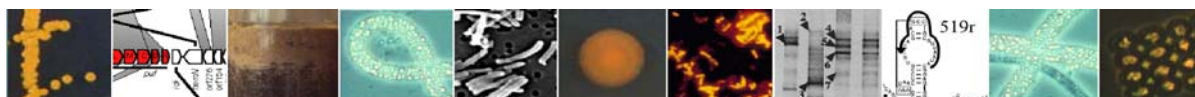
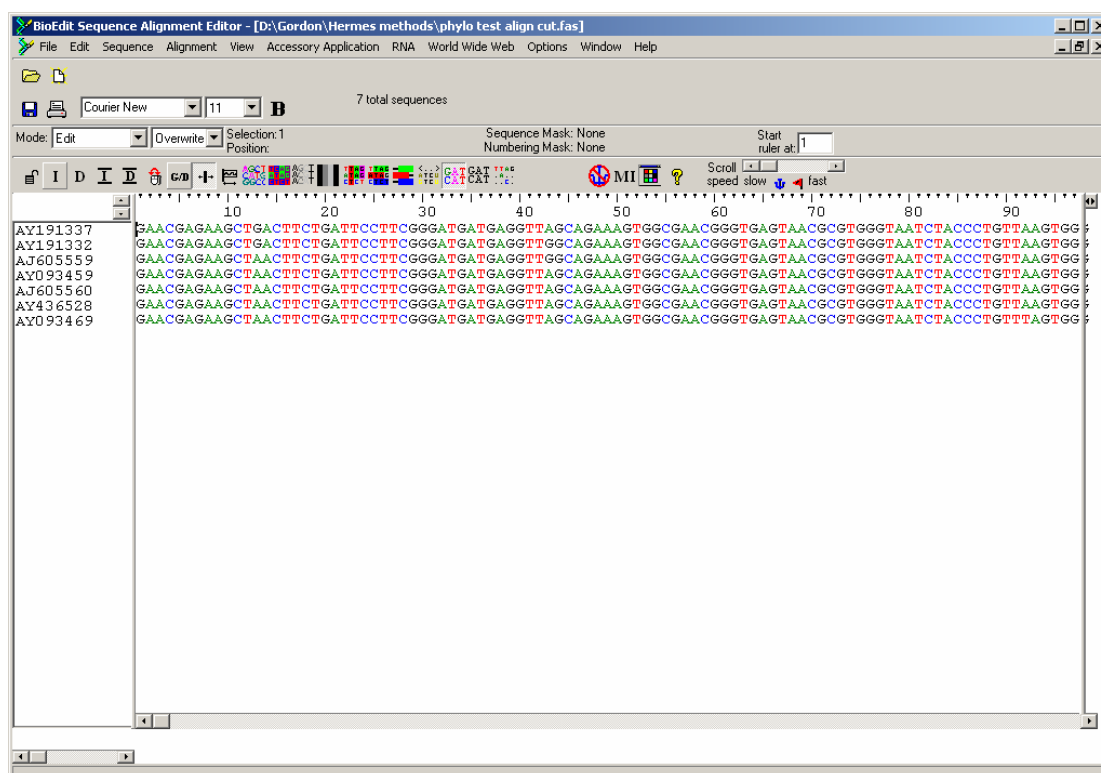
There are currently 4 primary methods of constructing phylogenetic trees from protein and nucleic acid data sets and here we will concentrate on distance methods.

1. Distance methods, Neighbor-Joining (NJ) is the favoured method.
2. Maximum parsimony (MP).
3. Maximum likelihood (ML).
4. Bayesian (BAY).

MEGA is an excellent software tool for quickly inferring phylogenetic trees for both nucleotide and protein sequences. This program also allows automatic and manual sequence alignment, mining web-based databases, estimating rates of molecular evolution, and testing evolutionary hypotheses. MEGA can be downloaded for free at <http://www.megasoftware.net/index.html>.

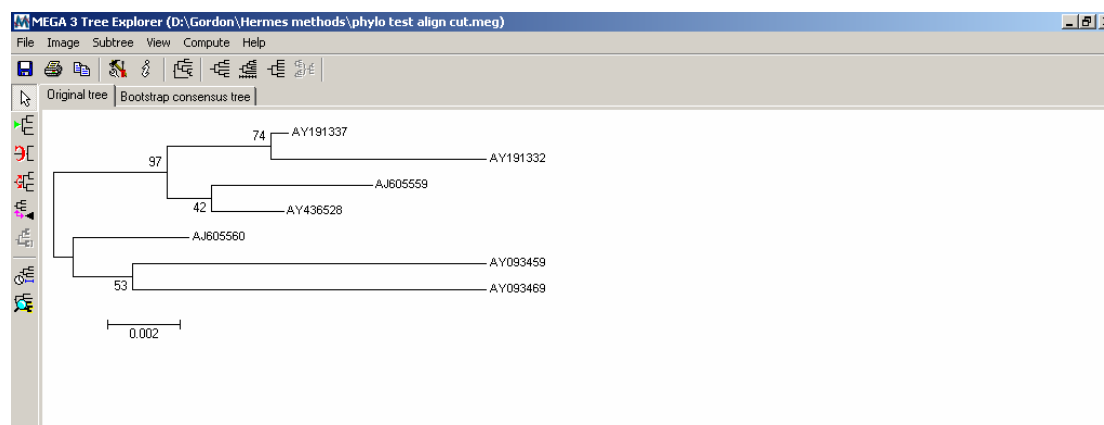
The above ClustalX alignment (Fig. 3) was imported into MEGA after manually editing in BioEdit to ensure all sequences were the same sequence length (Fig. 4). The aligned data set was used to construct a NJ tree with the Jukes and Cantor correction algorithm with bootstrapping (1000 replicates) as a test of phylogeny (see Fig. 5).

**Fig. 4. Alignment displayed in BioEdit after editing to the same sequence length**





**Fig. 5. Bootstrapped tree of the seven sequences from Fig. 4 constructed and displayed in Mega**



However, more robust distance models other than Jukes and Cantor (JC) are also used for analysis of nucleic acid sequences. JC assumes that all sites can change at the same rate. Whereas, LogDet/paralinear distances deal with unequal base frequencies in different sequences (Lake, 1994). Although, LogDet/paralinear distances does assume that all sites can change, it is therefore important to calculate and remove the number of sites that cannot change (invariable sites) (Lockhart *et al.*, 1996). Such analyses can be implemented in the excellent inexpensive computer software package PAUP (Swofford, 1998). See website at <http://paup.csit.fsu.edu/>

Additionally it is also recommended to use the Linux based software ARB: <http://www2.mikro.biologie.tu-muenchen.de/arb/>. The ARB software is a graphically oriented package comprising various tools for sequence database handling and data analysis. A central database of processed (aligned) sequences and any type of additional data linked to the respective sequence entries is structured according to phylogeny or other user defined criteria.

#### References:

- Graur, D. and Li, W-H. (2000). *Fundamentals of Molecular Evolution*. Sinauer Associates Inc, Sunderland MA.
- Hall, B.G. (2001). *Phylogenetic Trees Made Easy: A How-to Manual for Molecular Biologists*. Sinauer Associates Inc, Sunderland MA.
- Hall, T.A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symp. Ser.* 41, 95-98.
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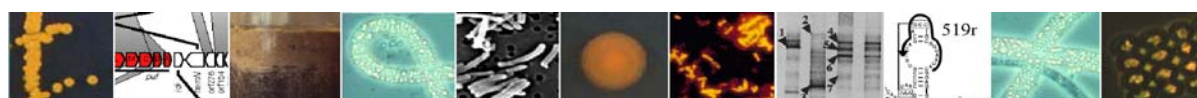
Lockhart, P. J., Larkum, A.W.D., Steel, M.A., Waddel, P.J. & Penny, D. (1996). Evolution of chlorophyll and bacteriochlorophyll: the problem of invariant sites in sequence analysis. *Proc. Natl. Acad. Sci. USA* 93, 1930–1934.

Thompson, J.D., Gibson, T.J., Plewniak, F., Jeanmougin, F. & Higgins, D.G. (1997). The ClustalX-Windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucl. Acids Res.* 25, 4876-4882.

Swofford, D. L. (1998). PAUP\*: Phylogenetic Analysis Using Parsimony (\*and other methods). Sinauer Associates, Sunderland, Mass.

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## 7. Molecular Methods for Investigating Diversity: Profiling Methods

### 7.1. Denaturing gradient gel electrophoresis (DGGE) for 16S rRNA genes

The aim of DGGE is to separate DNA fragments based on sequence composition. So in microbial ecology PCR amplified products of 16S rRNA genes from a mixed community of prokaryotes can be separated as distinct bands on a denaturing gradient gel with this approach. Each band is normally derived from the 16S rRNA genes of a single phylotype within the community sampled. DGGE is ideal for comparing profiles of prokaryotic diversity from different samples because the bands in several lanes can easily be compared. DGGE using the method described below can be used on products obtained by direct amplification from an environment or by nested PCR using primers producing a large product in the first round of amplification. The Muyzer *et al.* (1993) primers described below amplify a wide range of bacteria when checked for efficacy by the Oligocheck program described earlier. There are also suitable primers for amplification and DGGE of *Archaea* not described here. See also Schafer & Muyzer (2001) for a good general review of DGGE methodology.

#### PCR amplification of 16S rDNA fragments:

Primers complementary to conserved regions were used to amplify a 194-bp fragment of the 16S rDNA corresponding to nucleotides GC-341F to 534R in *Escherichia coli* sequence (Muyzer *et al.*, 1993). The nucleotide sequence of the forward primer, which is specific for eubacteria (5'-CCT ACG GGA GGC AGC AG-3'), contains at its 5' end a 40 - base GC clamp (5'-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3') to stabilize the melting behavior of the DNA fragments (Sheffield *et al.*, 1989). The universal consensus sequence (5'-ATT ACC GCG GCT GCT GG-3') was used as a reverse primer. A typical 50- $\mu$ l PCR mixture contained 20 pmol of each forward and reverse primer, 250  $\mu$ M (each) of deoxynucleotide triphosphates, 1 U of Biotaq DNA polymerase (Bioline), PCR buffer (Bioline), 1.5 mM MgCl<sub>2</sub>, and approximately 250 ng of template DNA. PCR was performed using a MJ Research PTC-200 machine and analysed on a 1.5 % agarose gel. The cycles used were as follows: 1 cycle at 95 °C for 10 min; 19 cycles at 95 °C for 1 min, 65 °C for 45 s, and 72 °C for 45 s; 19 cycles at 95 °C for 1 min, 55 °C for 45 s, and 72 °C for 45 s; followed by 1 cycle at 72 °C for 10 min. To increase the specificity of the amplification and to reduce the formation of spurious products, a "touchdown" PCR was performed. A touchdown PCR is a PCR in which the annealing temperature is set 10 °C above the expected annealing temperature (65 °C) and decreased by 0.5 °C every second cycle until a touchdown of 55 °C (Röllerke *et al.*, 1996). All PCR products (5 $\mu$ l volumes) were analysed by electrophoresis in 2% (w/v) agarose gels before DGGE analysis was performed.

#### Analysis of PCR products by DGGE:

DGGE was performed with Dcode Universal Mutation Detection System (Bio-Rad Laboratories) which is based on the Bio-Rad Protean II system and PCR products were separated using this system. 1 mm-thick (16 $\times$ 10 cm glass plates) polyacrylamide gels made of 10% (w/v) polyacrylamide gradient [acrylogel 2.6 solution; acrylamide-*N,N'*-methylenebisacrylamide (37.5:1); BDH Laboratory Supplies, Poole, UK]. DGGE gels contained a 30 to 60% denaturant gradient of urea and formamide solution increasing in the



direction of electrophoresis [100% denaturant is defined as 7 M urea with 40% (v/v) formamide]. Gels were polymerized with ammonium persulphate (APS) (Fisher Scientific, UK) and *N,N,N',N'*-tetramethyl-ethylenediamine (TEMED) (Sigma, UK) according to manufacturer's instructions. Gels were poured with the aid of a Model 475 gradient delivery system (Bio-Rad, Hercules, CA, U.S.A.) and prepared with, and electrophoresed in, 1× TAE buffer (pH 8.0; 40 mM Tris base, 20 mM acetic acid, 1 mM EDTA) and run at 60 °C for 5 h at 200 V. After electrophoresis, the polyacrylamide gels were stained for 20 min in 1× TAE containing SYBRGold nucleic acid gel stain 10000× concentrate in DMSO (Molecular Probes, Leiden, The Netherlands) and the UV gel image was captured by using a Gene Genius Bio Imaging System (Syngene, Cambridge, UK).

#### Reference:

Muyzer, G., de Waal, E.C. & Uitterlinden, A.G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59**, 695-700.

Sheffield, V.C., Cox, D.R., Lerman, L.S. & Myers, R.M. (1989) Attachment of a 40-base pair G+C rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single-base changes. *Proc Natl Acad Sci USA* **86**, 232-236.

Röllerke, S., Muyzer, G., Wawer, C., Wanner, G. & Lubitz, W. (1996) Identification of bacteria in a biodegraded wall painting by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl Environ Microbiol* **62**, 2059-2065.

Schafer, H., & G. Muyzer. (2001). Denaturing gel electrophoresis in marine microbial ecology, p. 425-468. *In* J. H. Paul (ed.), *Marine Microbiology*, vol. 30. Academic Press, San Diego.

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### 7.1.1. Sequencing bands from DGGE gels

Removing and sequencing DGGE bands from gels enables identification of the prokaryote from which the band was derived. In most cases the sequence will be short and so accurate identification by BLAST or phylogenetic analysis is not reliable. However, a good idea of the phylum and perhaps class/family of the prokaryote from which the sequence came can be obtained. The method described below is from Schafer & Muyzer (2001).

#### Method:

15. Transfer the DGGE gel to a UV-table.
16. Wipe a scalpel blade with ethanol and switch on UV-table, cut out band of interest and pick it up with the blade.
17. Immediately switch off the UV-source to minimize the damage to the DNA bands in the gel. Always cut the faintest bands out first and work quickly so the gel is exposed to the UV light for as little time as possible.
18. Transfer the gel piece to the labelled PCR tube.
19. Continue excising bands as described in steps 2-4, until all bands have been excised.
20. Add 100  $\mu$ l of DNase/RNase free water and leave for 20-30 min to wash the band.
21. Discard water and dry in the air for 5-10 min.
22. Mashed up the gel with a clean pipette tip.
23. Add 15  $\mu$ l DNase/RNase free water to bright bands or 10  $\mu$ l to faint bands.
24. Incubate at 4  $^{\circ}$ C overnight.
25. Use water from the supernatant as template for re-amplification with same primes as for the PCR for DGGE, store the remainder at -20  $^{\circ}$ C.
26. Check the PCR product by DGGE to make sure it is the proper band and to see it is single band.
27. The re-amplified products are purified with Microcon filter (YM-50) as described at DNA extraction method and sequenced with 341F or 534R primer.

#### Reference:

Schafer, H., & G. Muyzer. (2001). Denaturing gel electrophoresis in marine microbial ecology, p. 425-468. *In* J. H. Paul (ed.), *Marine Microbiology*, vol. 30. Academic Press, San Diego.

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### 7.1.2. Analysis of DGGE profile data

It is the aim of this section to describe how DGGE profile data can be analysed statistically to provide maximum information and value.

#### Background:

DGGE is an ideal technique for producing profiles of diversity across a number of sites, depths or times. This approach has been used for time series of samples in water columns, for depth series in sediment cores and for many other purposes, where temporal or spatial comparisons are required. Scientists then analyse these profiles in a variety of ways. For example, selected bands are sequenced and the resulting phylotypes are identified and compared along the profile. Bands can also be compared visually and major differences commented upon. However, these approaches are generally unsatisfactory because they are essentially qualitative and are open to various interpretations by different scientists. So in principle if profiles are to be interpreted a more quantitative approach is preferred.

Quantitative approaches to DGGE gel analysis have been described before (e.g. Schafer & Muyzer, 2001). However, here I will give a brief explanation of how we do this type of analysis in Cardiff. It should be pointed out that there is no perfect solution to such analyses, as there are lots of steps which involve qualitative judgements and subjective decisions to be made. This means that two people will rarely get exactly the same results. However, if the analysis and the data are robust similar overall conclusions should be drawn. If the analysis leads to sensible conclusions and different approaches lead to the same overall result, then the analysis is likely to be correct.

#### Considerations when running the DGGE gel for analysis:

- Run all the samples to be compared on one gel.
- Run marker lanes at least on each side of the gel and preferably in the middle as well.
- Only analyse gels that have run straight and as evenly as possible across the gel.
- Use reasonably high contrast gel images for the analysis if at all possible.
- Select spatial or temporal samples for your profiles for which you have other information if at all possible, as this makes it easier to interpret the gel analysis in terms of other variables.

#### Scoring the bands on a gel:

Bands can be scored either fully quantitatively, semi-quantitatively or by presence/absence as follows.

- Fully quantitative scoring. Scan the lanes in the gel image with a densitometer to obtain a quantitative trace of absorption readings taken over very small spatial intervals.
- Presence/absence scoring. Here you need to decide whether a particular band is present or absent across the whole gel for each lane, score 1 for presence and 0 for absence.
- Semi-quantitative scoring. The same as presence/absence except you can assign a relative intensity score to each band scored. So, for example, score 5 for a very bright band (the brightest on the gel) and 0 for no band at all. To do this it is best to have a





set of standards with which to compare your bands. A range of 0-3 is easy, but 0-5 is possible.

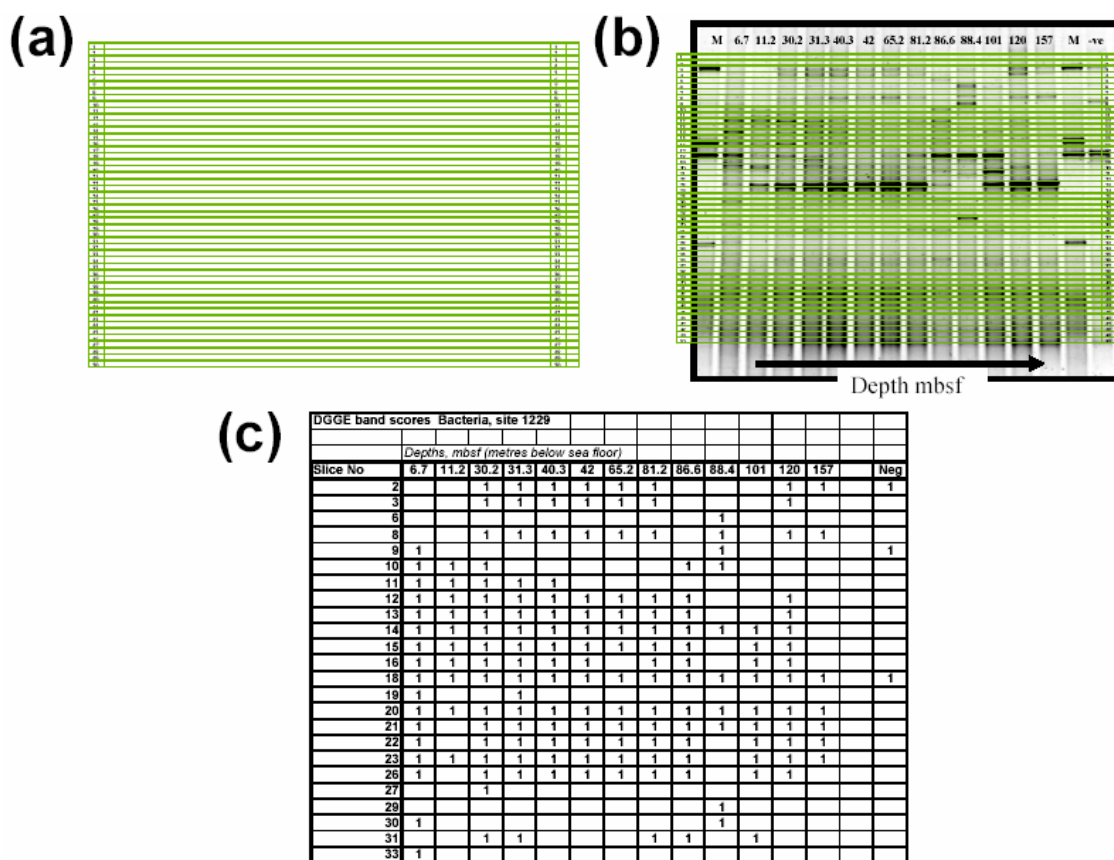
Our experience at Cardiff with several such studies indicates that presence/absence gives results that are generally easiest to interpret. So this is the approach I will describe here. I will use one DGGE gel image as an example. This is for *Bacteria* from 13 sediment samples between 6.7 and 157 metres below the sediment surface from site 1229 from Ocean Drilling Program Leg 201 taken from the Pacific Ocean near the coast of Peru (Parkes *et al.*, 2005).

Protocol for presence/absence band scoring:

This can be done satisfactorily in MS Powerpoint and is illustrated in Fig. 1 and described below.

- First make a grid for identifying the bands, the grid needs to be transparent but have lines in a colour through which you can see the bands it also helps to have numbers down the side so that you can score the bands easily. My grid was prepared in MS

**Fig. 1. Illustration of the steps in scoring bands on DGGE gel images as present or absent. (a) Green coloured grid, (b) grid superimposed on DGGE gel image adjusted for contrast and brightness and (c) resulting score matrix (1 = band present, blank = band absent). Note that the bands in the negative control lane are omitted from the analysis and that the picture seen in MS Powerpoint has far more detail than in this image used here for illustration only.**



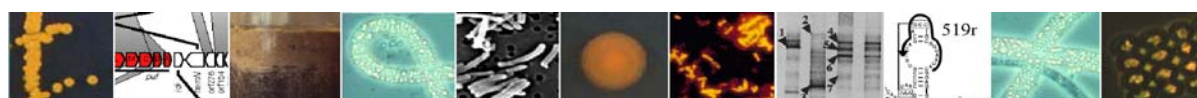
Word using the table generating features of this package it had fifty slices. The grid lines were coloured bright green (use Format/Borders and shading command) and the spaces between the grid lines are empty.

- Copy the grid from Word to Powerpoint using the Edit/Paste Special command. Experiment with different formats for copying until the grid is transparent. Once done keep this safe.
- Keep the original grid on one page of a Powerpoint file.
- Put your original DGGE gel image into another page of Powerpoint and copy it into a third page. You will work on the image in the third page so that you retain the original image unaltered.
- Copy the grid over the DGGE gel image in the third page.
- Adjust the grid via the Drawing toolbar commands so that the gap between each set of lines is about equal to the thickness of a typical DGGE band.
- Adjust the contrast and brightness of the gel image so that you can easily see bands that are present but do not have difficulty detecting whether a band is absent. Note that this takes a little practice and experimentation before you can score gels consistently.
  - Note that this must be done separately for each gel image as the degree of contrast/brightness adjustment varies greatly between gels.
- Record the presence absence data in a matrix. You can use MS Excel for this, for my example you need the depths to be the columns and the band positions to be the rows (see Fig. 1).
  - Note in this example there was so little DNA in the DNA extraction that an *Escherichia coli* band in all the gel lanes could not be avoided (see Webster *et al.*, 2003 for explanation). So equivalent band positions in the depth lanes for bands in the negative control lane with the nested PCR used had their scores removed before analysis. This removed the effect of *E. coli* contamination from the *taq* polymerase used in the PCR amplification.
  - Note also bands below the lowest marker lane were not scored as they were diffuse and inconsistent.

#### Analysing the presence/absence matrix:

You need some graphical way of viewing the relationships between different lanes on the DGGE gel. This can be done either by either a clustering approach or an ordination approach (e.g. principal component analysis, factor analysis). Clustering approaches produce dendrograms like phylogenetic trees and ordination approaches produce two or three dimensional plots. Some methods give both types of output (e.g. multi-dimensional scaling). It is beyond the scope of this protocol to describe these in details but they are explained in Fry (1993) and other multivariate statistical books. I have found both useful, but here I will briefly describe an ordination approach, with the statistical package Minitab 14.2.

- Copy the data matrix into Minitab.
  - Note replace the blanks in Fig. 1c with zeros and remove rows with 1 in the negative control lane, before analysis.
- Use the PCA command to do principal component analysis
  - Use the correlation matrix option
  - Extract no more than 5 components
- The output that you get gives eigenvalues, proportions and cumulative proportions for each component.



- In this case the first two components accounted for 70% of the variation in the band diversity of the lanes and the first 3 components accounted for 79% of the diversity.
- Scores/loadings for each depth are also given for each of the extracted components. These are values for new variables produced by the analysis (principal component 1 etc..., or often PC1), which between then explain much of the diversity.
- The scores/loadings for each component for each depth can then be used as new diversity variables to compare with other variables obtained for those depths (e.g. in this example total counts, activity measurements, geochemistry). Such comparisons can be done with statistical approaches such as multiple regression (see Fry, 1993) and for details of the results for this example gel see Parkes et al. (2005).

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## 7.2. Benthic diversity profiling of *Bacteria* using ARISA

Automated Ribosomal Intergenic Spacer Analysis (ARISA) cultivation-independent method allows a quick and reliable evaluation of bacterial richness (as numbers of phylotypes or OTU) and community structure in aquatic environments (Fisher & Triplett, 1999). Compared to other fingerprinting methods, such as the T-RFLP, which are based on the analysis of the 16S rRNA gene, ARISA is expected to provide a more accurate estimation of phylotype richness and community composition, due to the higher resolution power of the 16S-23S ITS region.

Undisturbed sediment samples are typically collected using a mini- or a multi-corer. A subsample (ca. 2-5 cm<sup>3</sup>) is transferred into sterile Falcon test tubes (50 ml) using an aseptic spatula or either a 10-ml sterile syringe from which the tip has been removed. The tube is then frozen at -20°C (preferably at -80°C) and kept stored until DNA extraction.

The DNA is extracted from a 1-g aliquot of sediment using one the available protocols of DNA recovery. Commercial kits for fast DNA recovery from soil samples are also available (e.g., UltraClean Soil DNA Isolation kit; MoBio Laboratories Inc., California, USA; Luna *et al.*, 2005).

The purity of the DNA extracts is checked by measuring the 260/280 nm absorbance ratio. Extracted DNA is quantified spectrophotometrically or, preferably, spectrofluorimetrically using Sybr Green I (Corinaldesi *et al.*, 2005) or other fluorescent molecules.

The DNA extracted is then amplified using universal bacterial primers 16S-1392F (5'-GYACACACCGCCCGT-3') and 23S-125R (5'-GGGTTBCCCCATTTCRG-3'), which amplify the entire ITS1 region in the rRNA operon plus ca. 282 bases of the 16S and 23S rRNA (Hewson & Fuhrman 2004). One of the primers (generally the 23S-125R) must be fluorescently labelled at the 5' end with a phosphoramidite dye, such as FAM (6-carboxyfluorescein), HEX (6-carboxyhexafluorescein) or similar. PCR reactions are performed in a final volume of 50 µl using the MasterTaq® kit (Eppendorf), which helps reducing the effects of PCR-inhibiting contaminants, such as humic acids, which can be co-extracted with the DNA. Reactions can be conducted accordingly with the kit procedure, by preparing all reagents as a master-mix before addition of template DNA (in a volume of 1 µl, containing ca. 1 - 5 ng of DNA).

PCR is generally run for 30 PCR-cycles, consisting of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, preceded by 3 minutes of denaturation at 94°C and followed by a final extension of 10 minutes at 72°C. For each PCR reaction, a negative control (containing the reaction mixture without the DNA template) and a positive control (containing the reaction mixture plus genomic DNA of *Escherichia coli*) are run. After amplification, 5-µl aliquots of the PCR-products are checked for quality using electrophoresis on high-resolution agarose gel.

Two independent PCR replicates for each sediment sample are pooled together, with the aim of minimizing stochastic PCR biases (Polz & Cavanaugh, 1998). The pooled PCR products are purified using the Wizard PCR clean-up system (Promega, Madison, Wis.) or similar. Purified DNA is eluted in 50 µl and then quantified spectrofluorimetrically as described above. For capillary analysis, which is generally performed using an ABI Prism 3100



Genetic Analyzer (Applied Biosystems), about 5 ng of amplicon (in a volume of 1  $\mu$ l) are mixed with 14  $\mu$ l of internal size standard (GS2500-ROX; Applied Biosystems) in deionised formamide, then denatured at 94°C for 2 minutes and immediately chilled into ice. Fragments are analysed in GeneScan mode using 47cm x 50  $\mu$ m capillaries, POP-4 polymer, 40seconds injection time, 15kV injection voltage, 15kV run voltage and 60°C capillary temperature (parameters can be varied according to fluorescence signal intensity). ARISA fragments in the range 390 - 1400 bp are then determined using Genescan analytical software 2.02 (ABI) or similar.

For the analysis and interpretation of ARISA profiles, Genescan numerical outputs from each electropherogram are then extracted and transferred to Microsoft Excel. From each profile, peaks which are less than 1.5 bp apart from a larger peak are considered “shoulders” peaks and omitted from the analysis. Furthermore, peaks which are not present in both replicates (i.e., “non-producible” peaks) are considered as artefacts and removed. For discriminating bacterial OTU (Operational Taxonomic Units) from baseline fluorescence, the total integrated height of each profile (by summing the height of each peak in the T-RFLP electropherogram) has to be calculated. Then the relative percentage of each peak is calculated (as a percentage over the total integrated height) and only peaks accounting for more than 0.16% of the total integrated height are considered to be OTU.

After data processing, bacterial richness is defined as the number of OTUs. Bacterial diversity indices (such as the Shannon–Wiener index and others) or the Pielou index (evenness) can be calculated assuming that the number of OTU represents the species number and the peak height associated to each peak represents the relative abundance of each bacterial OTU. Data from ARISA profiles can also be utilized for analyses of  $\beta$ -diversity and comparisons of samples in terms of species composition and community structure.

## References.

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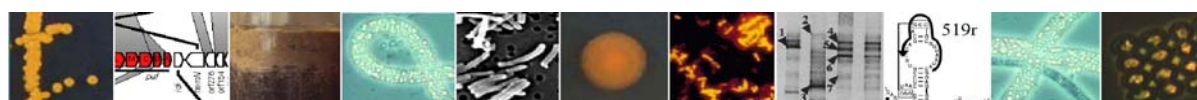
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### 7.3. Terminal restriction fragment length polymorphism (T-RFLP) analysis

#### 7.3.1. Benthic diversity profiling of *Bacteria* using T-RFLP

This fingerprinting method is based on a cultivation-independent, molecular approach, allowing a quick and reliable evaluation of bacterial richness (as number of ribotypes, also referred to as phylotypes or OTU) and community structure in marine sediments (Liu *et al.*, 1997).

Undisturbed sediment samples are collected using a mini or multi-corer or a ROV. A sub-sample (ca. 5 cm<sup>3</sup>) is transferred into sterile Falcon test tubes (50 ml) using an aseptic spatula or either a 10-ml sterile syringe from which the tip has been removed. The tube is then frozen at -20°C (preferably at -80°C) and kept stored until DNA extraction.

In the laboratory, sediment DNA is extracted from a 1-g aliquot of sediment using one of the available protocols of DNA recovery. It is possible to utilise commercial kits for fast DNA recovery from soil samples, such as the UltraClean Soil DNA Isolation kit (MoBio Laboratoires Inc., California, USA). This kit, indeed, provides similar results of diversity than other time-consuming laboratory protocols (Luna *et al.*, 2005).

The purity of the DNA extracts is then checked by measuring the 260/280 nm absorbance ratio with a spectrophotometer. Extracted DNA is quantified spectrophotometrically or, preferably, spectrofluorimetrically using Sybr Green I (Corinaldesi *et al.*, 2005) or other fluorescent molecules.

The 16S rRNA eubacterial gene is amplified using universal eubacterial primers, such as the 27F and 1492R (Lane 1991). For T-RFLP analyses, one of the primers (generally the 27F forward) must be fluorescently labelled at the 5' end with a phosphoramidite dye, such as FAM (6-carboxyfluorescein), HEX (6-carboxyhexafluorescein) or similar. PCR reactions are performed in a final volume of 50 µl using the MasterTaq® kit (Eppendorf), which helps reducing the effects of PCR-inhibiting contaminants, such as humic acids, which can be co-extracted with the DNA. Reactions can be conducted accordingly to the kit procedure, by preparing all reagents as a master-mix before addition of template DNA (in a volume of 1 µl, containing ca. 1 - 5 ng of DNA).

PCR is generally run for 30 PCR-cycles, consisting of 94°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes, preceded by 3 minutes of denaturation at 94°C and followed by a final extension of 10 minutes at 72°C. For each PCR reaction, a negative control (containing the reaction mixture but no DNA template) and a positive control (containing the reaction mixture plus genomic DNA of *Escherichia coli*) are run. After amplification, 5-µl aliquots of the PCR-products are checked on agarose-TBE gel (1%), containing ethidium bromide for DNA staining and visualization. The agarose run also allows checking the presence of unwanted or unspecific PCR products.

Two independent, PCR replicates for each sediment sample are pooled together, to help minimize stochastic PCR biases (Polz & Cavanaugh, 1998). The pooled PCR products are purified using the Wizard PCR clean-up system (Promega, Madison, Wis.) or similar. If unspecific PCR products are not observed, it is possible to purify directly the PCR product





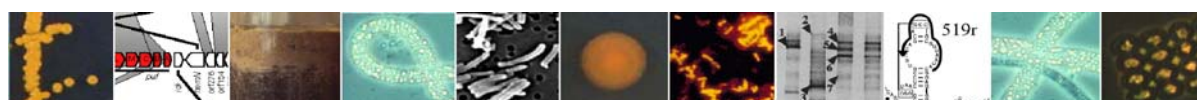
from the test tube. If unspecific products are observed, PCR products are separated by electrophoresis, the band of proper size is excised and then purified from the gel, using the same clean-up system or similar. Purified DNA is eluted in 50  $\mu$ l and then quantified spectrofluorimetrically as described above.

About 50 ng of purified 16S rDNA amplicons are digested in duplicate reactions containing 10 U of the enzyme Rsa I or Alu I (Promega, Madison, Wis.). Reactions are conducted at 37°C for 3 hours. Restriction digestions are then stopped by incubating at 65°C for 20 minutes and the samples stored at -20°C until capillary analysis is performed using an ABI Prism 3100 Genetic Analyzer (Applied Biosystems). Before capillary analysis, 1  $\mu$ l (or more, if signal intensity is too low) of each digest is mixed with 14  $\mu$ l of internal size standard (G1000-ROX; Applied Biosystems) in deionised formamide, then denatured at 94°C for 2 minutes and immediately chilled into ice. Fragments are analysed in GeneScan mode using 47cm x 50 $\mu$ m capillaries, POP-4 polymer (Perkin-Elmer), 40seconds injection time, 15kV injection voltage, 15kV run voltage, and 60°C capillary temperature (parameters can be varied according to fluorescence signal intensity). Terminal restriction fragment sizes between 47 and 946 bp are determined using Genescan analytical software 2.02 (ABI) or similar.

For analysis and interpretation of T-RFLP profiles, Genescan numerical outputs from each electropherogram are extracted and transferred to Microsoft Excel. From each profile, peaks which are less than 1.5 bp apart from a larger peak (“shoulders” peaks) are eliminated. As a subsequent step, peaks which are not present in both the two replicates (irreproducible peaks) are considered to be artefacts and thus removed. Then, for discriminating bacterial OTU (Operational Taxonomic Units) from the fluorescence baseline, several approach can be utilised. One consists in calculating the total integrated height of each profile (by summing the height of each peak in the T-RFLP electropherogram), then the relative percentage of each peak is calculated (as a percentage over the total integrated height) and only peaks accounting for at least 0.17% of the total integrated height are considered to be OTU.

Bacterial richness is expressed by the number of all OTU left after data processing. A typical one gram - sediment sample will yield several tens of OTUs. Bacterial diversity indices (such as the Shannon–Wiener index and others) or the Pielou index (evenness or equitability) can be calculated, assuming that the number of OTU represents the species number and the peak height associated to each peak represents the relative abundance of each bacterial ribotype or OTU. For calculating diversity indices it is possible to utilise the PRIMER software (Plymouth Marine Laboratory; Clarke, 1993).

Data from T-RFLP profiles can also be utilized for analyses of  $\beta$ -diversity and comparisons of samples in terms of species composition and community structure. To do this, the PRIMER software can be used, which allow performing the ANOSIM analysis (which tests the statistical significance in the similarity between groups of community samples), the SIMPER analysis (which examines the contribution of each species to the average Bray-Curtis dissimilarity between groups of samples) and the MDS analysis (which performs a non-metric Multi Dimensional Scaling ordination).



References:

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### 7.3.2. T-RFLP 16S rRNA gene based methods for methanogens

This is a protocol for T-RFLP (terminal restriction fragment length polymorphism) analysis using ABI-3100 using methods for methanogens as the example (Banning *et al.*, 2005). The methods described here are adapted from the Gruntzig *et al.* protocol on the RDPII website at [rdp8.cme.msu.edu/html/t-rflp\\_jul02.html](http://rdp8.cme.msu.edu/html/t-rflp_jul02.html).

#### DNA extraction:

Use preferred DNA extraction protocol. For sediment samples we use the FastDNA Spin Kit for Soil (Bio101, Vista, CA, USA) with the modifications detailed by Webster *et al.* (2003). Replicate extractions are pooled, concentrated and purified by dialysis with sterile water in Microcon YM-100 centrifugal filters (Millipore, MA, USA) to give a final volume of 100  $\mu$ L.

#### Polymerase chain reaction (PCR) using fluorescently labelled primers:

1. Use PCR protocol as designed for your unlabelled primers, but with forward and/or reverse primers fluorescently-labelled. Dye Set 'D', as commonly used on ABI-3100, is for ROX (6-carboxy-X-rhodamine, red), FAM (5-carboxyfluorescein, blue), HEX (6-carboxy-2',4',7',4,7-hexachlorofluorescein, green), NED (yellow). Size standards (e.g. ROX-500) are labelled with ROX, so we normally use FAM or HEX labelled primers, ordered from MWG.

**Notes:** (a) PCR amplification efficiency using labelled primers may be lower than with equivalent unlabelled primers.

(b) Keep labelled primers and labelled PCR products/digests in the dark as much as possible.]

2. *Example T-RFLP PCR using methanogen-specific 16S rRNA gene primers 355F(HEX)/1068R (after Banning *et al.*, 2005).*

2.1 PCR reaction mixtures contained 1X reaction buffer (Bioline Ltd., London, UK), 20 pmol of primers (355F (HEX-labelled)/1068R (unlabelled) synthesized by MWG, Germany), 0.5 mM each dNTP, 3 mM MgCl<sub>2</sub>, 1.25 U Taq DNA polymerase (Bioline), 10  $\mu$ g bovine serum albumen (Promega; only used when amplifying environmental DNAs) and 1.0  $\mu$ L of undiluted template DNA made up to 50  $\mu$ L with molecular grade water.

2.2 The PCR conditions used for 355F(HEX)/1068R were 30 cycles of 94°C for 1 min, 52°C for 1 min and 72°C for 1 min 30 s.

#### Purification and quantification of PCR products:

1. Replicate reactions (if done) are pooled and products purified using Wizard® PCR Preps DNA Purification System (Promega: catalogue no. A7170). DNA is eluted in final volume of 50  $\mu$ l ddH<sub>2</sub>O. (If non-specific/artefact PCR products are observed gel purification is advisable.)

2. Agarose gel electrophoresis with quantifiable molecular markers (e.g. Bioline Hyperladder I) can be used to estimate concentrations of PCR products, or alternative quantification procedures can be used; e.g. SYBER Green fluorescence, Genequant, NanoDrop, etc.



Restriction digestion of PCR products:1. *Example digest***Table 1 – Example restriction digest**

Reagent	Volume ( $\mu\text{L}$ )	Final concentration
PCR product (known conc.)	1-10	~ 200ng
Restriction endonuclease <sup>a</sup> (10 U/ $\mu\text{l}$ )	0.5-2	5U - 20U
BSA (10 $\mu\text{g}/\text{mL}$ )	0.2	2 $\mu\text{g}$
10X Buffer	2	1X
ddH <sub>2</sub> O	up to 20	
<b>Total volume</b>	<b>20 <math>\mu\text{L}</math></b>	

<sup>a</sup> E.g. *TaqI*; recognition sequence: 5'-T<sup>^</sup>CGA-3'; *RsaI*; recognition sequence: 5'-T<sup>^</sup>CGA-3'

2. Make up master mix without restriction enzyme or DNA. Dispense into 0.2 mL PCR tubes. Add purified PCR product (step 3 above). Add enzyme and mix by pipetting (all solutions containing restriction enzymes must be mixed gently to avoid denaturation). Incubate tubes at 65°C (for *TaqI*) for 3 h. Place digests on ice and store at -20°C (*TaqI* is not heat inactivated).

3. Double digests may be useful; e.g. incubate with *TaqI* at 65°C (as above) for 2 h followed by addition of *RsaI* and further incubation at 37°C for 2 h. Digestion prepared in appropriate buffer. *RsaI* can be heat inactivated at 65°C for 15 min prior to storage.

Desalting of restriction endonuclease (RE) digest (Microcon):

Dilute restriction products up to 500  $\mu\text{L}$  with ddH<sub>2</sub>O and transfer to a YM-10 filter (Microcon). Follow manufacturer's instructions summarised briefly as follows. Centrifugation of sample at 14000 xg ca. 35 min. Transfer filter to a new sterile tube. Add 480  $\mu\text{L}$  of ddH<sub>2</sub>O. Centrifuge as previously for 35 min. Recover sample by inverting filter and placing in new collection tube, followed by centrifugation at 1000 x g for 3 min.

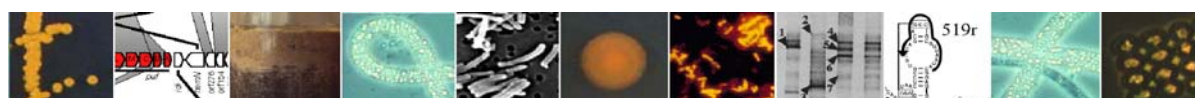
Capillary electrophoresis:

1. Prepare samples for loading onto ABI-3100, as follows: 1  $\mu\text{L}$  desalted RE digest (from step 5 above diluted as necessary; optimal final DNA concentration in injected sample is 1.4 ng/ $\mu\text{L}$ ); 0.5  $\mu\text{L}$  ROX-500 (or other size standards); 8.5  $\mu\text{L}$  of Hi-Di formamide (reagents as supplied by Applied Biosystems)

**Note:** (a) Prepare ROX-500 and Hi-Di formamide as master mix, then dispense 9  $\mu\text{L}$  into each sample tube/well (x17 master mix = 8.5  $\mu\text{L}$  ROX-500 in 144.5  $\mu\text{L}$  Hi-Di)

2. Heat the sample at 95°C for 5 min (use a PCR block) and place immediately on ice for a minimum of 2 min. Ensure there are no air bubbles in the samples.

3. For ABI-3100 runs with 50 cm capillaries (and POP-6) use an injection time (IT) of 22 s and injection voltage (IV) of 1.5 kV. Samples take ~2 h to run with run



module for ROX-500 standards. Alternative run protocols and polymers may be used, but will need to be standardised accordingly.

### Viewing results using GeneScan software (version 3.7, Applied Biosystems):

1. Basic viewing procedure
  - 1.1. Open new file: FILE → new
  - 1.2. From 'Create New' box select Project
  - 1.3. PROJECT → add sample files. If text box comes up with message, 'sample files are locked', and the sample file names appear in italics you have to right click on each .fsa file select properties and remove 'read only' check. Then start again at 7.1.1.
  - 1.4. Two windows should appear- Analysis Control and Results Control. Analysis Control should already be set up with correct size-standards and parameters. Use Results Control to view sample T-RFLPs.
  - 1.5. Check size standards in all sample profiles first (can view maximum of 8 at a time with one colour only) to check that peaks have been sized correctly and profiles do not contain spurious peaks.
2. Altering analysis parameters
  - 2.1. To change horizontal axis to desired range (~6000-24000) for all samples, in Analysis Control box select samples by clicking on far left button for each, right click on Analysis Parameters button and change from collection setting to Analysis Parameters.
  - 2.2. In menu bar go to Settings → Analysis Parameters → put in new analysis range. Select Analyse button in top left hand corner of Analysis Control box.
  - 2.3. ALTERNATIVELY, to save new Analysis Parameters for future use go to main menu File → New → select Analysis Parameters box.
3. Peak detection parameters in Analysis Parameters
  - 3.1. *Smooth Options*: smooths peaks in electropherograms but does NOT change values for data processing (i.e. peak height, area, etc). Therefore, can select heavy smoothing if it improves appearance of peaks
  - 3.2. *Peak amplitude thresholds*: increasing this does not change electropherogram but reduces the number of small peaks processed and presented in data table. 50 is the default value, but this can be increased if the T-RFLP profile is complex.
  - 3.3. *Min. Peak Half Width*: defines the smallest width (at half maximum height) that constitutes a peak. The default is 3, but can be increased for T-RFLPs to 10. All true peaks seem to be above this width.
  - 3.4. *Polynomial degree*: most significant factor is determining sensitivity of peak detection. Range is 2-5 and default is 4. Higher numbers increase sensitivity. If peaks are not very clean and it is desirable to include shoulder peaks into 1 peak for analysis, reduce to degree 2.





3.5. *Peak window size*: Higher peak window sizes (in data points) values smooth out the polynomial curve, which decreases the sensitivity. Default is 19 points, but this can be increased to deal with “noisy” peaks.

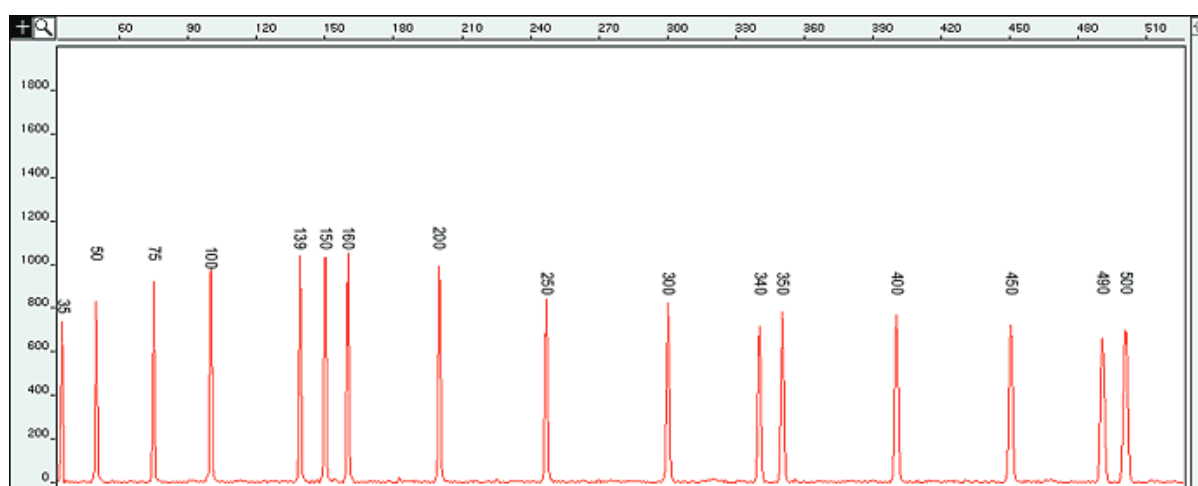
3.6. *Slope thresholds*: leave at 0.0.

**Note:** To go back to original analysis parameters you have to re-analyse samples with “GS500Analysis.gsp” selected for all samples under analysis parameters column in Analysis Control window.

4. Note use of T-RFLP Analysis Program (TAP) for analysis of profiles and as a tool to assist with T-RFLP primer design; see references below.
5. Printing T-RFLP profiles

Printing from Results Control window gives selected graphs (with or without tables) showing whole display range on horizontal axis. To print a zoomed-in graph (as shown on in Fig. 1) select File → print from the menu bar with the display box as the from window.

**Fig. 1 ROX-500 size standards for comparison and sizing of T-RFLPs in profiles**



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Anon. RDPII - T-RFLP Analysis Program (TAP) at [rdp8.cme.msu.edu/docs/tap\\_doc.html](http://rdp8.cme.msu.edu/docs/tap_doc.html)

Contact:

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## 8. Culture Methods

### 8.1. Isolation methods

#### 8.1.1. Media for sulphate-reducing bacteria

Chemically defined, transparent media that do not contain organic nutrients other than the substrate of interest (e.g. fatty acid salt, alcohol, hydrocarbon) are preferred over complex media or media containing precipitates.

All non-reacting, heat-stable components (e.g.  $\text{NH}_4\text{Cl}$ ,  $\text{MgCl}_2$ ) are autoclaved together in solution (= basal salt medium). Components that undergo chemical changes or volatilization in the heat are added from separately sterilized stock solutions after autoclaving and cooling of the basal salt medium.

Oxygen is excluded as far as possible during preparation of the medium. An anoxic chamber is useful but not obligatory. With appropriate anoxic gassing devices such as a syringe connected to a nitrogen bottle and tubes with fitted stoppers and fixing caps (to avoid loss of stoppers), all steps can be done at a normal laboratory bench. (This is also known as “Hungate technique”.)

#### Stock solutions:

##### 1. Trace element mixtures

	Acidic	Chelated
Distilled water	987 ml	1000 ml
HCl (25% = 7.7 M)	13 ml	–
EDTA, disodium salt	–	5.2 g
$\text{H}_3\text{BO}_3$	10 mg	10 mg
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	5 mg	5 mg
$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}^*$	4000 mg	2100 mg
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	190 mg	190 mg
$\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$	24 mg	24 mg
$\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$	2 mg	10 mg
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	200 mg	144 mg
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	36 mg	36 mg
pH	leave acidic	6.0 (with NaOH)

\*Only fresh, greenish crystals should be used. Brownish grains indicate weathering and oxidation.

The trace element solutions are autoclaved (preferentially anoxically under  $\text{N}_2$ ).



## 2. Bicarbonate solution

Distilled water (final volume)	1000 ml
NaHCO <sub>3</sub>	84 g

The solution is saturated with CO<sub>2</sub> (by shaking in a stoppered bottle under a head space of CO<sub>2</sub>) and autoclaved in closed tubes or bottles with fixed stoppers (butyl rubber or Viton) under a head space of CO<sub>2</sub> ( $\geq 1/4$  of total volume).

## 3. Vitamin mixture

Sodium phosphate, 10 mM, pH 7.1	100 ml
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
D(+)-Pantothenic acid, calcium salt	5 mg
Pyridoxine dihydrochloride	15 mg

## 4. Thiamine solution

Sodium phosphate, 10 mM, pH 3.4	100 ml
Thiamine chloride dihydrochloride	10 mg

5. Vitamin B<sub>12</sub> solution

Distilled water	100 ml
Cyanocobalamin	5 mg

All these vitamin solutions are filter-sterilized (pore size, 0.2  $\mu$ m) and stored in the dark (preferentially in bottles of brown glass) at 4 °C.

## 6. Sodium sulfide solution

Distilled water (final volume)	100 ml
Na <sub>2</sub> S · 9H <sub>2</sub> O	48 g

Only clean crystals of sodium sulfide are used. Sodium sulfide is auto-oxidizable. Sodium sulfide is dissolved by stirring under an N<sub>2</sub> atmosphere. The solution is autoclaved under a head space of N<sub>2</sub> ( $\geq 1/4$  of total volume).

Preparation of media:

## Preparation of the basal salt media

Depending on the physiological type of microorganisms to be cultivated and the salinity of the original source, one of the following basal mineral media is prepared. For many marine isolates, the full marine medium can be replaced by the saltwater medium that tends to form less inorganic precipitates.



	Freshwater		Saltwater		Full marine	
Distilled water	1	l	1	l	1	l
NaCl	0.5	g	20.0	g	26.0	g
MgCl <sub>2</sub> · 6H <sub>2</sub> O	0.5	g	3.0	g	5.0	g
CaCl <sub>2</sub> · 2H <sub>2</sub> O	0.1	g	0.15	g	1.4	g
Na <sub>2</sub> SO <sub>4</sub>	3.0	g	3.0	g	4.0	g
NH <sub>4</sub> Cl	0.3	g	0.3	g	0.3	g
KH <sub>2</sub> PO <sub>4</sub>	0.2	g	0.2	g	0.1	g
KCl	0.5	g	0.5	g	0.5	g

Media should be prepared in special flasks with tubes for anoxic sterile gassing and a closable outlet that allows distribution of the complete medium to smaller cultivation tubes or bottles.

After subsequent dissolution of the salts, the solution is autoclaved. After autoclaving, redissolution of oxygen is prevented by cooling under an N<sub>2</sub>-CO<sub>2</sub> mixture. Then, the following sterile stock solutions are subsequently added (amounts per liter of medium):

Trace element mixture	1.0	ml
NaHCO <sub>3</sub> solution	30.0	ml
Vitamin mixture	1.0	ml
Thiamin solution	1.0	ml
Vitamin B <sub>12</sub> solution	1.0	ml
Reductant: Na <sub>2</sub> S solution	5.0	ml

The pH is adjusted to 7 with sterile 1 M Na<sub>2</sub>CO<sub>3</sub> or H<sub>2</sub>SO<sub>4</sub> solution. The completed medium is distributed to culture tubes and bottles in which it is stored anoxically under a small head space of an N<sub>2</sub>-CO<sub>2</sub> mixture.

It is advisable to prepare the medium without an organic substrate (as above). Then, the medium can be used for various purposes (such as substrate utilization tests); the organic compound of interest is simply added individually to each tube or bottle.

Special procedures for cultivation with gases (e.g., H<sub>2</sub>, CH<sub>4</sub>) in the head space, precipitating long-chain fatty acids or the water-insoluble liquid hydrocarbons have been described in detail (Widdel & Bak, 1992; Widdel *et al.*, 2004).

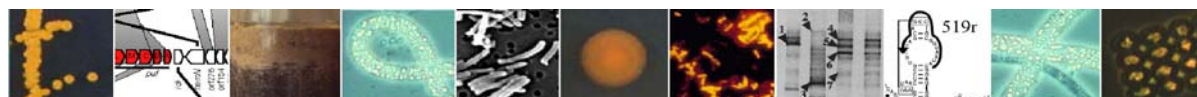
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## 8.2. Enumerating viable bacteria

### 8.2.1. High throughput MPN methods for anaerobic bacteria

The aim of this protocol is to describe a high throughput most probable number method (MPN) for counting viable prokaryotes in sediment. Besides total cell counts obtained by epifluorescence microscopy, cultivation-based methods can be used for the quantitative assessment of microbial communities. The latter can be designed to target certain physiological groups like sulphate-reducing bacteria or methanogens, but suffer from biases since not all microorganisms targeted will grow on the media chosen.

#### Preparation of MPN series for anaerobic microorganisms:

MPN series for anaerobic microorganisms are prepared in an anaerobic hood. For ship-based work an inflatable polyethylene chamber (e.g. AtmosBag, 280 l, Aldrich, Milwaukee, Wisconsin, USA) can be used instead. After all equipment necessary for the preparation of the MPN series is placed into the hood, the polyethylene chamber should be flushed with nitrogen gas, evacuated and filled with N<sub>2</sub> again. The procedure should be repeated up to five times to almost completely remove atmospheric oxygen (Süß et al., 2004). MPN series are set up in sterile deep well plates with 8 x 12 wells (e.g. made from polypropylene, Beckman, Fullerton, CA) and an approximate volume of 1500 µl per well. The final content of the wells should not exceed 1000 µl.

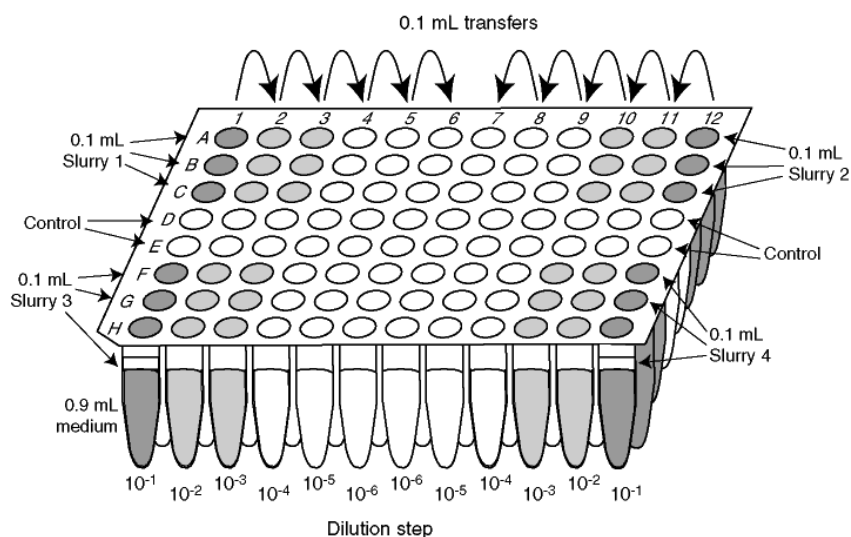
Medium can be filled into autoclavable small troughs with lids (e.g. Tip-Tub, Eppendorf, Hamburg, Germany) and transferred to the deep-well plates (Fig. 1) using multichannel pipettes (always use pipette tips with filters to minimise risk of contamination). Sediment slurry is added to each well of the first dilution. After mixing well, samples will be consecutively diluted into the following wells. At least one row of wells should not be inoculated but diluted like the others and serve as a control for aseptic conditions. After inoculation, the plates have to be covered with sterile lids (e.g. CAPMAT, Beckman, Fullerton, CA) that seal each well separately.

The MPN plates are then put into gas-tight plastic bags equipped with a gas generating and catalyst system for anoxic conditions, like the Anaerocult C mini and Anaerocult A mini systems by Merck (Darmstadt, Germany) or the AnaeroGen compact system by Oxoid (Basingstoke, UK). For controlling anoxic conditions, an indicator strip (e.g. Anaerotest, Merck, Darmstadt, Germany) is also placed in plastic bag before sealing. The commonly used plastic bag systems are usually sufficient for one plate per bag. MPN series should be incubated for at least six weeks to allow sufficient growth yield for microscopic or fluorimetric detection.

Most common are MPN series with three or five parallels. MPN series performed with coastal and deep-sea surface sediments should at least comprise eight dilutions (Köpke et al., 2005), allowing three different series and three controls on one plate or two series and two controls, respectively. For subsurface sediments or pelagic water samples six dilutions appear to be sufficient. In this case four MPN series with parallels and three controls can be placed on one plate (Fig.1).



**Fig. 1. Pipetting scheme for four MPN series with three parallels and six dilutions on a 96-well plate. For each sample, one row remained uninoculated as a control. (From Proc. ODP, Init.Rep. Vol. 201, Suppl. Material)**



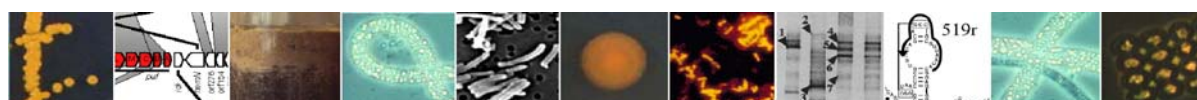
#### Growth detection in MPN series:

In MPN experiments growth detection is often hampered by low growth yield, in particular from subsurface samples and by microorganisms sticking to particles like FeS (Süß et al., 2004). Therefore, turbidimetric growth analysis is not applicable. Growth can be analysed by epifluorescence microscopy after staining with nucleic acid dyes like SybrGreen II and DAPI or by fluorimetric analysis, a method that is very sensitive and fast to perform (Martens-Habbenha and Sass, 2005).

The gas-tight plastic bags containing the MPN plates are transferred into an anaerobic hood and the plates taken out of the bags. The lid is removed entirely or is cut into smaller pieces and carefully lifted using tweezers (these should be sterile if subculturing is planned and must not touch the inner surface of the wells or the nubs on the lower side of the lid). After the lid is removed, the plate should be covered with a sterile lid (e.g. microtiterplate lid) to avoid airborne contamination.

For epifluorescence microscopical analysis 10 µl of culture are transferred from each well into a cavity of a diagnostica microscope slide. To each sample 2 µl of a solution of a nucleic acid dye are added. Most recommendable are DAPI (0.3 µg·ml<sup>-1</sup>) and SybrGreen II (1:100, Molecular Probes, Leiden, Netherlands). Sample and dye are carefully mixed with a pipette tip and the stained samples incubated for at least 1 h in the dark prior to microscopic examination. For epifluorescence microscopy an UV excitation filter set for SybrGreen II (BP450-490, FT510, LP515) or DAPI (BP365, FT395, LP397) is required.

For fluorimetric analysis, 100 µl are transferred from each well into a black microplate (Nunc 237108, VWR International, Darmstadt, Germany) and 25 µl of a Sybr Green I (Molecular Probes, Leiden, The Netherlands) solution (2000 fold dilution in TE buffer: 200 mM Tris and





50 mM EDTA, pH 8) are added. The plates are covered by lids and are incubated for at least 12 h in the dark at 4°C to achieve optimal fluorescence yield. Fluorescence is measured on a fluorescence microplate reader.

The MPN counts can be calculated by hand from tables published by de Man (1977). However, the reduced volume of inoculum, as compared to the classical procedure in tubes, has to be taken into account. Alternatively the MPN calculator software of Klee (1993) or of Briones and Reichardt (1999) can be used.

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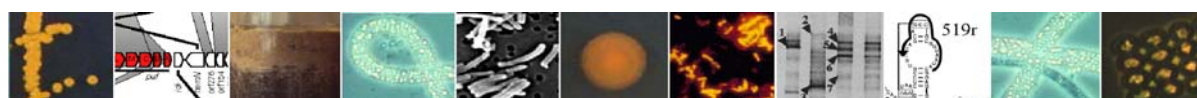
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