UNIVERSITY OF SOUTHAMPTON

FACULTY OF ENGINEERING , SCIENCE AND MATHEMATICS

School of Ocean and Earth Science

The role of microbial populations in the cycling of iron and manganese from marine aggregates

by

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Thesis for the degree of Doctor of Phylosophy

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BIOTRACS

Bio-transformations of trace elements in aquatic systems

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ABSTRACT

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THE ROLE OF MICROBIAL PROCESSES IN MARINE AGGREGATES ON THE CYCLING OF IRON AND MANGANESE

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Marine aggregates play an important role in the cycling of carbon, nutrients and trace metals. Within aggregates the oxygen depleted by aerobic microbial respiration may not be replaced rapidly, generating anoxic or suboxic microzones. Reduced compounds that are unstable in the oxygenated water column have been previously found associated with marine snow. Therefore, in the experiments described in this thesis, artificial aggregates were made in the laboratory from senescent phytoplankton material and incubated to investigate the role of the associated microbial populations to the biogeochemical redox cycling of iron and manganese and to the degradation of organic matter.

The release of dissolved iron from artificial aggregates which did not contain any measurable (~10 µm) anoxic microzones, was demonstrated under dark conditions. The rate of release was controlled by the amount of reducible Fe(III) available, and appears to be limited by the competing oxidation of Fe(II). Moreover highly significant releases in reduced Mn were detected from aggregates incubated under a constant velocity shear, although the same aggregates did not affect the dissolution of iron. A possible reason is likely associated with the higher stability of Mn(II), compared to Fe(II) in aerobic environments.

Molecular (16S rRNA gene) analyses showed the bacterial community associated with artificial aggregates to be similar to that found in natural aggregates and dominated by (predominantly uncultured) *α- and γ-Proteobacteria, Bacteroidetes, Planctomycetes and Cyanobacteria*. It was possible to culture NO_3^--, Fe(III)- and Mn(IV)-reducing bacteria from the artificial aggregates, and marine particles incubated with Fe(III) under anaerobic conditions contained a range of γ- and *δ-Proteobacteria* known to respire Fe(III) and in most cases Mn(IV). Moreover several microorganisms belonging to *γ-Proteobacteria* were isolated from marine aggregates and strains affiliated to the genera *Amphritea*, *Marinobacterium* and *Marinobacter,* were demonstrated to grow through the reduction of Fe(III), with *Marinobacter* also capable of respiring Mn(IV). Whilst the precise mechanism of reduction is not clear, it is evident that marine aggregates can be a source of Fe(II) and dissolved Mn, in coastal waters and most probably other natural water systems.

Fatty acid analyses revealed the prevalence of saturated over unsaturated fatty acids indicating that aggregates were already partially degraded when incubation started. Nonetheless, the lipids in the artificial aggregates were rapidly degraded further as indicated by a depletion in short chain (<20) saturated and monounsaturated fatty acids. In contrast, the concentrations of linear and branched, saturated long chain (>20) fatty acids fluctuated, suggesting that some of these lipids could have been produced *in situ* by marine microorganisms rather than deriving from

higher plant debris. In addition, a bacterial branched monounsaturated fatty acid (11-methyl-octadecenoic acid), which has not previously been found in marine particles was present in artificial aggregates. *Roseobacter litoralis* found among the aggregate-attached bacteria contains 11-methyl-octadecenoic acid, and other bacteria present in artificial aggregates have the potential to produce long-chain saturated and polyunsaturated fatty acids. Thus, the fatty acid assemblage appears to reflect both organic matter degradation, including selective preservation, but also changes in the microbial assemblage.

A range of future studies are suggested to elucidate the mechanisms for Fe(III) and Mn(IV) reduction in aggregates. These include microscale analyses of dissolved species and evaluation of the presence of metal binding ligands associated with aggregates. Moreover it is important to assess the activity of the Fe(III)- and Mn(IV)-reducing bacteria present in aggregates *in situ* and the production of long chain fatty acids in degrading aggregates.

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has been produced under the supervision of the following persons

Supervisors:	Prof. Peter J Statham Dr Richard D Pancost Prof. Jonathan R Lloyd
Chair of advisory panel:	Dr Duncan Purdie

Table of contents

List of Tables

List of Figures

DECLARATION OF AUTHORSHIP

I, Sergio Balzano, declare that the thesis entitled "The role of microbial processes in marine aggregates on the cycling of iron and manganese" and the work presented in it are my own. I confirm that:

- This work was done wholly while in candidature for a research degree at the "University of Southampton".
- Where any part of this thesis has previously been submitted for any qualification at this university or other institutions, this has been clearly stated.
- Where I have consulted the published work of others, this is always clearly attributed.
- Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work.
- I have acknowledged all main sources of help.
- Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself.
- Part of this work have been published as:

Balzano S, Statham PJ, Pancost RD, Lloyd JR (2009) Role of microbial populations in the release of reduced iron to the water column from marine aggregates. Aquatic Microbial Ecology 54:291-303

- Part of this work has been submitted to the journal Marine Chemistry as:

Balzano S, Pancost Rd, Lloyd JR, Statham PJ. Changes in fatty acid composition in degrading algal aggregates

Signed……..

Date…….

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List of abbreviations

ATP-Adenosine-5'-triphosphate

BIOTRACS- Biotransformations of trace elements in aquatic systems

BLAST- Basic local alignment search tool

BLP-Bacteria-like particles

β-OH-FA-β-hydroxy fatty acids

brFA-Branched-chain fatty acids

C32HA-Bishomohopanoic acid

Chl-a- Chlorophyll a

DAPI-4',6-diamidino-2-phenylindole

DBL-diffusive boundary layer

DCM- dichloromethane

DNA- Deoxyribonucleic acid

DNRA- Dissimilatory nitrate reduction to ammonium

DOM- Dissolved organic matter

DON- Dissolved organic nitrogen

δ-MnO_2- Synthetic vernardite

EDTA- Ethylenediaminetetraacetic

FA- Fatty acids

Fe(III)-NTA- Ferric nitrilotriacetate

f/2- Provasoli-Guillard marine water enrichment solution

GC- Gas chromatography

GC-MS- Gas chromatography-mass spectrometry

GDGT- Glycerol dialkyl glycerol tetraethers

HNLC- High Nutrient Low Chlorophyll

IUPAC- International union of pure and applied chemistry

LbrFA-Long chain (>17 carbon atoms) branched fatty acid

MBA-Marine Biological Association of the United Kingdom, Plymouth

Me11,C18:1- methyloctadecenoic acid

MSc-Master of Science

MQ- Millipore Quality

MUFA- Monounsaturated fatty acids

NADH- Reduced nicotinamide adenine dinucleotide

NCBI- National centre for biotechnology information

*n*LCFA- Long-chain n-alkanoic acids

NOCS- National Oceanography Centre, Southampton

*n*SCFA- Short-chain (C_{14} to C_{20}) *n*-alkanoic acids

odd/even-ratio of odd over even numbered fatty acids

OGU- Organic Geochemistry Unit, University of Bristol

OM- Organic matter

PCR- Polymerase chain amplification

Phaeo-a- Phaeophorbide a

PLY- The Marine Biological Association of Plymouth

POM- Particulate organic matter

PUFA- Polyunsaturated fatty Acids

PVC- Polyvinyl chloride

RFLP- Restriction fragment length polymorphism

rpm- Revolutions par minute

rRNA- ribosomal ribonucleic acid

SbrFA- Short chain (≤17 carbon atoms) branched fatty acid

SEAS- School of Earth, Atmospheric and Environmental Sciences, University of Manchester.

SRB-Sulfate-reducing bacteria

Teflon- Polytetrafluoroethylene

TEP- Transparent exopolymer particles

TSB- Triptone soy broth

UNS/SAT-ratio of unsaturated to saturated FA

VLP- Virus-like particles

Chapter 1. Introduction

1.1 Importance of biogeochemical cycling in the water column

Primary production is crucial for the carbon cycle as it removes carbon dioxide from the atmosphere and incorporates it in the biosphere. Nutrients and trace metals are actively assimilated by phytoplankton and removed from the water column. As a significant portion of the plankton biomass sinks towards the seafloor through the biological pump (Fig. 1.1), the upper water column is continuously depleted in nutrients and trace metals which become a limiting factor for phytoplankton growth. Several parts of world's Ocean contain high nutrients but low chlorophyll concentrations (HNLC), in these areas the low concentrations of trace metals, especially iron, are limiting factors for the growth of phytoplankton (Boyd et al. 2000; Coale et al. 1996; Hutchins and Bruland 1998; Martin et al. 1994).

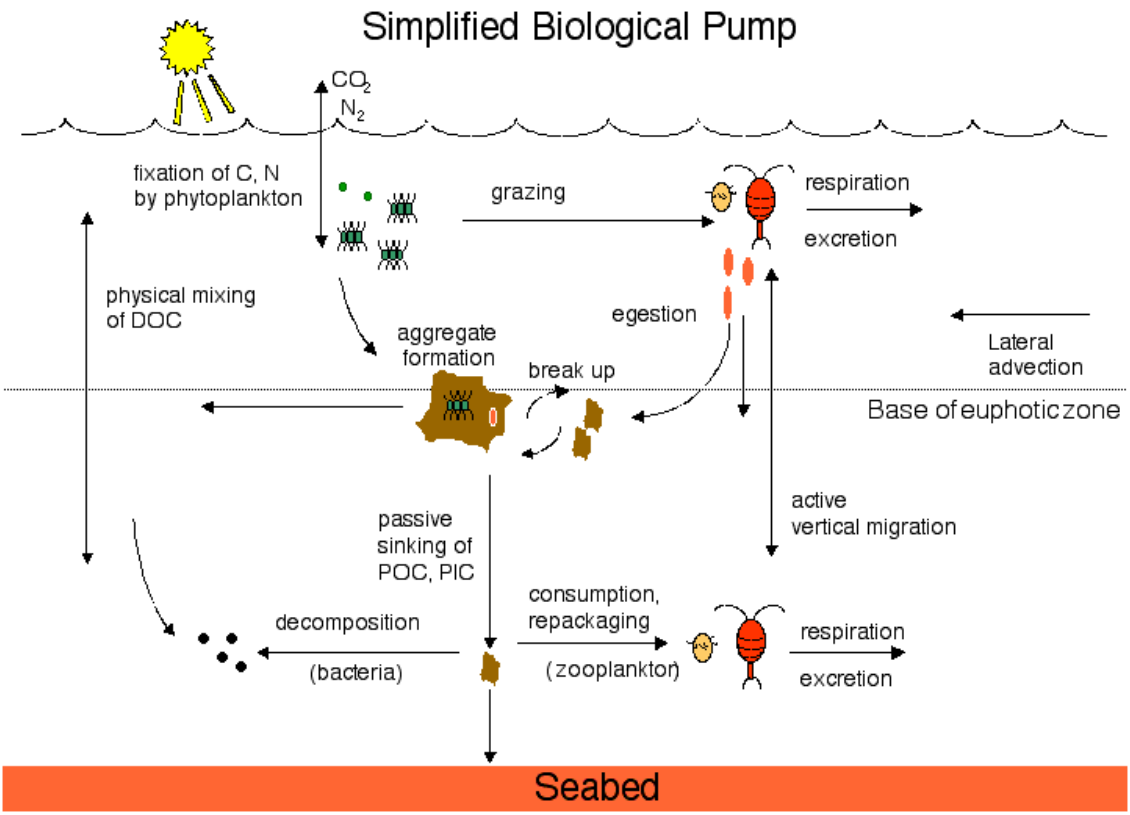


Figure 1.1. Simplified scheme of the biological pump, image from http://www.msrc.sunysb.edu/octet/biological_pump.html

1.1.1 Iron and manganese

In the present thesis, both dissolved iron and dissolved manganese are operationally defined as the metals present in material passing through 0.2 µm filters.

Availability of trace metals to plankton depends on their chemical form in seawater. The form of iron and to a lesser extent manganese in the sea is not yet well known; according to the pH and redox conditions of seawater (pH ~ 8.1; pE ~ 12.5), these metals are expected to be present as Fe(III) and Mn(IV) (Stumm and Morgan 1993), but their solubility is very low. The reduced forms, Fe(II) (ferrous iron) and Mn(II) are more soluble in seawater but are unstable and tend to be oxidized under aerobic conditions (Millero et al. 1987; Stumm and Morgan 1993).

1.1.2 Impact of sinking material on trace metal cycles

In the oxidised form iron and manganese are insoluble and thus likely to sink inside larger particles towards the seafloor leaving the upper water column and becoming unavailable for biological uptake. Sinking particles remove also carbon and nutrients from the upper water column and are the main vehicle for vertical material transport in the ocean (Fowler and Knauer 1986). However some of the elements sinking in marine snow flocs, may be dissolved and released back in the seawater as a consequence of microbial activities. Essential elements can be thus recycled and taken up again by plankton.

Anaerobic processes in the sediment play an important role in reducing Fe and Mn to dissolved forms that can diffuse back into the water column. A minor role in the dissolution of trace metals may be played by marine aggregates.

1.2 Phytoplankton growth and aggregate formation

High primary production events may lead to particle aggregation in seawater (Eisma and Li 1993; Giani et al. 2005; Gorsky et al. 2003; Graham et al. 2000; Kiorboe et al. 1998; Lampitt et al. 1993a; Pilskaln et al. 1998; Pilskaln et al. 2005; Shanks 2002; Silver and Alldredge 1981; Trent et al. 1978; Walsh et al. 1997).

1.2.1 Aggregation of particles in the water column

The coagulation of particles into larger aggregates and their consequent sedimentation on the seafloor (Fig. 1.2) has been widely described (Beaulieu 2002;

Simon et al. 2002; Turner 2002). Three main factors lead to particle aggregation: physical features of the particles (shape, size, concentration and distribution), the number of collisions between particles and the stickiness coefficient (Alldredge and Silver 1988; Kiorboe 2001; Kiorboe et al. 1994). The physical processes bringing particles together are Brownian motion, shear, and differential settling (Mccave 1984). Brownian motion is due to collision of particles with fluid molecules and generates a random movement of particles. Brownian motion contribution is not negligible for small (<8µm) particles whereas velocity shear is significant for larger (>8µm) particles (Eisma 1993; Mccave 1984). Velocity shear occurring in seawater increases around the fronts, as well as when strong winds blow on the seawater surface. The rate of cell divisions and death is also suggested to be an important factor in the aggregation of phytoplankton cells (El Saadi and Bah 2007). Moreover particle scavenging contributes to the increase in particle size while aggregates are sinking. In particular abandoned larvacean houses and pteropod webs can scavenge a significant number of cells while sinking (Kiorboe et al. 1996). Zooplankton activities including seawater filtration, direct grazing on phytoplankton and faecal pellet ejection also contribute to flocculation events (Lampitt et al. 1990; Pomeroy and Deibel 1980).

Particles may then stick together after collision, they are thought to be sticky if the stickiness coefficient, defined as the ratio between successful and total collisions, exceeds 0.9 (Hansen and Kiørboe 1997; Jackson 1990; Kiorboe et al. 1994; Mccave 1984). The stickiness coefficient is variable in different species and high stickiness coefficients were measured for *Chaetoceros*, *Rhizosolenia*, *Thalassiosira*, *Skeletonema* and *Phaeocystis* species (Hansen and Kiørboe 1997). The stickiness coefficient is also dependent on the physiological state of cells (Riebesell 1991). During diatom growth, the highest sticking coefficient is shown by cells at the start of their steady-state (Kiorboe et al. 1994) or during the biological break-down of the bloom (Engel 2000).

Particle flocculation is promoted by organic molecules likely exuded by phytoplankton such as transparent exopolymer particles (TEP) (Alldredge et al. 1993). In addition a range of metabolites including polysaccharides (Mopper et al. 1995), Coomassie-stained proteins (Long and Azam 1996), unidentified yellow 4',6-diamidino-2-phenylindole (DAPI)-stained compounds (Brachvogel et al. 2001) as well as humic and fulvic acids (Giani et al. 2005) have also been suggested to act as important binding agents in marine aggregates (Simon et al. 2002). A recent study highlighted that carbohydrates and proteins are also involved in the aggregation

processes in seawater whereas the role of lipids in the same process appears to be insignificant (Mecozzi et al. 2009).

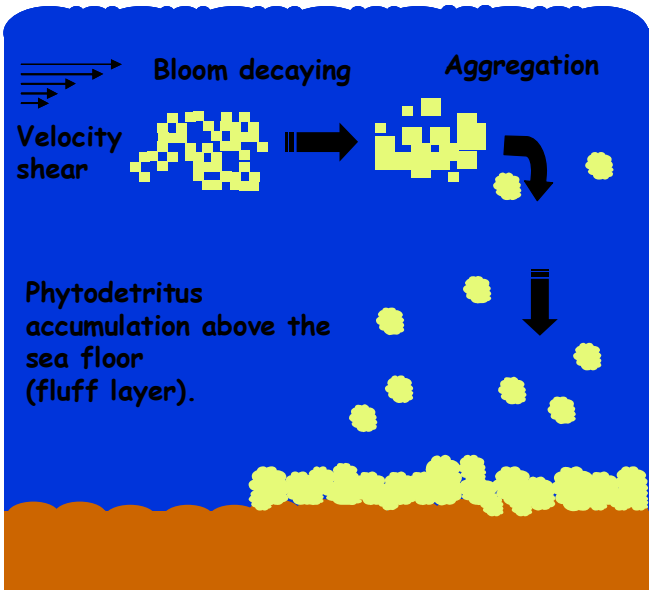


Figure 1.2. Formation and sedimentation of marine snow.

1.2.2 Transparent exopolymer particles (TEP)

TEP are one or two dimensional shaped gels with high interstitial water content (Alldredge et al. 1993), constituted mainly by carbohydrates. Their size ranges from 3 to 100 μm (Alldredge et al. 1993). TEPs are the main agents leading to marine snow formation (Alldredge and Jackson 1995; Logan et al. 1995) and their maximal concentrations are usually registered during the stationary growth phase of phytoplankton cells, in the laboratory (Mopper et al. 1995; Passow 2002) as well as in natural waters (Engel 2004; Prieto et al. 2006). The stickiness coefficient calculated for TEP is three orders of magnitude higher than that of phytoplankton cells (Passow and Alldredge 1999).

TEP are exuded by phytoplankton and usually constitute the matrix which sticks together bacteria, microalgal cells, organic debris and minerals in marine snow (Biddanda 1986). Bacteria can promote TEP production from phytoplankton (Passow et al. 2001) and are also involved in its degradation, controlling TEP dynamics and more generally particle aggregation (Passow and Alldredge 1995). A minor contribution to TEP production in seawater derives also from bacteria (Passow 2002) and macroalgal

detritus (Thornton 2004). TEP are produced via coagulation of dissolved polysaccharides which have been exuded by phytoplankton and bacterial cells (Alldredge et al. 1993; Decho 1990; Passow 2002). TEP surfaces are highly colonised by bacteria and may also provide surfaces for adsorption of a variety of chemical species in seawater (Alldredge et al. 1993; Biddanda 1986)

1.2.3 Marine aggregates: composition and classification

The principal constituents of marine aggregates (Fig. 1.3) are living and dead algal cells, empty diatom frustules, bacteria, protozoa and zooplankton debris, faecal pellets, macrophyte detritus, clay and silt minerals, TEP, carbohydrates, proteins, lipids and more complex and refractory organic substances (Alldredge and Silver 1988; Simon et al. 2002; Turner 2002). Aggregates (Fig. 1.3) are usually distinguished as microaggregates (<0.5mm) and macroaggregates or marine snow (>0.5mm) (Alldredge and Silver 1988).

Marine aggregates are often composed of different diatoms species including *Asterionellopsis*, *Chaetoceros, Detonula, Ditylum, Fragilariopsis*, *Odontella*, *Pseudonitzschia*, *Rhizosolenia*, *Skeletonema, Thalassionema, Thalassiosira*, and *Thalassiotrix* species (Alldredge et al. 2002; Beaulieu 2002; Pilskaln et al. 2005). Coccolithophorids such as *Emiliana huxleyi* are also common in marine snow collected from temperate and arctic waters (Conte et al. 1995). In addition cyanobacteria, dinoflagellates and *Phaeocystis* colonies may also contribute to important aggregation events (Beaulieu 2002; Engel et al. 2002; Simon et al. 2002).

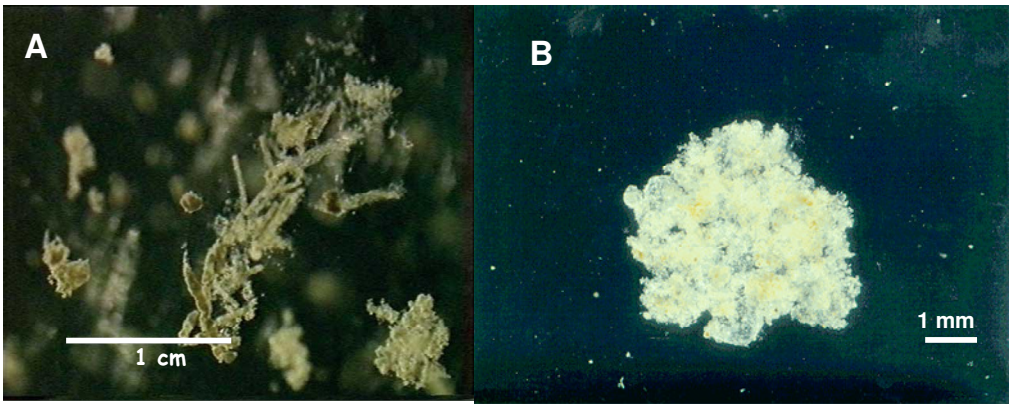


Figure 1.3. (**A**) A rain of marine snow. Modified from www.niwa.co.nz). (**B**) A marine aggregate. Photo from RS Lampitt.

Sinking rates of macroaggregates range between 5 and 200 m d^{-1} (Alldredge and Cohen 1987; Kiorboe et al. 1994). Aggregation usually enhances the sinking rate of particles (Kiørboe et al. 1996), although aggregates dominated by cyanobacteria exhibit very low sinking rates or do not sink (Grossart and Simon 1997). A particular class of marine aggregates is the mucilaginous type (Degobbis et al. 1995; Herndl and Peduzzi 1988); they are composed mainly of refractory organic matter and are less dense than marine snow. Mucilaginous aggregates usually do not sink but accumulate on the isopycnal in equilibrium with their density. The accumulation of mucilaginous aggregates in the water column is not known to be common in the ocean but does occur periodically in the Adriatic Sea (Najdek et al. 2002).

1.3 Artificial marine aggregates

1.3.1 Sampling natural marine snow: difficulties and limitations

Due to their fragility marine aggregates are very difficult to sample and transport, and they may be disrupted very easily if collected with plankton nets or filtration devices. Material collected from the same sediment trap may contain aggregates of different origin and histories, and their original shape is also affected. Moreover formaldehyde or other fixing agents are usually present in the sediment traps to prevent zooplankton grazing and microbial degradation, making the aggregates collected unsuitable for laboratory incubations.

To minimise human manipulations, aggregates were often directly collected by scuba divers using syringes (Alldredge 1998; Rath et al. 1998; Shanks 2002). Samplers able to collect large volumes (100-1000L) of seawater have been occasionally used for collecting marine snow (Lampitt et al. 1993b) and devices which allow the careful collection and video-documentation of aggregate size and abundance are also available (Lunau et al. 2004).

However model aggregates were often generated artificially in the laboratory from senescent plankton or debris and used for a range of different studies (Azua et al. 2007; Moriceau et al. 2007; Passow et al. 2003; Ploug and Jorgensen 1999; Ploug et al. 1997).

1.3.2 Previous attempts to aggregate organic matter

The first attempt to form artificial marine aggregates was using clay sediment and organic matter derived from seaweed, fish and plankton (Kranck and Milligan 1980). Another attempt in forming marine snow was made by growing bacteria on dissolved organic matter (DOM) from ground seaweed (Biddanda 1985). However natural substrates from which marine snow is formed were not used in these experiments, thus aggregates were not representative of natural marine snow.

Shanks and Edmondson (1989) developed a method for the formation and the maintenance of intact marine snow in the laboratory: unfiltered seawater, collected during a phytoplankton bloom (associated with an aggregation event) and therefore rich in plankton, was used as the medium from which to make marine snow. Cylindrical bottles were filled of this seawater and rotated on a roller table for about 3.5 hours at 15 revolutions per minute (rpm). Natural marine snow was also collected from the same station at the same time. The species composition, as well as the physical features (size and shape distribution, porosity) of natural and artificial aggregates were then measured and compared. The aggregates made in the laboratory were morphologically, biologically, and, within limited set of measurements and parameters, physically similar to the marine snow sampled in the field (Shanks and Edmondson 1989).

1.4 Impact of sinking particles on nutrients and trace metal cycles

Some of the nutrients and trace metal assimilated by plankton accumulate in marine snow flocs and sink towards the seafloor, leaving the upper water column and becoming unavailable for biological uptake.

1.4.1 Trace elements in seawater

An element dissolved in a solution is defined as a trace element if its concentration does not exceed 10^{-2} mM. Due to their very low concentration, dissolved trace metal behaviour does not affect the main properties (pH, pE) of the solution which can be considered constant. Element speciation, defined as the distribution of the element between different chemical species (Tessier and Turner 1995), is affected by the solubility of salts, hydroxides and complexes that the element can form in seawater. Trace metal speciation in seawater with major ions is quite clear while the interactions

between trace metals and DOM are not yet well known. Trace metal speciation may be influenced by association with other compounds, called ligands, present in trace concentration. In seawater compounds deriving from refractory phytodetritus, such as humic or fulvic acids, are able to act as ligands and can complex trace metals (Rose and Waite 2003).

Inorganic and organic particles in seawater affect the solute distribution by absorbing some dissolved compounds and releasing other chemical species. In seawater the effect of adsorption is negligible for major elements but it becomes more significant for solutes present at trace concentrations. The particulate organic matter (POM) may thus act as a source or a sink for trace elements affecting their speciation and availability to organisms (Benes and Steinneš 1995). The rate of trace metal adsorption onto surfaces often increases with pH for those cations which don't form any colloidal complex (Benes and Steinneš 1995). Complexed metals adsorbed onto marine aggregates can be rapidly transported downward (Cowen and Silver 1984).

1.4.2 Trace metal chemistry

Whilst the importance of trace metals to organisms is very clear, on the other hand mechanisms of trace metal recycling in seawater, are less well known. As metals are accumulated in biogenic matter it is the release from POM through biological activities and other processes that is an essential link in understanding their cycling.

Colloidal material derived from mucus exuded by phytoplankton and bacterial cells, has been shown to interact with trace metals (Morel and Price 2003). Trace metals in seawater may thus be assimilated and released by marine particles which provide an area of increased nutrient availability. Inorganic and organic particles play a dominant role in controlling the trace element distribution in riverine (Sokolowski et al. 2001; Wen et al. 2008) estuarine (Breuer et al. 1999; Kowalski et al. 2009; Morris 1986; Turner 1996; Wen et al. 2008), and marine environments (Ellwood 2004; Norisuye et al. 2007; Wen et al. 2006).

If within macroaggregates, hypoxic and anoxic microenvironments are generated, and anaerobic metabolism dominates the biological activity, a range of compounds, including Fe(III) and Mn(IV), may be reduced and then diffused into the surrounding oxic seawater, if re-oxidation is slow. Iron and manganese are important for biological processes such as primary production and bacterial degradation of organic matter. Plankton tends to assimilate these metals mainly in the dissolved

reduced forms Fe(II) and Mn(II) (Hudson and Morel 1990; Maldonado and Price 2000; Rose and Waite 2003).

1.4.3 Biogeochemistry of iron

Iron is an essential element for cell growth, it is present in various redox enzymes and iron-sulfur proteins critical for photosynthetic electron transport (Lehninger 1975). Protein-bound iron complexes act as vital electron mediators in many metabolic processes in living systems (Ussher et al. 2004), iron complexes have important functions in metabolic processes such as intracellular respiration, aerobic and anaerobic photosynthesis (photosystem I, photosystem II, cytochrome), Adenosine-5'-triphosphate (ATP) synthesis, nitrogen fixation, reduction of nitrate, nitrite and sulfate (Ussher et al. 2004) and Deoxyribonucleic acid (DNA) synthesis (Weinberg 1989).

Iron is the fourth most abundant element in the Earth's crust, exceeded only by oxygen, silicon and aluminium but despite its crustal abundance it is found at trace concentrations in marine environments, becoming a limiting factor in HNLC regions (Boyd et al. 2000; Coale et al. 1996; Hutchins and Bruland 1998; Martin et al. 1994; Martin and Gordon 1988). The major pathway for transport of iron particles to the surface ocean is the wet deposition of dust particles (Fig. 1.4) (Jickells and Spokes 2001; Johnson et al. 1997; Spokes et al. 2001).

The availability of trace metals to plankton depends on their chemical form in seawater, most of which are not available to organisms (Miller and Kester 1994). The form of iron in the sea is not yet well known; in the oxygenated water column iron would be expected to be present as Fe(III) oxides and hydroxides, but their solubility is very low (Sunda 2001): dissolved Fe(III) is highly reactive with respect to hydrolysis, adsorption and complex formation, inorganic Fe(III) thus exists in solution mainly as iron oxides and iron hydroxides. These species are sparingly soluble and undergo progressive dehydration and crystallisation which decreases their solubility. Fe(III) oxides and hydroxides tend to be scavenged in seawater by settling particles (Fig. 1.4) (Kuma et al. 1996; Sunda 2001). However more than 99% of dissolved iron in seawater is bound to organic chelators (Gledhill and Vandenberg 1994; Rue and Bruland 1995; Rue and Bruland 1997; Wu and Luther 1995) which keep it stable in a dissolved form. Organic complexation of iron increases its residence time through enhancing its solubility and inhibiting scavenging (Johnson et al. 1997).

Iron is taken up by organisms as dissolved iron (Hudson and Morel 1990;

Maldonado and Price 2000) often bound to organic ligands (Chen and Wang 2008; Maldonado and Price 1999; Maldonado and Price 2001) although some microalgae can also incorporate the particulate iron present in ingested bacteria (Maranger et al. 1998). Reduction of Fe(III) complexes is thus very important in surface seawater and reductive dissociation may provide a source of bioavailable iron to phytoplankton. Fe(III) in seawater can be reduced to Fe(II) (ferrous iron) which is much more soluble, kinetically labile and forms much weaker organic chelates (Sunda 2001). The processes leading to Fe(III) reduction include photoreduction (Ozturk et al. 2004; Rijkenberg et al. 2005), bioreduction at cell surfaces (Maldonado and Price 1999; Maldonado and Price 2000; Maldonado and Price 2001) and microbial reduction in suboxic or anoxic environments (Lovley et al. 2004). Reduced iron is however unstable and tends to be oxidised in the oxic water column (Stumm and Morgan 1993). This oxidation may be catalysed by organic matter (Rose and Waite 2003).

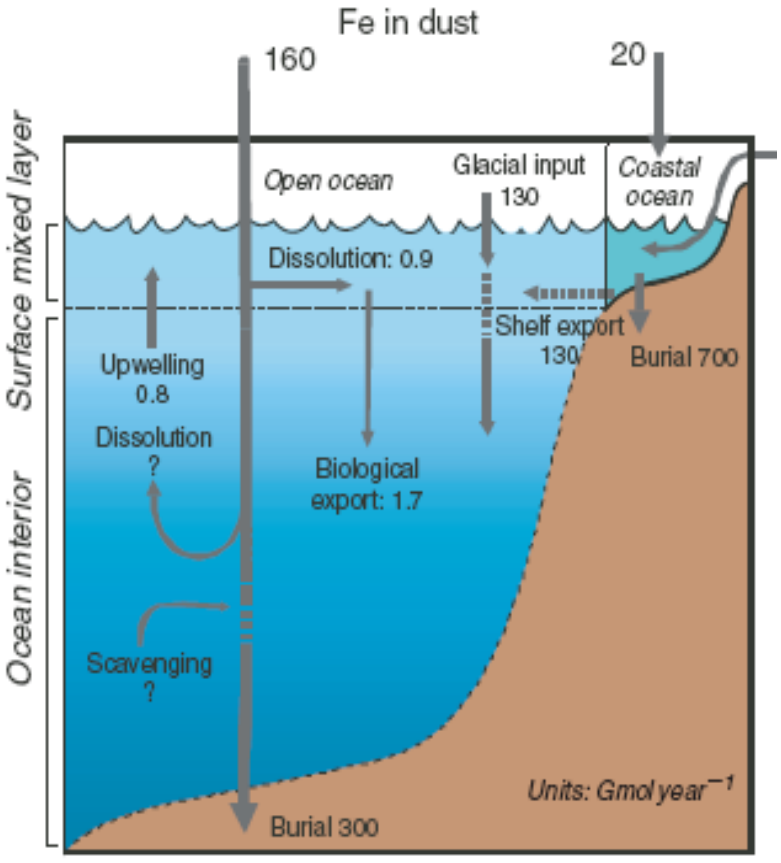


Figure 1.4. Simple box model of the global iron cycle. From Hunter and Boyd 2007.

The upper water column is dominated by siderophore-like ligands (Fig. 1.5) likely produced by bacteria. In contrast deep waters are dominated by a range of organic ligands (including breakdown products of chlorophyll such as tetrapyrrols) likely derived from the degradation of refractory substances (Barbeau et al. 2001; Hunter and Boyd 2007). Eukaryotic phytoplankton uses ferrireductase systems that preferentially access iron chelated by tetradentate porphyrins (Hutchins et al. 1999). In contrast bacteria, in particular cyanobacteria, efficiently assimilate iron bound to siderophores (Hutchins et al. 1999).

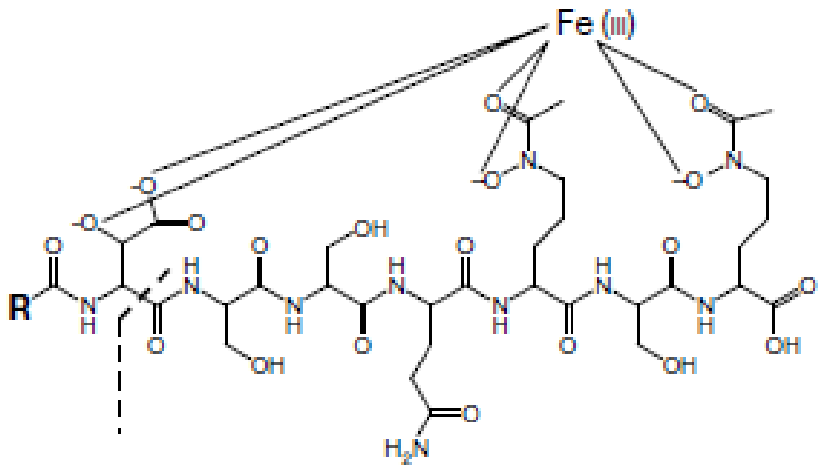


Figure 1.5. Example of ferric iron complexation with aquachelin, a marine siderophore. Modified from Barbeau et al. 2001.

Fe(III) oxides may encounter photo-induced reductive dissolution when in the photic zone which produces bioavailable ferrous iron to organisms in fresh and marine surface waters and accelerates the biogeochemical cycling of iron and other reactive elements (Mcnight 2004; Ozturk et al. 2004; Rijkenberg et al. 2005; Zuo 1995). Fe(III) photoreduction in seawater can be promoted by the presence of diatoms (Rijkenberg et al. 2008) and dinoflagellates (Shaked et al. 2002).

In addition non-dissimilatory Fe(III) reduction is thought to be the main mechanism for Fe acquisition by microalgae. Diatoms have been shown to produce superoxides (Kustka et al. 2005) and extracellular enzymes (Maldonado and Price

2000; Salmon et al. 2006; Shaked et al. 2005) which reduce Fe(III) to Fe(II).

Fe(II) has a very different behaviour from Fe(III) with its complexation being negligible compared to its oxidation at circumneutral and acidic pH (Rose and Waite 2003). At alkaline pH, inorganic complexation may occur with carbonate and hydroxyl anions leading to the formation of $FeCO_3$ (siderite) and Fe(II) hydroxides (Ussher et al. 2004).

1.4.4 Biogeochemistry of manganese

Manganese is the fifth abundant element in the Earth crust, and it is an essential micronutrient for most organisms: it is present in photosystem 2 of all photosynthetic organisms and can also be found in numerous enzymes and other proteins (Crowley et al. 2000; Sigel and Sigel 2000).

In seawater, manganese occurs in both particulate and dissolved forms. Particulate manganese is dominated by Mn(IV), with Mn(III) being also present. Under anaerobic conditions particulate manganese tends to be reduced to Mn(II) which is soluble in seawater (Tebo et al. 2007). Mn(II) is largely produced in the anaerobic sediment and released in the aerobic water column (Burdige 1993; Thamdrup 2000) where it is unstable and tends to be oxidised to particulate manganese (Stumm and Morgan 1993). The oxidation of Mn(II) in seawater is an autocatalytic process (Sung and Morgan 1981) which occurs at a very slow rate (Von Langen et al. 1996). Bacteria and fungi can enhance this oxidation process (Tebo 1991; Tebo et al. 2005).

Dissolved manganese was initially thought to exist in seawater only as Mn(II), however recent studies revealed that under low oxygen concentrations a significant proportion of dissolved manganese is present as Mn(III) (Johnson 2006; Luther et al. 1994; Trouwborst et al. 2006). Mn(III) is an intermediate of both Mn(IV) reduction (Duckworth and Sposito 2005a) and Mn(II) oxidation (Duckworth and Sposito 2005b; Webb et al. 2005) and can be stabilised by complexation with pyrophosphate (Kostka et al. 1995) and organics (Klewicki and Morgan 1998) including siderophores (Duckworth and Sposito 2005a; Duckworth and Sposito 2005b). Particulate manganese can be also reduced by photo-induced reduction, within the photic zone (Chase et al. 2005; Mcnight 2004).

1.5 Microbial colonisation of marine aggregates

1.5.1 Marine microbes

Prokaryote and eukaryote microbes are ubiquitous in the worlds' ocean, their size ranges from 0.2 to 200 µm and a wide morphological, phylogenetic and metabolic diversity occurs within them. Marine microbes are involved in the production, transformation and remineralisation of organic matter and mediate biogeochemical cycles (Sherr and Sherr 2000). A significant portion of marine microbes (prokaryotes and eukaryotes) have not yet been characterised by microbiologists, most of them being unculturable with the current techniques available.

Bacteria constitute an important domain of microbes, they are the most important biological component involved in the decomposition of OM in the ocean (Cho and Azam 1988) and are primarily distinguished as free-living (or bacterioplankton) and particle-attached bacteria. Free-living bacteria are usually smaller than particle-attached bacteria; they are thought to grow at very slow rates and to be adapted to the oligotrophic conditions occurring in large areas of the ocean. In contrast, particle attached bacteria are larger in size, and can grow rapidly when high nutrient concentrations occur in the environment (Giovannoni and Rappe 2000). Most of bacteria in seawater are not active but present in a senescent state. Only a small part of the microbial community can respond to an increase in substrate availability becoming active (Rappe and Giovannoni 2003).

1.5.2 Interactions of heterotrophic eukaryotes with marine particles

Marine snow constitutes an important ecological niche for several microorganisms: many metazoan swimmers, including copepods and larvae of other animals, actively detect and feed on marine snow particles and on microorganisms attached to it (Kiorboe 2000). In addition, a range of heterotrophic microorganisms are temporarily or permanently attached to aggregates and are involved in the degradation of organic matter (OM) and the biogeochemical cycling of elements. Protozoans, mainly ciliates and flagellates, are also present in marine snow (Turley and Mackie 1994; Zimmermann-Timm et al. 1998) and play an important role in the cycling of nitrogen and in the control of bacterial populations (Grossart and Ploug 2001; Kiørboe 2003; Ploug and Grossart 2000). Although fungi are important in the degradation of

detrital material in the marine environment (Fell and Newellm 1998), the colonisation of marine snow by fungi has never been investigated.

1.5.3 Prokaryotes attached to marine aggregates

Aggregate-attached bacteria (Fig. 1.6) have been studied extensively. Prokaryote abundance in aggregates exceeds that of seawater by 2 to 3 orders of magnitude (Delong et al. 1993; Simon et al. 2002; Turley and Mackie 1994). Microbial communities inside sinking and settled particles have therefore a significant impact on ocean element cycles. In addition the bacterial communities attached to marine aggregates are phylogetically different from free-living bacterial assemblages (Acinas et al. 1999; Delong et al. 1993; Hodges et al. 2005). In natural systems, the bacterial diversity of particle-attached bacteria is highly variable, and in different studies it has been reported to be either higher (Rooney-Varga et al. 2003) or lower (Ghiglione et al. 2007) than that of free-living bacteria. Molecular analyses of 16S rRNA gene diversity suggested that the most abundant bacteria present in marine snow belong to the groups of *Bacteroidetes* and *γ-Proteobacteria,* with *α-Proteobacteria* and *Planctomycetes* often accounting for lower proportions of the community (Abell and Bowman 2005; Bidle and Azam 2001; Delong et al. 1993; Grossart and Ploug 2000; Moeseneder et al. 2001; Rath et al. 1998; Selje and Simon 2003; Simon et al. 2002). However, as most pelagic bacteria are thought to be inactive (Rappe and Giovannoni 2003), it is not clear whether groups of aggregate-attached bacteria are active or senescent.

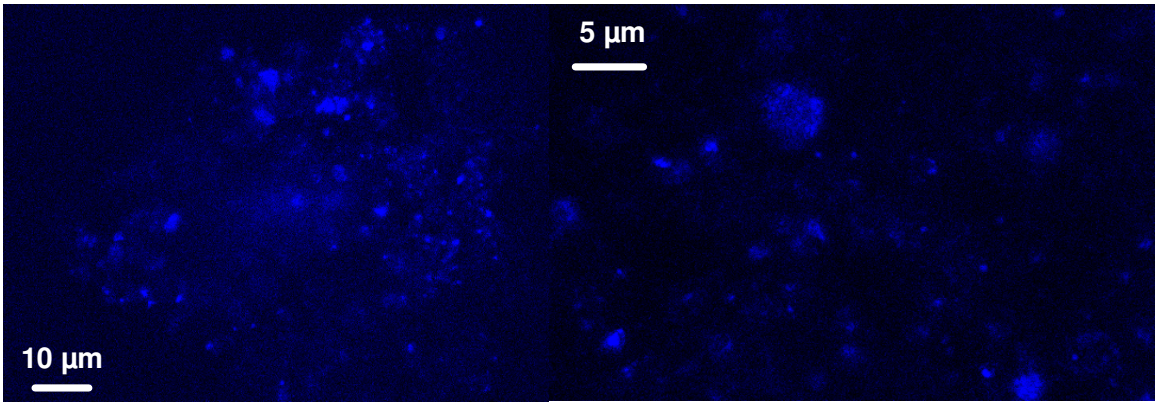


Figure 1.6. Fragment of artificial marine aggregate intensively colonized by bacteria that have been stained blue with the fluorochrome DAPI. The fragment was photographed using a confocal microscope.

The abundance and activity of particle-attached bacteria is highly variable in the marine environment (Turley and Stutt 2000) but can account for an important proportion of the total bacterial number in estuarine systems (Byron and John 2000; Zimmermann 1997) and occasionally in pelagic waters (Mevel et al. 2008). The abundance of attached bacteria on mucilaginous aggregates varies with aggregate size, with smaller particles supporting higher bacterial activities (Crump and Baross 2000; Ploug et al. 1999), and age, with younger particles being more colonised than older ones (Bongiorni et al. 2007). However, recent studies in the North West Mediterranean Sea have also shown significant seasonal and diel variations in the abundance and activity of bacteria colonising suspended particles (Ghiglione et al. 2007; Mevel et al. 2008).

Bacterial production rates in aggregates that were actively mixed using a plankton wheel, were reported to be up to 1 order of magnitude higher than in a static system (Grossart and Ploug 2001; Ploug and Grossart 1999; Ploug and Grossart 2000). Fluid flow around sinking aggregates is thus highly important for the chemical microenvironment and growth condition of the aggregate-associated bacteria and other microorganisms.

1.5.4 Ecological successions in marine snow

The rapid successional changes in microbial populations suggest that any labile matter available in newly formed aggregates is quickly consumed by bacteria (Alldredge and Silver 1988). The bacterial community attached to sinking or settled aggregates changes along with their degradation, such that populations adapted to metabolise the increasingly refractory compounds become increasingly dominant.

Microbial communities associated with marine snow undergo complex successional changes on time scales of hours to days, which significantly alter the chemical and biological properties of the particles (Alldredge and Silver 1988). The composition of bacteria attached to diatom aggregates incubated in the laboratory under dark conditions changed over time, with a decrease in *α-Proteobacteria* and an increase in *Bacteroidetes* and *γ-Proteobacteria* (Fig. 1.7) (Grossart and Ploug 2001; Tamburini et al. 2006). In contrast, *α-Proteobacteria* abundances increased along with γ-*Proteobacteria* in dark incubated mucilaginous aggregates (Zoppini et al. 2005).

Flocculation events generate microhabitats enriched in nutrients, and promote the growth of bacteria and consequently protists: aggregates were reported to be

initially colonised by small bacterivorous protists such as choanoflagellates, then by sarcodines and ciliates and finally by large carnivore protists such as amoebae and large flagellates (Artolozaga et al. 1997). The high number of protists registered in aggregates was suggested to be an efficient way to transfer energy to higher trophic levels (Artolozaga et al. 1997).

Control on bacterial colonisation by zooplanktonic grazers depends on many factors such as colonisation, detachment, growth and predator-prey interactions on the surface of the particle, effects of sinking on colonization rates and the fractal nature of aggregates (Kiørboe 2003). Bacterial colonisation on particles was found to be controlled by flagellate grazing and a rapid turnover on microbial populations due to the continued colonisation and detachment was predicted (Kiørboe 2003). Marine aggregates were found to be highly enriched in ciliates and slightly enriched in flagellates (Grossart et al. 2003). Aggregation was thus reported to enhance protozoan grazing of bacteria (Alldredge and Silver 1988; Biddanda 1986), although TEP was also suggested to provide bacteria with a refuge against grazers (Alldredge et al. 1993).

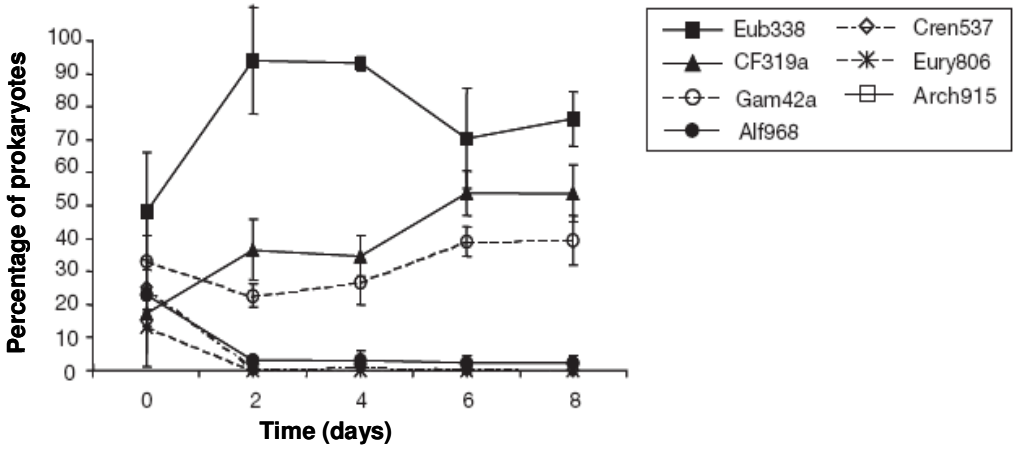


Figure 1.7. Example of bacterial succession in a set of diatom aggregates incubated the laboratory under dark conditions. Eub338: Bacteria; CF319a: Cytophaga–Flavobacter cluster of the Cytophaga–Flavobacter–Bacteroides division; Gam42a, Alf968: γ-subclass and α-subclass of Proteobacteria; Cren537: Crenarchaea; Eury806: Euryarchaea; Arch915: Archaea. Modified from Tamburini et al. 2006.

1.6 Degradation of marine particles

1.6.1 Freshness and lability of organic matter in aggregates

The organic matter present in seawater has a highly heterogeneous composition, a range of compounds, including cellulose and terrestrially derived lignin, are very refractory to chemical and microbial degradation and can persist in seawater for long periods. In contrast a number of compounds can be rapidly degraded and remineralised by microorganisms. The organic matter in seawater undergoes a number of compositional changes associated with zooplankton reworking and microbial degradation.

In previous studies the organic matter in seawater has been divided into four classes (Lee et al. 2004; Wakeham et al. 1997b) according to their distribution across the water column:

- Chlorophylls and polyunsaturated fatty acids, which are very labile components. They are diagnostic of plankton in surface waters and are rapidly lost from particles in the euphotic zone.
- Zooplankton diagnostic compounds such as oleic acid and C_{27} sterols. Phytoplankton derived pheophorbide and C_{28} sterols. These compounds usually exhibit their highest concentration in deep waters.
- Compounds of bacterial origin including branched fatty acids and higher plants derived C_{29} sterols. They usually reach the highest concentration at the water-sediment interface.
- Compounds characteristically resistant to microbial degradation or produced in the sediment by anaerobic processes, they accumulate inside the sediment. and include the long-chain fatty acids, long-chain fatty alcohols, alkanes and alkenones, squalene and glicine.

1.6.2 Indicators of diagenetic status of organic matter

The concentrations of photosynthetic pigments, the most important of which is chlorophyll a (Chl-a), are measured to evaluate phytodetritus freshness. Chl-a is constituted by a magnesium atom surrounded by a chlorin ring which is linked to a phytol chain (Fleming 1967). It is a very labile compound and tends to be rapidly degraded. A combination of degradation products of chlorophyll, usually dominated by

pheophorbide a (Phaeo-a) is often found in sinking particles and phytodetritus (Beaulieu 2002). Specifically, the percentage contribution of Chl-a to the total chloropigments indicates the freshness of phytodetritus (Duineveld et al. 2000) and the Phaeo-a to Chl-a ratio has been used to indicate the quality of deep-sea phytodetritus (Rice et al. 1986). In addition the ratio between the two dominant microalgal carotenoids, fucoxanthin and peridinin, over their degradation products, fucoxantinol and peridinol have often been used as indicators of bacterial degradation (Lee et al. 2004; Wakeham and Lee 1993).

The ratios between unaltered labile amino acids (e.g. arginine, aspartic and glutamic acid) and their degradation products (ornithine, β-alanine and γ-aminobutyric acid, respectively) are also used to infer the degradation status of organic matter in seawater (Ittekkot et al. 1984; Wakeham and Lee 1993). Moreover muramic acid is a component of prokaryotic cell membranes and has been used to estimate bacterial biomass associated with marine particles (King and White 1977; Moriarty 1977).

1.6.3 Alteration of organic matter with depth

As the depth increases the fluxes of POM, fatty acids and amino acids decrease, most of the organic carbon is recycled in the first few hundreds meters and only a small percentage of it reaches the seafloor (Fig. 1.8) (Knauer and Martin 1981; Knauer et al. 1979; Lee et al. 1988; Wakeham and Lee 1993). Amino acids can make up 25-50% of POM in surface water; while sinking most of them are easily degraded (Lee and Cronin 1984; Lee et al. 2004).

The relative proportion of organic carbon that is chemically uncharacterisable at the molecular level (not easily recognisable as a specific compound by standard techniques) increases with depth and accounts for most of the bulk carbon in the sediment (Fig. 1.8) (Lee et al. 2004). Aggregates while sinking tend thus to be depleted in fatty acids and amino acids whereas the relative proportion of carbohydrates is constant (Lee et al. 2004). Substantial alterations of organic matter thus occur when aggregates reach the seafloor.

1.6.4 Fate of phytodetritus on the seafloor

The formation of a phytodetritus layer above the seafloor has often been reported in the ocean (Beaulieu 2002). Its remineralisation causes a solute flux from sediment to the water column. In shallow waters phytodetritus may be resuspended by

currents encountering a further mineralization (Beaulieu 2002).

Fresh phytodetritus abundantly accumulates where metabolic activity of microorganisms is low, especially in hypoxic and anoxic basins such as Baltic Sea and Black Sea (Beaulieu 2002). Detritus accumulation on the sea floor and consequent high organic matter consumption and biological oxygen demand are likely to drive the environment into anoxia.

1.6.5 Degradation of lipids in marine particles

The bacterial community attached to sinking or settled aggregates changes along with their degradation, such that populations adapted to metabolise the increasingly refractory compounds become increasingly dominant. Simultaneously, microbial processes affect the chemical structure of marine aggregates, resulting in a loss of total OM, a relative decrease in both amino acids and lipids and a relative increase in uncharacterised OM over time (Lee et al. 2004; Panagiotopoulos et al. 2002; Wakeham et al. 1997b).

Sinking aggregates are initially dominated by lipids derived from phytoplankton and sometimes higher plant debris. Subsequent zooplankton grazing and microbial reworking generate further lipids following a selective consumption of labile components, and produce compounds which may be taxonomically specific biomarkers (Lee et al. 2004). The lipid composition of natural aggregates has been extensively investigated in sinking marine snow (Burns et al. 2004; Burns et al. 2003; Conte et al. 1998; Goutx et al. 2007; Kiriakoulakis et al. 2001; Marchand et al. 2005; Tolosa et al. 2004; Wakeham et al. 1997a) as well as in suspended mucilaginous flocs (Blazina et al. 2005; Najdek 1996; Najdek et al. 2002). The fatty acid component is typically dominated by hexadecanoic (C16:0, Fig. 1.9), hexadecenoic (C16:1), octadecanoic (C18:0) and octadecenoic (C18:1, Fig. 1.9) acids which are widespread in all living organisms and commonly attributed to phytoplankton and zooplankton (Killops and Killops 2005; Tolosa et al. 2004; Volkman 1986; Zimmerman and Canuel 2001). The neutral fraction of the lipids typically includes alcohols containing 13 to 28 carbon atoms, phytol, and a wide range of sterols containing 26 to 30 carbon atoms (Burns et al. 2004; Kiriakoulakis et al. 2001; Marchand et al. 2005; Wakeham et al. 1997a).

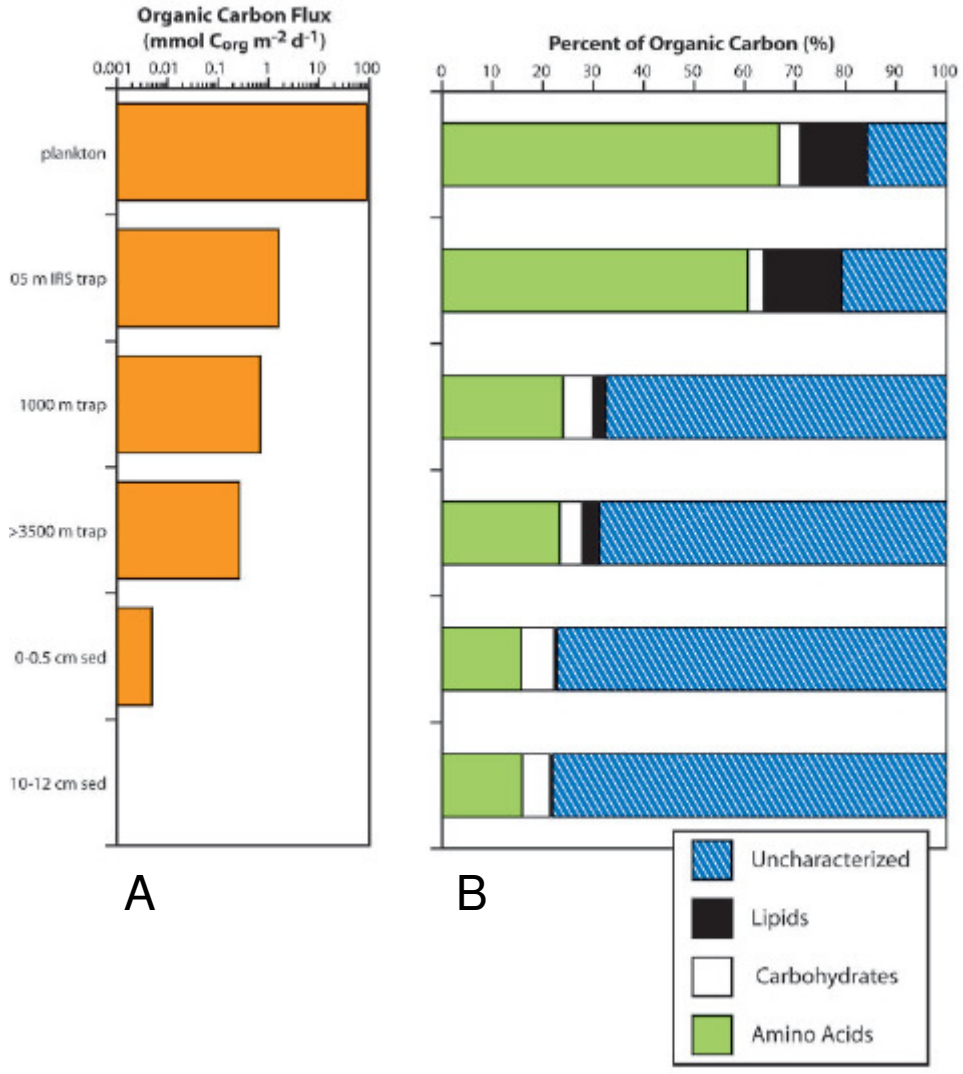


Figure 1.8. (**A**) Flux of organic carbon and (**B**) major biogeochemical classes, present in the plankton, sediment traps and sediment in the Equatorial Pacific Ocean. Modified from Lee et al. 2004.

Phytoplankton derived polyunsaturated fatty acids (PUFA) eicosapentaenoic (C20:5, Fig. 1.9) and docohexanoic (C22:6) acids are abundant in fresh aggregates (Goutx et al. 2007; Wakeham et al. 1997a) or flocs suspended in the photic zone and, therefore, hosting an active photosynthetic population (Blazina et al. 2005; Najdek 1996). Sterols are more resistant than fatty acids to degradation, being found in oxic and anoxic deep waters (Kiriakoulakis et al. 2001; Wakeham 1995; Wakeham et al. 1997a), and the phytoplankton-derived C_{28} and C_{30} sterols (Nichols et al. 1984; Volkman 1986) and zooplankton-derived C_{27} sterols (Volkman 1986) often have been used to infer the origin of OM in marine particles (Tolosa et al. 2003; Tolosa et al. 2008; Volkman et al. 2007; Yoshinaga et al. 2008; Zimmerman and Canuel 2001).

Lipids specific to bacterial membranes such as branched chain fatty acids (Fig. 1.9) (Kaneda 1991; Parkes and Taylor 1983) and hopanoids (Rohmer et al. 1984) are common in marine particles and attributed to bacteria (Yoshinaga et al. 2008; Zimmerman and Canuel 2001). Slow sinking marine snow typically contains a higher bacterial and zooplankton signature compared to fast sinking flocs (Goutx et al. 2007; Yoshinaga et al. 2008). Saturated straight chain fatty acids with more than 20 carbon atoms (*n*LCFA) in marine particles and sediment are typically ascribed to waxes of higher plants and are often associated with terrestrial inputs of OM (Cranwell 1982; Meyers 1997).

While marine aggregates sink in the oxic water column, PUFAs are quickly depleted being present in very low concentrations in deep sea particles (Burns et al. 2004; Wakeham et al. 1997a); in contrast the relative proportion of *n*LCFA and branched-chain fatty acids (brFA) increase, whereas sterols, monounsaturated fatty acids (MUFA) and straight short-chain fatty acids (12 to 20 carbon atoms, *n*SCFA) concentrations are constant reflecting the increase in bacteria and zooplankton derived components and the decrease in algal lipids (Kiriakoulakis et al. 2001; Wakeham et al. 1997b). Similar trends were also found in degrading *Thalassiosira* phytodetritus aerobically incubated in the laboratory under dark conditions (Harvey and Macko 1997).

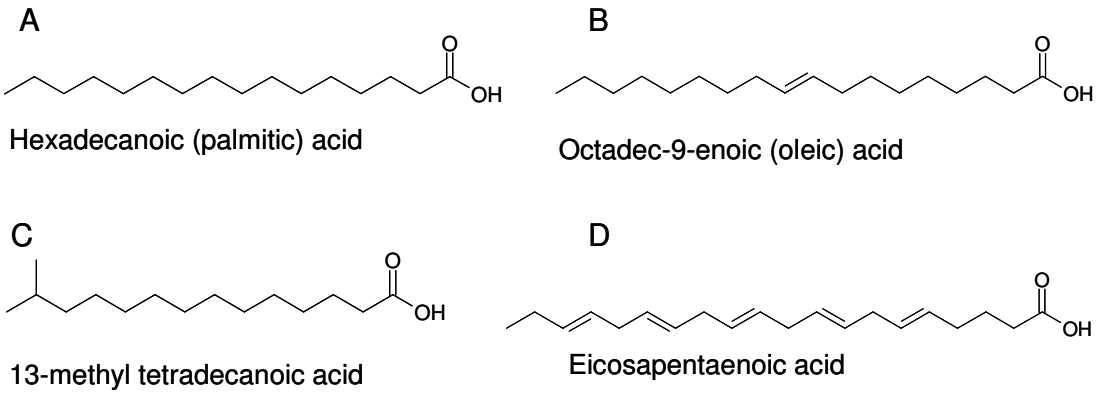


Figure 1.9. Fatty acids common in marine environment. (**A**) saturated straight chain, (**B**) monounsaturated, (**C**) saturated branched chain and (**D**) polyunsaturated fatty acids.

1.6.8 The particle decomposition paradox

On marine snow, the carbon demand of attached bacteria is too small to consume all the organic carbon which aggregates lose in a reasonable time (Simon et al. 1990). This discrepancy between the observed rapid decrease in POM and the dense populations of microorganisms on marine snow and the surprisingly low bacterial carbon demand of the attached bacteria has been stated as a "particle decomposition paradox" in the ocean (Karl et al. 1988). The particle decomposition paradox was explained assuming that part of marine snow is grazed by zooplankton and thus put into the food chain becoming available to higher trophic levels and that a large fraction of carbon and nutrients is released from aggregates as DOM and is processed and assimilated by free-living bacteria in the surrounding water rather than by the microbial community attached to the particles (Karl et al. 1988). Successive studies demonstrated faster respiration rates for diatom aggregates (Grossart and Simon 1998) and detritus obtained from gelatinous zooplankton (Sempere et al. 2000) suggesting a high variability in the consumption of organic matter in aggregates (De La Rocha and Passow 2007).

Aggregate degradation involves thus a release of DOM which is partially decomposed by free-living bacteria in the surrounding seawater (Simon et al. 2002). Aggregate-attached bacteria release enzymes which break down POM producing DOM. The DOM production rate was found to exceed the ability of aggregate-attached bacteria to consume it (Smith et al. 1992) so that sinking particles leave a rich plume of DOM behind them. Free-living bacteria in the plume consume the DOM present and grow at very high rates (Azam and Long 2001; Kiørboe and Jackson 2001).

1.7 Boundary layers and anoxia in marine aggregates

Intense bacterial metabolism occurs inside the aggregates, leading to a partial degradation of the organic matter present and thus the release of dissolved compounds to the surrounding seawater (Azam and Long 2001) in a form that can be again assimilated by phytoplankton. The degradation of organic matter may thus lead to the release of dissolved elements including nitrogen and phosphorus as well as metals such as iron and manganese.

1.7.1 Implications for microbial respiration in aggregates

The microbial processes occurring in sinking aggregates lead to strong heterogeneities in the distribution of solutes. Microbial respiration in aggregates may be important enough to deplete the particles of oxygen, if the rate of oxygen consumption in aggregates exceeds that of oxygen diffusion. The production of CO_2 associated with aerobic respiration affects the internal pH of aggregates. Gradients in the pH and the concentration of solutes are thus generated (Fig. 1.10) (Alldredge and Cohen 1987; Ploug and Jorgensen 1999; Ploug et al. 1997).

The size of the oxygen boundary layer in aggregates is controlled mainly by the rate of solute diffusion through it as well as the dynamic of the water column. Sinking aggregates can be a source of recycled nutrients as well as trace metals, and therefore lead to heterogeneous distributions of these elements within and around the particles (Alldredge and Cohen 1987). They have been reported to have a thin boundary layer due to different solute gradients (Alldredge and Cohen 1987; Alldredge and Silver 1988; Kiørboe and Jackson 2001; Ploug et al. 1999; Ploug and Jorgensen 1999; Shanks and Reeder 1993).

The rate of consumption of oxygen in aggregates is related to the biomass present and increases with increasing aggregate volume. In contrast, the rate of diffusion of oxygen to the centre of aggregates from the surrounding seawater is proportional to the aggregate surface. Large particles have thus a low surface/volume ratio and are more likely to become anoxic compared to small particles.

Studies based on microelectrodes (Alldredge and Cohen 1987) and chemical redox indicators (Shanks and Reeder 1993) demonstrated the presence of persistent oxygen and pH gradients around and within marine snow. The pH was found to decrease from 8.2 in the surrounding seawater to 7.4 in the centre of an anoxic aggregate (Ploug et al. 1997). Diatom aggregates suspended by a net-jet flow system showed also decreasing oxygen concentrations towards the centre (Fig. 1.10) (Ploug and Jorgensen 1999). The concentration of oxygen in the centre of aggregates was found to decrease when the aggregates were transferred from the net-jet flow system to a simple container (Fig. 1.10, Ploug and Jorgensen 1999).

While organic matter is degraded in aggregates the microbial respiration causes a decrease in the pH, phosphate is released to the surrounding seawater and the low pH conditions (~ 7) can limit the dissolution of biogenic silica (Passow et al. 2003).

Ammonium, likely originated from the degradation of organic matter, was found to accumulate in descending particles collected from surface waters (Karl et al. 1984). The same particles were then depleted in ammonium by nitrification at greater depths (Karl et al. 1984).

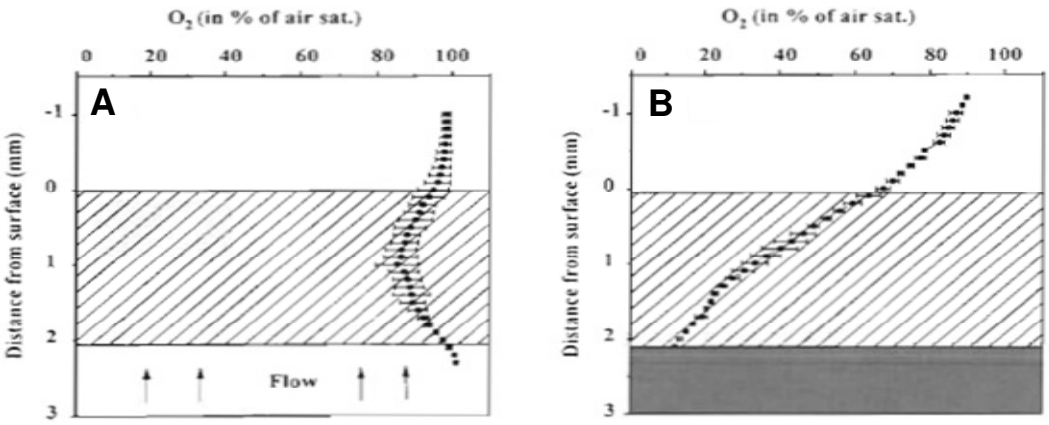


Figure 1.10. Oxygen distribution in a (**A**) sinking aggregate and (**B**) when it is sitting on a solid surface. From Ploug and Jørgensen 1999.

1.7.2 Reduced species and anaerobic bacteria in aggregates

A range of reduced species which are not present at important concentrations in the oxygenated water column, have been found to be associated with marine snow. Methane has been found in faecal pellets and sinking particles in the North Pacific (Karl and Tilbrook 1994). Marine particles were suggested to be a source for the paradoxically high concentrations of methane present in oceanic waters (Brooks et al. 1981; Burke et al. 1983; Rusanov et al. 2004; Seifert et al. 1999). Consistent with this methanogenic bacteria were found to be present in marine particles (Bianchi et al. 1992; Marty 1993). Methanogenic bacteria appear to grow in the digestive tract of zooplankton and to survive in faecal pellets and aggregates. However a recent study highlighted an additional (and more likely) source for the methane supersaturation observed in seawater: methane was demonstrated to be formed aerobically from the microbial breakdown of phosphonate (Karl et al. 2008).

Sulfide has been detected in both laboratory-made and field-collected marine aggregates (Shanks and Reeder 1993) and sulfate-reducing bacteria (SRB) have been sequenced from both riverine (Bockelmann et al. 2000; Grossart and Ploug 2000) and

marine aggregates (Grossart and Ploug 2001). Aggregates may thus contribute to the high concentrations of sulfide occasionally detected in aerobic waters (Cutter and Krahforst 1988; Luther and Tsamakis 1989).

A key role is played by aggregates in the global nitrogen cycle: the respiration of nitrate was demonstrated to occur in aggregates settled in aerobic bottom water of the Pacific (Wolgast et al. 1998). Moreover cyanobacterial aggregates collected from the Baltic Sea were found to contain nitrate and nitrite reductase genes although the denitrification activities were found to be negligible (Tuomainen et al. 2003). Recent molecular analyses on particle-attached microorganisms from the Namibian Upwelling System revealed the presence of anaerobic ammonium oxidisers (Anammox) in both anaerobic and aerobic environments (Woebken et al. 2007). Anammox is likely to occur in the anoxic microenvironments inside aggregates; ammonium produced by *Bacteroidetes*-mediated amino acid degradation and nitrite produced by *Crenarchaeota*-mediated nitrification were suggested to be used as sources for anaerobic ammonium oxidation (Woebken et al. 2007).

1.8 Anaerobic degradation of organic matter

1.8.1 Electron acceptors alternative to oxygen

Across a redoxcline, as the oxygen concentration decreases microorganisms use a succession of alternative electron acceptors to oxidise organic matter (Fig. 1.11) (Stumm and Morgan 1993). Oxidation of organic matter is observed to occur first by reduction of oxygen, followed by reduction of nitrate to nitrite and nitrite to gaseous nitrogen (denitrification sensu stricto) (Stumm and Morgan 1993; Zumft 1997). Reduction of MnO_2 should occur at about the same pE level followed by dissimilatory nitrate reduction to ammonium (DNRA) (Zumft 1997) and at a pE higher than that required for dissimilatory Fe(III) reduction to Fe(II) (Stumm and Morgan 1993). Fermentation occurs at different pE levels. When a sufficiently negative pE level is reached reduction of SO_4^{2-} and methanogenesis may occur almost simultaneously (Stumm and Morgan 1993). Ordered successions of anaerobic communities that exploit these reactions for their metabolism have been largely described in the sediment (Canfield et al. 1993; Finke et al. 2007; Jensen et al. 2003; Thamdrup 2000; Thamdrup et al. 2000) and may also be present inside marine aggregates, becoming active in those microzones where oxygen has been depleted by significant aerobic respiration.

The presence in marine snow of a range of reduced species (Karl et al. 1984; Karl and Tilbrook 1994; Shanks and Reeder 1993) and the occurrence of different reducing processes (Woebken et al. 2007; Wolgast et al. 1998) highlights the very low pE which may occur inside these aggregates. Anaerobic bacteria are likely involved in the mentioned reducing processes under suboxic or anoxic conditions. The presence of anaerobic microorganisms in marine snow has been poorly investigated and has mainly focused on SRB (Bockelmann et al. 2000; Grossart and Ploug 2001; Grossart and Ploug 2000) and thus the importance of direct bacterial reduction of, for example Fe(III) or Mn(IV), in comparison to the indirect reduction (e.g. via biologically derived sulfide) is not yet known.

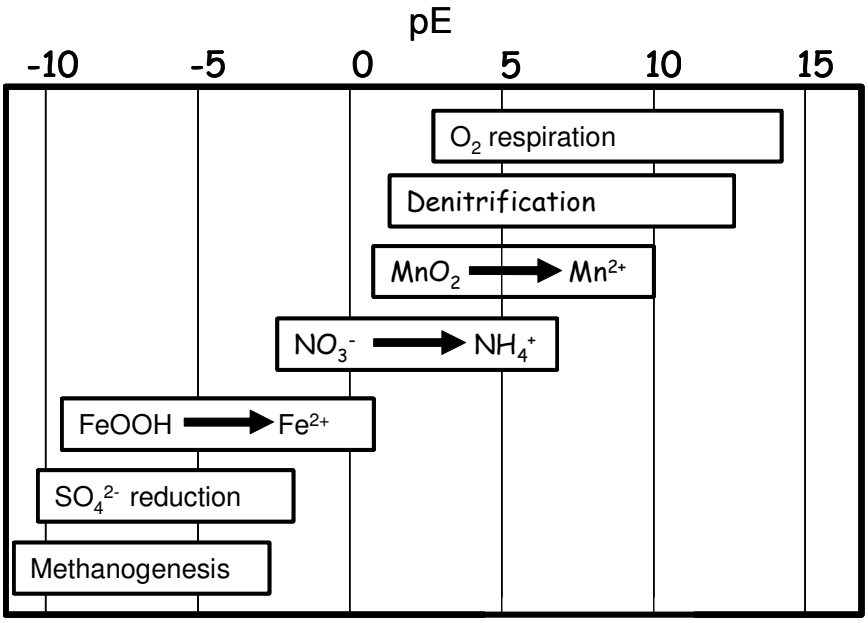


Figure 1.11. Sequence of microbially mediated redox processes. Modified from Stumm and Morgan 1993.

1.8.2 Fermentation and its implications on trace metal cycling

A range of microorganisms are able to conduct fermentation under hypoxic and anoxic conditions and have been described in sediment (Rossello-Mora et al. 1999; Rusch et al. 2005). Some fermentation products such as ethanol, acetate, lactate and formate may be used as electron donors for the dissimilatory reduction of Fe(III) (Lloyd 2003; Lovley et al. 2004). Moreover other microorganisms not directly involved in substantial Fe(III) reduction may also use Fe(III) to oxidise reduced nicotinamide

adenine dinucleotide (NADH) produced in excess during the fermentation process (Dobbin et al. 1999; Lovley 2000).

1.8.3 Microbial reduction of iron and manganese

A wide range of prokaryotes can conserve energy though the reduction of Fe(III) to Fe(II), many of these microorganisms are also able to grow through the reductive dissolution of Mn(VI) (Lloyd 2003; Lovley 1991; Lovley et al. 2004; Thamdrup 2000). Ferric iron is the dominant electron acceptor in many subsurface environments, the reduction of Fe(III) may have been an early form of respiration on Earth (Lovley and Chapelle 1995). Iron and manganese biologically mediated reduction in sediments have been well described (Canfield et al. 1993; Thamdrup 2000), bacteria can gain energy from the reduction of Fe(III) and Mn(IV) using a wide range of organic substrates (Lloyd 2003; Lovley et al. 2004). The most common electron donors are organic acids such as acetate and lactate (Lovley et al. 2004). Inorganic substrates such as H_2S and H_2 can also act as electron donors for Fe(III) and Mn(IV) reductions (Burdige and Nealson 1986; Burdige 2006). Similarly the reduction of Mn(IV) can also be associated with the Fe(II) oxidation as reported for marine and lacustrine sediments (Thomsen et al. 2004; Wijsman et al. 2002).

Some SRB including *Desulfovibrio* and *Desulfotomaculum* species have also been shown to reduce Fe(III) and other metals (Coleman et al. 1993; Li et al. 2004; Li et al. 2006; Lloyd et al. 2001; Lovley 1993; Tebo and Obraztsova 1998).

The first organisms that were shown to conserve energy for growth through the reduction of Fe(III) or Mn(IV) were *Shewanella oneidensis* and *Geobacter metallireducens* (Lovley 1989; Lovley et al. 1987; Myers and Nealson 1988).
The mechanisms of Fe(III) and Mn(IV) reduction have been studied in detail in these two species and include direct electron transfer on the outer cell membrane or by secreted electron shuttles (Fig. 1.12), or nanowires protruding from the cell surface (Bonneville et al. 2006; Gorby et al. 2006; Lloyd 2003; Lovley et al. 2004; Reguera et al. 2005; Von Canstein et al. 2008). However the presence in marine snow of Fe(III)- and Mn(IV)-reducing bacteria has never been investigated.

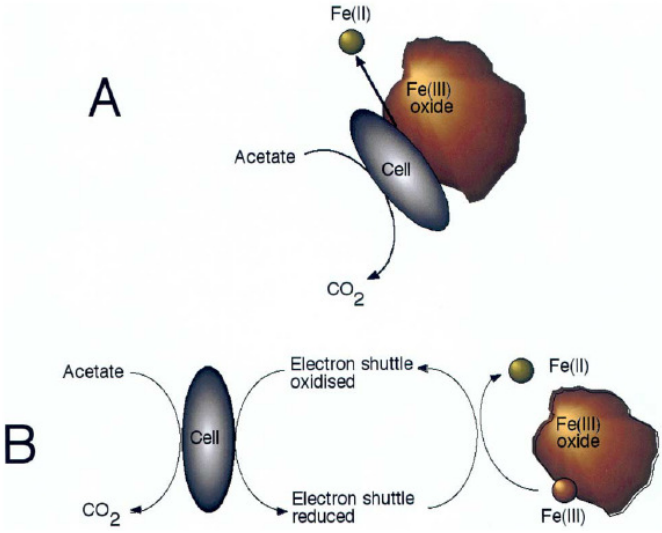


Figure 1.12. Mechanisms of reduction of insoluble Fe(III) oxides, via (A) direct contact with the surface of the cell or (B) an extracellular electron shuttle. Image from Lloyd 2003.

1.9 Aims and objectives

1.9.1 The BIOTRACS project

The present PhD is part of a European project focused on the biotransformations of trace elements in aquatic systems (BIOTRACS). The BIOTRACS project aims to evaluate the interactions of microbiological and geochemical processes. Microbial organisms interact with trace element redox transformations in all aquatic systems involving profound implications for the global carbon cycle and the fluxes of carbon dioxide within the Earth-Ocean system. These bio-transformations, central to many key environmental processes, are poorly understood. An interdisciplinary approach is thus required to understand the links between microbiology and aquatic geochemistry.

The overall aim of the present PhD is to better understand the mechanisms of trace element recycling from particles in the ocean water column. Mn and especially Fe are essential for biological growth in the ocean; their recycling from particles may be an important mechanism for maintaining phytoplankton populations in the upper ocean. Thus there may be implications for biological controls on carbon transfers to the deep ocean.

1.9.2 Objectives

This thesis aims to examine the impact of marine aggregates on the cycling of nutrients and trace metals and to investigate the microbial community involved in these processes. The following hyptheses were tested in this research:

(1) the presence of an oxygen gradient within marine aggregates leads to the reduction of iron and manganese; (2) these reducing processes are driven by anaerobic bacteria which are present within marine particles.

(3) most of the organic matter present in aggregates is degraded aerobically

(4) Artificially generated aggregates undergo compositional changes in lipids similar to those reported for natural particles sinking in oxic waters.

Specific objectives are listed as follows.

- To assess the presence of anoxic microzones and the occurrence of reducing processes, within aggregates settled on the seafloor.
- To evaluate the impact of aggregates suspended in the water column, on the release of reduced iron and manganese.
- To investigate the presence, in marine aggregates, of anaerobic bacteria likely involved in the production of reduced species.
- To assess the compositional changes in fatty acid composition during the degradation of aggregates with particular attention devoted to anaerobic biomarkers.
- To understand the impact of phytodetritus deposition on the seafloor to the cycling of iron and manganese.

Chapter 2. Materials and methods

For this PhD work, experiments were carried out at National Oceanography Centre, Southampton (NOCS), Organic Geochemistry Unit (OGU), University of Bristol and School of Earth, Atmospheric and Environmental Sciences (SEAS), University of Manchester.

2.1. Artificial aggregates as model particles for biochemical studies

Due to the difficulty in the prediction of natural flocculation events, successful collection of fragile marine snow, and correct manipulation and transport, studies on marine snow are often carried out on model particles formed in the laboratory. Artificial marine snow is usually obtained by slowly rotating seawater that is rich in senescent phytoplankton on a roller table for few hours (Shanks and Edmondson 1989), thus generating the velocity shear necessary for the flocculation of particles into larger aggregates. A number of studies including aggregation experiments (Ziervogel and Forster 2005), microscale measurements inside flocs (Ploug and Jorgensen 1999; Ploug et al. 1997), biogeochemical studies of silica dissolution (Moriceau et al. 2007; Passow et al. 2003), and estimates of bacterial activities (Azua et al. 2007) have been carried out on artificial marine snow formed using a roller table.

2.2. Formation of artificial marine aggregates

2.2.1 Cleaning procedures

All the plasticware (containers, tubing, pipettes) used to collect seawater, cultivate phytoplankton, form artificial aggregates and consequently manipulate them for the determination of Fe and Mn, were made in polytetrafluoroethylene (Teflon), polyvinyl chloride (PVC) or polycarbonate and were washed one week in a 2% Neutrocon (Decon Laboratories Ltd., Hove, East. Sussex, UK) solution and 3-4 days in 10% HCl (Fisher, UK) to avoid any trace metal contamination from the containers. In addition the plasticware was sterilised by autoclaving (22 minutes, 120 ºC) before being used to prevent microbial contamination.

2.2.2 Method overview

The formation of marine aggregates in the ocean occurs when the concentration of plankton and POM are high, and the particles involved are sticky and move at a precise shear velocity (Engel 2000; Passow and Alldredge 1995; Simon et al. 2002) such that the coagulation of particles after collision exceeds their disruption. The flocculation of living and dead cells into larger aggregates usually occurs during the decay of phytoplankton blooms (Alldredge et al. 2002; Engel 2000; Prieto et al. 2002). In the present research artificial marine aggregates were formed in the laboratory (Figs 2.1-2.2) using a roller table and following published methods (Shanks and Edmondson 1989).

Artificial marine aggregates were made from senescent phytoplankton material of mixed populations as well as four selected species *Thalassiosira weissflogii*, *Alexandrium tamarense*, *Phaeocystis globosa* and *Chaetoceros gracilis* (Appendix 1). *Thalassiosira* species are often involved in phytoplankton blooms leading to aggregation events (Alldredge et al. 2002; Kiorboe et al. 1996; Tiselius and Kuylenstierna 1996) and were used in previous studies to form artificial aggregates (Grossart et al. 2006a; Grossart et al. 2006b; Moriceau et al. 2007). In addition *Alexandrium, Phaeocystis* and *Chaetoceros* species are often involved in phytoplankton blooms (Anderson 1997; Jago et al. 2007; Kim et al. 2002; Kremp et al. 2009; Lee et al. 2003; Oyama et al. 2008; Seuront and Vincent 2008).

2.2.3 Cultivation of phytoplankton strains

Phytoplankton strains were purchased from Plymouth Culture Collection or obtained from Dr DA Purdie at NOCS (Table 2.1). Strains of *T. weissflogii* and *A. tamarense*, *Phaeocystis globosa* and *Chaetoceros gracilis* were used in the present work (Table 2.1).

Table 2.1. List of the strains cultivated in the present work.

Strain ID	Species	Provenance	Samples obtained
PLY 541	*Thalassiosira weissflogii*	MBA*	T3, TA1, TA2, TA7
PLY 173	*Alexandrium tamarense*	MBA	A1, A3, TA1, TA2. TA7
PLY 147	*Phaeocystis globosa*	MBA	P1, P2, P3, P4
PLY 666	*Chaetoceros gracilis*	MBA	Co1, Co2, Co3, Co5, Co6, Co7

* The Marine Biological Association of the United Kingdom, Citadel Hill, Plymouth, PL1 2PB Devon UK.

All cultures were maintained at the NOCS, in f/2 medium (Guillard 1975) or f/20 medium (obtained by diluting 10 fold f/2 medium in seawater). Cultures were kept in 0.25 or 0.5L flasks and 1 to 2L tanks at 20 ºC and a 12:12 light:dark cycle. The f/2 medium was prepared by diluting a marine water enrichment solution purchased from Sigma Aldrich (GG 9903) in 5-µm filtered and autoclaved seawater to reach the typical concentrations of the medium (Guillard 1975). The f/2 medium contains a mixture of inorganic nutrients, trace metals and vitamins (Appendix 1): nitrates, nitrites, phosphates and silicates are present at concentrations exceeding by one order of magnitude those naturally present in Southampton Water and in the Solent (Howard et al. 1995; Iriarte and Purdie 1994). The concentrations of iron and manganese are instead very low relative to those reported for the same coastal system (Fang 1995; Head 1971). The f/2 medium also contains organics such as ethylenediaminetetraacetic acid (EDTA), B_{12} vitamin, biotin and thiamine (Appendix 1), but none of these are likely to affect the lipid characterisation which was carried out on some of the aggregates.

The strains were cultivated until the cells reached the stationary-state phase of their growth (Fig. 2.1), when the cells are reported to release the highest number of exopolymers (TEP) involved in flocculation (Engel 2000; Passow and Alldredge 1995; Prieto et al. 2002). The stationary growth phase was determined after daily observations and cell counting using a light microscope "Leika DM IRB/E".

2.2.4 Cultivation of mixed phytoplankton assemblages

Cultures were also obtained from mixed phytoplankton assemblages harvested from Southampton Water and the Solent (Table 2.2). The seawater was collected, and filtered using 20-µm Whatman® cellulose filters, to remove large zooplankton. The marine water enrichment solution (Sigma Aldrich, UK) was added to obtain typical f/2 concentrations (Guillard 1975). The phytoplankton assemblages were observed under the same light microscope as above and they were often dominated by diatoms. The mixed phytoplankton assemblages were cultivated until the number of total cells became constant over time, reflecting a senescent state (stationary growth phase) of most of the species present (Fig. 2.1).

Using a seawater enrichment obtained from a natural sample rather than monoclonal phytoplankton cultures as background material for the formation of

artificial aggregates, makes the mesocosm experiment more realistic and the artificial aggregates produced more comparable to natural particles.

Table 2.2. List of the enrichment cultures obtained from mixed phytoplankton assemblages.

Sample	Provenance*	Collection date	Aggregates obtained
ENR1	Southampton Water	10/04/2006	N1, N2, N5, N6, N7
ENR3	Southampton Water	23/01/2006	N3, N4
ENR10	Southampton Water	27/04/2006	N10
ENR14	Southampton Water	14/06/2006	N14
ENR15	Southampton Water	14/09/2006	N16, N17, N18, N19, N20
ENR16	Solent Water	09/07/2007	Co1, Co2, Co3
ENR18	Solent Water	02/10/2007	Co5, Co6, Co7
ENR21**	Solent Water	17/11/2007	A, B, C, D, E, F, G

* Seawater was collected and marine water enrichment solution (Sigma) was added until reaching typical f/2 (Guillard 1975) concentrations in order to promote the growth of phytoplankton assemblages naturally present in seawater.

** In this enrichment the marine water enrichment solution (Sigma) was not added, but the seawater was fertilised with 880 µM $NaNO_3$, 100 µM $Na_2SiO_3 \cdot 9H_2O$ and 35 µM $NaH_2PO_4 \cdot 2H_2O$.

2.2.5 Formation of artificial marine aggregates

Several sets of aggregates were formed from senescent algal cultures from selected strains (Table 2.1) or enrichments of mixed phytoplankton assemblages (Table 2.2). The former cultures were mixed with a 10-µm filtered nearshore seawater sample from Southampton Water (Salinity 30) in a ratio 9 to 1. Seawater was added to the monoclonal cultures to include in the microcosm a typical near-shore microbial community, because the bacterial communities colonising a non-axenic phytoplankton culture may not reflect the real distribution of prokaryotes in seawater.

The senescent cultures were then transferred in the dark at 20 ºC and kept for 2-3 days prior to flocculation experiments to pre-degrade the cells, thus promoting the release of TEP and other compounds involved in the flocculation. Artificial aggregates were obtained as shown in Fig. 2.1.

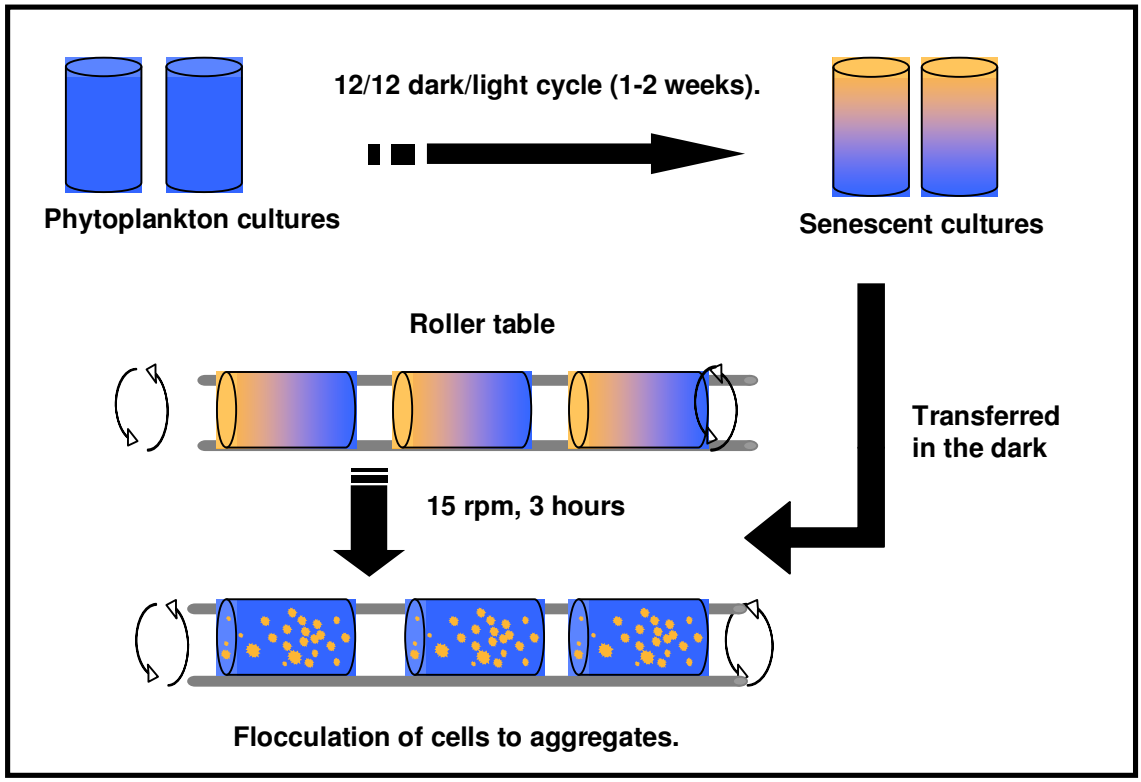


Figure 2.1 Experimental design of the method applied to obtain artificial aggregates in the present study.

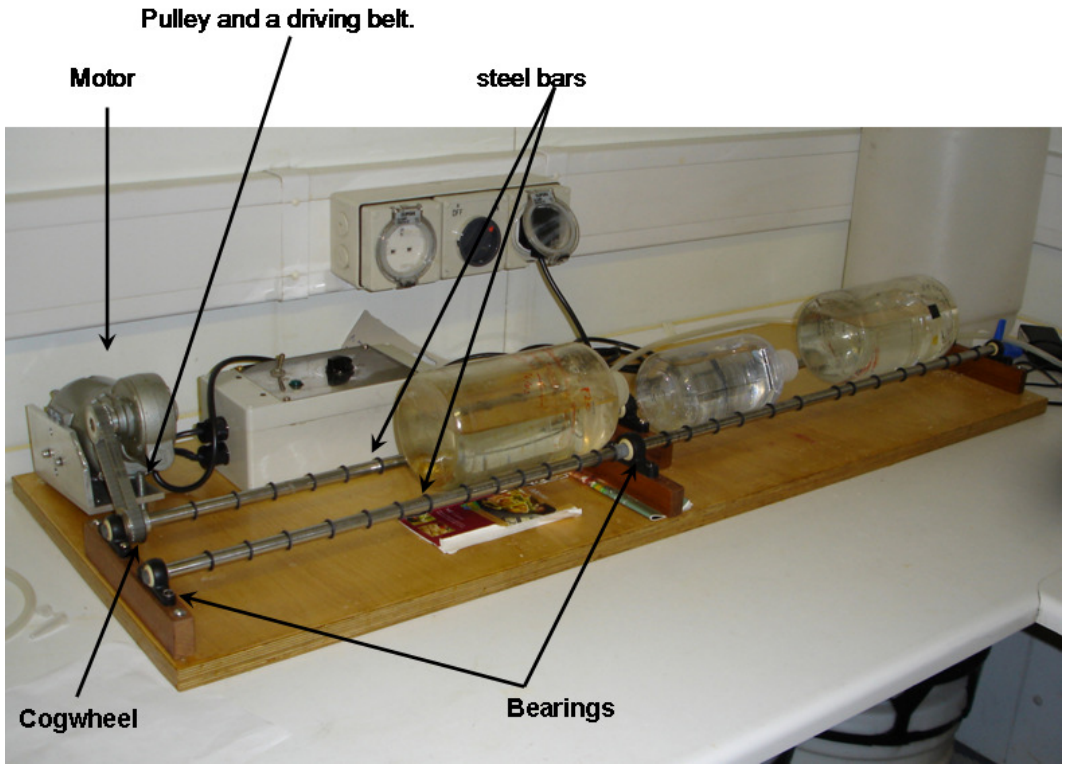


Figure 2.2. Roller table used to form artificial aggregates after 2-3 hours of rotation at 10-15 rpm.

Flocculation of living and dead cells into larger aggregates was obtained after rotating 1 or 2L polycarbonate cylindrical tanks containing the senescent cultures on a roller table (Shanks and Edmondson 1989) constructed in the laboratory (Fig. 2.2). Briefly the roller table consisted of a wooden frame on which rested two parallel steel bars supported bearings. One steel bar was connected by a driving belt to a variable speed motor (Fig. 2.2). Cultures were rotated at 15 rpm for 2-3 hours until marine snow flocs were visible.

Aggregates were then incubated under dark conditions for different studies:

1) Release of nutrients, dissolved iron and dissolved manganese (Chapters 3-4).
2) Assessment of the attached bacterial community and cultivation of the anaerobic bacteria present (Chapters 3-4).
3) Compositional changes in lipids during aerobic degradation (Chapter 5).
4) Simulation of a phytodetritus deposition event on the sediment (Chapter 6).

2.3 Release of reduced iron, fatty acids and microbial populations in aggregates

In the experiments described below the artificial aggregates were obtained from senescent cultures of *Thalassiosira weissflogii* and *Alexandrium tamarense* as well as from mixed phytoplankton assemblages. Once formed these aggregates were incubated to measure the release in nutrients and reduced iron and to investigate the lipid composition and the microbial populations attached to aggregates (Table 2.3).

In order for the releases in nutrients and reduced iron from aggregates to be measurable and distinguishable from the natural fluctuations in chemical species occurring in seawater, the aggregates incubated for this purpose, were transferred with their surrounding seawater to smaller flasks. Aggregates were transferred using a sterile wide-mouth plastic pipette. Other aggregates were formed and incubated following the same method as that described here, results are shown in Appendix 3 and Appendix 4.

2.3.1 Aggregates from cultures of Thalassiosira and Alexandrium

0.9 L of material from a culture of *T. weissflogii* were mixed with 0.9 L of material from a culture of *A. tamarense* and 200 mL of 10-µm filtered Southampton Water, the

mixture obtained was homogenised on a shaker and then transferred in two 2L cylindrical polycarbonate tanks. The tanks were then rotated on the roller table to form artificial aggregates as described above. The aggregates obtained from one of these tanks were transferred with their surrounding seawater to a smaller (0.5 L) flask (Flask TA1); 0.5 L of the medium from the same tank was 10-µm filtered and transferred to Flask TA2. The other tank was incubated in parallel (Tank TA7) and used to investigate the lipid composition of degrading aggregates (Table 2.3).

2.3.2 Aggregates from natural seawater samples

Aggregates N1 and N7 were made from Southampton Water (ENR1, Table 2.2) and were dominated by *Skeletonema* sp. The enrichment ENR1, containing senescent cells was transferred to two 2L cylindrical polycarbonate tanks. Aggregates from one of these tanks were transferred to a 0.5L flask (Flask N1) and 0.5 L of the medium from the same tank was 10-µm filtered and transferred to Flask N2. The parallel 2L tank (Tank N7) was incubated to assess the lipid composition of degrading aggregates (Table 2.3).

Table 2.3. Sample abbreviations, type and origin of cultures.

Sample	Provenance	Vol.[a]	Sample type	Type of study	Results shown
N1	ENR1[b]	0.5L	Artificial aggregates	Nutrients and Fe(II) releases, microbiology	Chapter 3
N2	ENR1	0.5L	Filtered medium	Nutrients and Fe(II) releases	Ch. 3
N7	ENR1	2L	Artificial aggregates	Fatty acids	Ch. 5
N10	ENR10[b]	2L	Artificial aggregates	Fatty acids, microbiology	Ch. 5
TA1	90% algal cultures[c] and 10% Southampton Water[d]	0.5L	Artificial aggregates	Nutrients and Fe(II) releases	Ch. 3
TA2	90% algal cultures and 10% Southampton Water	0.5L	Filtered medium	Nutrients and Fe(II) releases	Ch. 3
TA7	90% algal cultures and 10% Southampton Water	2L	Artificial aggregates	Fatty acids	Ch. 5

[a] Incubation volume
[b] Details in Table 2.2
[c] Senescent cultures of *Thalassiosira weissflogii* and *Alexandrium tamarense*
[d] 10-µm filtered Southampton Water.

From another enrichment of Southampton Water (ENR10) aggregates were formed in Tank N10. This enrichment was dominated by *Thalassiosira* sp. and different dinoflagellates, with lower abundances of *Skeletonema* sp. compared to ENR1. The algal composition in both enrichments reflects the phytoplankton distribution of Southampton Water during April (Howard et al. 1995).

2.3.3 Incubation of aggregates: nutrients and reduced iron

After their formation all the samples containing aggregates (N1, N7, N10, TA1 and TA7) and the seawater controls (N2 and TA2) were incubated in the dark at 20 ºC for a period of 1 to 2 weeks. The containers were only loosely capped to allow air exchange.

Changes in the concentrations of NO_3^-, NO_2^- and PO_4^{3-} in all the samples were followed over time by periodic subsampling after gentle stirring; subsamples were then frozen at -20 ºC until nutrient analyses (Section 2.8.2). The pH was measured regularly and was found to range between 6.5 and 7.0 without showing any obvious variation over time or between one sample and another. The salinity of the enriched seawater in which aggregates were incubated was 31. The concentration of dissolved oxygen in the overlying water was measured using a Unisense microelectrode (Revsbech 1989) and was found to be between 190 and 200 µM without showing any obvious variation over time or between one sample and another.

The concentration of dissolved ferrous iron was measured over time in N1, N2, TA1 and TA2 (Table 2.3) following the ferrozine method (Viollier et al. 2000). On day 5 individual aggregates were gently collected to measure the concentration of oxygen as described in Section 2.8.5.

2.3.4 Aggregate-attached bacteria and lipid composition

On day 5 artificial aggregates from N1, N10 and TA1 were collected, refrigerated at 4 ºC for bacterial cultivation techniques (Section 2.11) or frozen at -20 ºC for analysis of 16S rRNA gene (Section 2.12).

From the tanks N7, N10 and TA7, subsamples including aggregates and their surrounding seawater were collected at different times reflecting different degradation stages. To prevent OM contamination, furnace pre-combusted (450°) and sterile glass pipettes (5mm in diameter) and vials were used to collect and store aggregates. Artificial aggregates were sub-sampled immediately after the formation of aggregates (subsamples S1N7, S1N10 and S1TA7), five days (subsamples S2N7, S2N10 and

S2TA7) and ten days (subsamples S3N7, S3N10 and S3TA7) since their initial incubation, respectively. Sub-samples were rinsed in sterile seawater and frozen at -20ºC for lipid investigation (Section 2.9).

2.4. Addition of Fe(III) and Fe(III)-reducing microorganisms to aggregates

Colloidal Fe(III) was added to samples N5, N6 (Appendix 2) to assess if the presence of bioavailable Fe(III) can enhance the release in reduced iron from marine aggregates. The results from this study are shown in Appendix 3.

Artificial aggregates were also incubated with colloidal Fe(III) and a mixture of known Fe(III)-reducing bacteria belonging to the genus *Shewanella* (Appendix 2).

2.4.1 Colloidal Fe(III)

A colloidal solution of Fe(III) was prepared by adding 100 mmol $FeCl_3 \cdot 6H_2O$ to 1L MQ water, the solution was left at 20 ºC for one hour and then refrigerated at 4 ºC (Wells and Mayer 1991). The colloidal Fe(III) solution was added to some of the samples to obtain a concentration of Fe(III) of 100µmol/L. Colloidal Fe(III) was added to samples N5, N6 (Appendix 3) and to sample N17 and N19 as described below.

2.4.2 Shewanella species

Bacterial strains of *Shewanella oneidensis* MR-1 (Myers and Nealson 1988), *S. baltica* Os-155 (Ziemke et al. 1998) and *S. frigidimarina* NCIMB400 (Lee et al. 1977) were obtained from Dr H von Canstein at the Williamson research centre for molecular environmental sciences of the University of Manchester. The strains were cultivated aerobically in triptone soy broth (TSB) following the instructions of the supplier (Fisher, UK). The three strains were cultivated aerobically in TSB (Fisher Scientific, UK) and 20 mL from each culture were collected and mixed together.

2.4.3 Experimental design

The enrichment ENR15 (Table 2.2) containing senescent phytoplankton material was distributed in five 2L tanks and the experiments were designed as follows (Appendix 2):

1. N16: phytoplankton enriched seawater from ENR15.

2. N17: phytoplankton enriched seawater from ENR15, with 100 µmol L^{-1} colloidal Fe(III) added.
3. N18: phytoplankton enriched seawater from ENR15 with 100 mL bacterial mixture added.
4. N19: phytoplankton enriched seawater from ENR15 with 100 µmol L^{-1} colloidal Fe(III) and 100 mL bacterial mixture added.
5. N20: phytoplankton enriched seawater from ENR15.

Aggregates were formed by rotation of the bottles as described in 2.2.3, the sample N20 was then autoclaved (120 ºC, 20 minutes) and served as a sterile control. The tanks where aggregates were formed were removed from the roller table and incubated in the dark at 20 ºC. Aliquots of seawater from the tanks were then periodically collected and the concentration of dissolved Fe(II) determined (Section 2.8.3).

2.5 Aggregates from natural phytodetritus

In May 2007 a natural phytoplankton bloom occurred in Southampton Water and in the Solent. Fluffy particles suspended on the surface of the Estuary were visible although large aggregates were not observed.

Surface seawater with visible suspended particles from the Solent (50º33'00''N, 1º17'22''E; salinity 33.8) and Southampton Water (50º 52'47''N, 1º22'63''E; salinity 28.5) was collected on May 9th on board of the RV Callista and stored in acid cleaned 2L cylindrical polycarbonate tanks. The initial concentration of dissolved Mn present in the seawater collected at both sites was determined as described in Section 2.8.4.

The cylindrical bottles containing the collected seawater were then transferred in the laboratory within hours and rotated on the roller table (Fig. 2.2) as described in Section 2.2.3. After their formation aggregates were collected and transferred with their surrounding seawater into 250 mL acid cleaned polycarbonate flasks. Aggregates obtained from Southampton Water were transferred in Flask SOT1, whereas 250 mL of 10-µm filtered material from the same tank was transferred in Flask SOT2 which was used as control (Table 2.2). Following the same method, the flasks SOL1 and SOL2 were prepared from rotated Solent Water, with the former containing aggregates and the latter 10-µm filtered material (Table 2.2). The four flasks were then incubated at 20

ºC in the dark for 8 days and 25 mL of seawater were periodically collected for the future determination of dissolved Mn as described in Section 2.8.4.

Table 2.4. Samples obtained from natural phytodetritus*.

Sample**	Provenance	Sample type
SOT1	Southampton Water	Aggregates
SOL1	Solent Water	Aggregates
SOT2	Southampton Water	Control†
SOL2	Solent Water	Control

* The release in dissolved manganese was investigated from these aggregates. Results are shown in chapter 3
** All the samples were incubated in 250 mL flasks
† 10-µm filtered material

2.6 Incubation of aggregates during rotation on the roller table

2.6.1 Seawater collection and fertilisation

Seawater was collected from the Solent on 18/11/2008 (salinity 34.5) and transferred to seven 1.2L cylindrical polycarbonate tanks (Table 2.5).

The seawater collected was fertilised by adding nutrient salts, (Fisher Scientific, UK) at typical f/2 concentrations (Appendix 1): 880 µM $NaNO_3$, 105 µM $Na_2SiO_3 \cdot 9H_2O$ and 35 µM $NaH_2PO_4 \cdot 2H_2O$ were thus added to the tanks. Trace concentrations of iron were present in the salts used to fertilise seawater; the amount of iron present in the aliquots of nutrient solutions supplied to seawater was calculated (≈10 nM) and was over 10 fold lower than that typically present the Solent Water (Fang 1995).

Tanks A, B, C, D and E (Table 2.5) were incubated at 20 ºC in a dark/light cycle to promote phytoplankton growth until reaching the stationary growth phase (determined counting the total number of cells using light microscopy). In contrast the tanks F and G were kept in the dark to prevent phytoplankton growth and used as controls. After two weeks phytoplankton were visibly growing in tanks A to E and aliquots from them were collected and observed under a light microscope. Diatoms such as *Skeletonema*, *Thalassiosira* and *Chaetoceros* were mainly present.

Table 2.5. List of aggregates incubated under dynamic conditions[a].

Sample	Provenance	Sample type	Type of study
A	ENR21[b]	Aggregates	Release in dissolved Fe and Mn, microbiology
B	ENR21	Aggregates	Release in dissolved Fe and Mn, microbiology
C	ENR21	Aggregates	Release in dissolved Fe and Mn
D	ENR21+ sed.[c]	Aggregates	Release in dissolved Fe and Mn
E	ENR21+ sed.	Aggregates	Release in dissolved Fe and Mn
F	10-µm filtered Solent Water	Control	Release in dissolved Fe and Mn
G	10-µm filtered Solent Water + sed.	Control	Release in dissolved Fe and Mn

[a] All the aggregates listed in this table were incubated in the same 2L tanks were they had been formed while in rotation on the roller table. All the results from this study are shown in chapter 4.
[b] Details in table 2.2
[c] 140 mg of sieved surface sediment added to 1L of sample.

2.6.2 Addition of sediment

Prior to aggregation on the roller table a sediment suspension was added to the tanks D and E containing senescent phytoplankton and to the control tank G. Sieved surface sediment was collected and sieved as described in Section 2.7.1. Two weeks before the experiments started a suspension was obtained by mixing the sediment with MQ water. The suspension was then sparged in a wide container and air was bubbled on its surface using an aquarium pump, to promote the oxidation of reduced metals present. An aliquot of the suspension was then dried overnight at 50 °C for the determination of HCl leachable Fe and Mn present in the sediment: 100 mg of dry sediment were added to test tubes containing 5 mL of 1M HCl. Tubes were agitated on a rotating wheel for four hours, then centrifuged at 1000 rpm and the supernatant collected. Metals eluted in the supernatant were analysed by Flame Atomic Adsorption Spectrometry using a Varian SpectrAA 55 atomic absorption spectrophotometer.
The sediment suspension was kept at 20 ºC in the dark under aerobic conditions to prevent the photoreduction of Fe(III) and promote the oxidation of Fe(II). After two weeks the suspension was agitated on the shaker and 1.5 mL of it, which contained 140 mg of dry sediment, was supplied to samples D and E containing crashed phytoplankton and to the control sample G, prior to the rotation on the roller table.

2.6.3 Aggregate formation and incubation

Prior to the addition of surface sediment the concentration of dissolved Fe(II), dissolved Fe(III) and dissolved Mn in the bottles was determined (Sections 2.8.3-2.8.4). After the sediment suspension was added to samples D, E and G all the cylindrical polycarbonate bottles were incubated in the dark at 20 °C, on the roller table (Fig. 2.2) at 10 rpm for two weeks. Visible aggregates were formed within a few hours since the rotation started.

The pH and the concentrations of dissolved Fe(II), dissolved Fe(III) and dissolved Mn in the tanks were determined over time (Sections 2.8.3-2.8.4); the average size of aggregates and the abundance of free-living and particle-attached bacteria were also determined.

2.6.4 Aggregate size and microbial abundance

From each tank, 25-30 aggregates were periodically collected using a wide-mouth sterile pipette and the diameter measured using an inverted light microscope Leika DM IRB/E. Aggregates were then retransferred in the same incubation bottles or used for the estimate of the density of aggregate-attached bacteria. For the estimate of microbial density aggregates of known size were rinsed twice in sterile 1mL tubes containing 0.2-µm filtered and autoclaved seawater, which had been previously adjusted to the same salinity (34.5) as that where aggregates were incubated. Aggregates were then transferred into new sterile microcentrifuge tubes and autoclaved seawater was added until reaching a total volume of 1 mL.

The free-living bacteria present in the tanks were also enumerated: 1 mL of seawater was collected from each tank and transferred into a sterile microcentrifuge tube.

To the microcentrifuge tubes 50 µL of 37% paraformaldehyde was then added and samples frozen at -20 ºC until analyses by flow cytometry (Section 2.10) (Troussellier et al. 1995) performed on a FACsort flow cytometer (Becton Dickinson, Oxford, UK).

At the end of the incubations an aliquot of aggregates from tanks A and B was collected, rinsed twice in sterile seawater, and then transferred in sterile 60 mL bottles containing sterile seawater and refrigerated at 4 ºC for future cultivation of the anaerobic bacteria present (Section 2.11). Aggregates from tanks A and B collected and rinsed using the same method were also frozen at -20 ºC for molecular analyses of the 16S rRNA gene (Section 2.12). The microbial density has been reported to be 2 to 4

orders of magnitude higher in aggregates compared to seawater (Simon et al. 2002; Turley and Mackie 1994). The risk of contaminating a marine snow sample with bacterioplankton deriving from the seawater surrounding the aggregate, is therefore very low and probably it was not strictly necessary to rinse the particles in sterile seawater.

2.7. Sediment-core based experiments

The work shown in this Section was carried out at NOCS in collaboration with William B Homoky whose major contribution consisted of collecting and sieving the sediment and designing the mesocosm systems to which phytodetritus was added. Two different sediment-core based experiments were carried out during the present PhD, in July and October 2007 (Table 2.6).

2.7.1 Sediment sampling and processing

Sediment samples were collected from Southampton estuary on board RV Callista during a high-tide cycle in October 2006. Sediments were collected near Netley (50°53'01'' N, 1°24'25'' W) from a water depth of 3.1m. Sampling was achieved by duplicate Van Veen grabs that were subsequently transferred to plastic buckets and returned to NOCS.

Sediments were then manually passed through a 250 µm sieve to remove macro-faunal assemblages. The sieved sediment was transferred to a clean plastic box and homogenized by stirring with a plasticized, powered paint stirrer.

Table 2.6. Sediment-cores studied in the present work.

Core ID	Incubation date	Phytodetritus added	Aeration†
CO1	18/07/07	ENR16* + *Chaet.***	Yes
CO2	18/07/07	ENR16* + *Chaet.***	Yes
CO3	18/07/07	ENR16* + *Chaet.***	Yes
CO4	18/07/07	No (control)	Yes
CO5	11/10/07	ENR18* + *Chaet.***	No
CO6	11/10/07	ENR18* + *Chaet.***	No
CO7	11/10/07	ENR18* + *Chaet.***	No
CO8	11/10/07	No (control)	No

*Defined in Table 2.2
** Senescent culture of *Chaetoceros gracilis* PLY 666
† Cores were aerated using a PVC tube submerged in the seawater overlying the sediment cores, and connected with an aquarium pump.

2.7.2 Preparation of cores

Eight push cores 6 cm in diameter were sub-sampled to a depth of about 16 cm from the homogenised sediment using acid cleaned polycarbonate core tubes. Push cores were then stoppered using neoprene bungs and transferred to a controlled temperature laboratory (dark, 20 ºC). 300 mL of Solent Water (salinity 34.5) were then slowly added on top of the homogenised sediment cores using a plastic funnel taking care to minimise disturbance to the sediment surface. The cores were left to settle, with air being actively bubbled in the water using an aquarium pump, for two weeks before any subsequent experimental manipulation.

2.7.3 Phytodetritus

The Phytodetritus which was added to the sediment cores was obtained by mixing 50mL from a senescent culture of *Chaetoceros gracilis* (Table 2.1) with 50mL from enrichments of mixed phytoplankton assemblages obtained from Solent Water. Material from *C. gracilis* was mixed with ENR16 for the experiments carried out in July 2007, and ENR18 for the experiments carried out in October (Table 2.2). In both cases the mixture obtained was rotated on the roller table to coagulate cells into larger aggregates (Shanks and Edmondson 1989). The flocs formed were then collected using a wide-mouth pipette and transferred into a 100 mL flask so that aggregates were concentrated in a smaller seawater volume. The aggregates were then disrupted on a shaker until obtaining a homogeneous mixture.

2.7.4 Sediment-core incubation

At the start of the experiments, 30 mL of the phytodetritus mixture were gently added to 3 sediment cores. Phytodetritus was not added to another sediment core which served as control (Table 2.6). Two different experiments, for a total of 8 sediment cores, were designed as described above and carried out in July and October 2007, respectively.

For the first experiment the sediment cores were incubated for 1 week (dark, 20 ºC) and atmospheric air was actively bubbled through the seawater overlying the cores during the experiments (as described in 2.7.2). The pH, the dissolved Fe(II), Fe(III) and Mn concentrations as well as the oxygen profile were determined over time. For the second experiment the cores were pre-incubated for 2 weeks (dark, 20 ºC) with air actively bubbled as above, and then the artificial aeration was interrupted and

phytodetritus added as described above. Cores were incubated for 2 weeks (without artificial aeration) and the same chemical species as above were determined over time.

2.8 Determination of inorganic species

2.8.1 Collection and storage of samples

For nutrients analysis, 25-30 mL of seawater were collected from the flasks and the tanks where samples were incubated and frozen at -20 ºC. Measuring concentration of trace metals in a solution requires an extreme care as adsorption of them by containers and instruments may strongly affect the results (Benes and Steinneš 1995).

Reagents used for the preparation of samples for Fe and Mn measurements were either trace metal grade (Fisher) or prepared in the clean laboratory under trace metal free conditions. Samples were handled and stored using plasticware which had been cleaned as described in Section 2.2.1. The seawater samples stored for the determination of dissolved Fe and Mn were acidified with 0.1 % (v/v) 10M HNO_3 to prevent adsorption into the walls of the tubes and refrigerated at 4 ºC to minimise evaporation.

Analyses of dissolved ferrous iron were carried out (Section 2.8.3) immediately after the collection of seawater from the flasks, tanks and sediment cores. Three replicate seawater samples of 4mL or 20 mL were collected from the flasks or the tanks. For the sediment cores 3 replicate samples of 4 mL were carefully collected from the surface of the seawater overlying the cores. After collection seawater was filtered through 0.2µm acid cleaned cellulose nitrate membrane filters (Whatman®) into cleaned polystyrene test tubes and analysed using the ferrozine method (Viollier et al. 2000) as described in Section 2.8.3. The same subsamples were eventually used for the determination of the dissolved ferric iron (Section 2.8.3).

For the determination of dissolved manganese, seawater (8 mL) was collected from the samples, filtered through 0.2 µm cellulose nitrate membrane filters (Whatman®) into polystyrene test tubes, acidified as described above for the storage of samples for iron analysis.

2.8.2 Nutrients autoanalyser

Nitrate, nitrite and phosphate were analysed using a Burkard autoanalyser (Hansen and Koroleff 1999) at NOCS. A standard spectrophotometer supported with a

4 cm cell was used. The spectrophotometer was connected to an autosampler which sipped about 4 mL of each sample.

Nitrate standards of 200, 100 and 50 μM, nitrite standards of 10, 5 and 2 μM and phosphate standards of 5, 2 and 1 μM were made from stock solutions. Nitrates and phosphates were measured together, with the standards mixed in the same vial.

Three replicate samples (3 mL) were collected for each sampling point. Samples were transferred into 3 mL plastic vials for analysis in the Burkard Autoanalyser (Hansen and Koroleff 1999). No treatment was necessary for the samples. The autoanalyser measures the concentration of NO_2^- after reduction of all the nitrates to nitrites using a Cadmium reduction column. For the nitrite analysis, the Cadmium reduction column was removed to avoid the NO_3^- reduction to NO_2^-.

2.8.3 Dissolved ferric and ferrous iron: the ferrozine method

The ferrozine (monosodium salt hydrate of 3-(2-pyridyl)-5,6-diphenyl-1,2,4-triazine-p,p'-disulfonic acid) reagent reacts with Fe(II) to form a stable magenta complex (Stookey 1970; Viollier et al. 2000). Iron Spectrophotometric determination was used operating at a wavelength of 562 nm which is meant to provide the maximum absorbance (Viollier et al. 2000). Better accuracy is achieved by recording the absorbance within 10 minutes of adding the ferrozine reagent (Viollier et al. 2000).

A solution was prepared adding 0.51 g of ferrozine, purchased from Sigma Aldrich, into 100 mL of 10M ammonium acetate. The buffer solution was prepared adding primary grade ammonium acetate to MQ water; acetic acid was then added to achieve a pH ~ 5.5.

Ferrous iron standard solutions were used for the calibration of the spectrophotometer; a 40 μM solution of ferrous ammonium sulphate $[Fe(SO_4)_2(NH_4)_26H_2O]$ in MQ water was prepared and acidified with 100 μL of 10M HNO_3 to stabilise the standard solution for long time periods.

From the flasks, the tanks and the seawater overlying the sediment cores three replicate seawater samples (4 mL each) were collected and filtered through 0.2 μm acid cleaned cellulose nitrate membrane filters (Whatman®) into cleaned polystyrene test tubes containing 200 μL of ferrozine solution. After circa 10 minutes, the absorbance at 562 nM was measured using a Hitachi U-1500 spectrophotometer provided with 4 or 10cm cells. The concentration was thus measured from three independent replicates.

For the determination of the dissolved [Fe(II) + Fe(III)], the Fe(III) present was

pre-reduced to Fe(II) by adding a 1.4 M solution of hydroxylammine hydrochloride (Sigma Aldrich, UK) in 2M HCl in a ratio 1 solution to 5 sample. The samples were pre-reduced for two hours (Viollier et al. 2000), 0.6 M NH_4OH (Romil spA™) were then added to achieve a pH ~ 5.5 and absorbance at 562 nM was measured again. Standard Fe(III) solutions were prepared from $FeCl_3$ salt purchased from Fisher Scientific (UK). During this procedure, Fe(II) was not oxidized and Fe(III) was not reduced, as determined in the Fe(II) and Fe(III) standard solutions.

2.8.4 Dissolved Mn: the Flow Injection Analyser

A flow injection analyser for the measurement of dissolved manganese was designed at NOCS following a published method (Mallini and Shiller 1993). The reaction forming the basis of this method is the oxidation of Tiron (4,5-dihydroxi-1,3-benzenedisulfonic acid disodium salt) by H_2O_2 to form a coloured semiquinone that is detected at 440 nM (Otto et al. 1983). Under alkaline conditions Mn catalyses this reaction, especially if 2,2'-dipyridyl is present as an activator (Mallini and Shiller 1993). The reaction is highly sensitive to pH changes and the presence of salts also poses a problem. A chelator column was used for sample pre-concentration and salt removal. All the components of the flow injection analyser which entered in contact with the sample during the analyses (connectors, lines) were in plastic and had been previously cleaned as described in Section 2.7.1.

The borate buffer was prepared with 0.3 M boric acid and 65 mM sodium tetraborate decahydrate both purchased from Fischer Scientific (UK). To 200 mL of borate buffer 1.3 g of Tiron and 0.85 g of 2,2'-dipyridyl (both purchased from Sigma Aldrich, UK) were dissolved by microwave heating. Then 6M NH_4OH (Romil spA™, UK) was added to achieve a pH of 9.6 after mixing the former solution with 0.9 M HCl (in a ratio 1:1). The acid used for cleaning the column and eluting the metals consisted of 0.9 M HCl (trace grade, Fisher). A 0.2 M H_2O_2 solution was made daily.

All lines in the flow system were Teflon tubing (0.8 mm internal diameter) except for the pump manifold which used polyvinyl chloride (PVC) tubing. The buffered reagent, peroxide and elution acid were propelled by a peristaltic pump at the same rate (0.7 mL min^{-1}). Sample, cleaning acid and MQ water were connected to a three way distribution valve which was connected to a PVC tube entering the same peristaltic pump as above. The Mn present in the pH buffered (~8.0) samples was loaded on a column containing metal binding resin (Toyopearl 650M, Tosoh

Bioscience, Germany) which binds and concentrates transition metals while allowing alkaline and earth alkaline metals to pass through. The three way distribution valve allowed either sample, cleaning acid or MQ water to be pumped. A second 6 port 2 way valve (injection valve) allowed (1) the pumping of the liquid coming from the first valve to the resin and then waste, with the eluting acid being propelled and mixed with other reagents or (2) the pumping of the liquid coming from the first valve to waste while the eluting acid passed through the resin removing the adsorbed Mn and entered the mixing coil. The buffered (pH~8.0) sample was thus introduced in the continuous flow system where Mn was loaded, the resin was then rinsed by MQ water and the valve switched in the inject position such that the Mn was eluted by the acid. Buffered reagent and eluted samples were combined in a mixing coil, they were then combined with H_2O_2 in a second, longer (1m) mixing coil. The final mixed reagents and sample stream had a pH of 9.6.

The absorbance at 440 nM was recorded by a light emitting diode (LED) based detector connected to a computer based data acquisition system (Mallini and Shiller 1993). The valves were also connected to a computer and the software LabView was used to record the data and control the valves. Standard Mn(II) solutions were prepared from a certified standard solution purchased from Sigma Aldrich.

2.8.5 Dissolved oxygen

Measurements of oxygen were made in individual marine aggregates, isolated from samples TA1 and N1, as well as in the sediment cores (Section 2.6). The redox profile of a microenvironment might be affected by the piercing action due to the ingress on it of a sharp microelectrode. The oxygen profile measured might be therefore different from the one occurring before the microelectrode piercing action. However as both natural and artificial marine aggregates were shown to be 95-99% porous and previous measurements of solutes concentration in marine snow were also carried out using similar methods (Alldredge and Cohen 1987; Ploug and Jorgensen 1999; Ploug et al. 1997; Shanks and Reeder 1993) the application of microelectrode based instruments for the determination of dissolved oxygen in aquatic aggregates seems to be an appropriate technique.

Measurements of oxygen within individual aggregates in the present study were made using a Unisense© microelectrode (Revsbech 1989) provided with a sensing tip of 10 µm in diameter. A sensing tip of 50 µm in diameter was used for the sediment cores

to prevent any damage from the contact with sediment grains; oxygen was measured in the seawater overlying the water phytodetritus interface, in the phytodetritus layer and in the sediment underneath it.

Using a wide-mouth pipette individual aggregates were carefully collected from their flasks and transferred, along with surrounding medium, into a 50 mL glass beaker containing a layer of agar (the agar prevents damage to the microelectrode tip once it has fully penetrated the aggregate). The concentration of oxygen was measured across the aggregates and the sediment cores at steps of 100 µm, and the electrode signal was logged using a Unisense© picoammeter connected to a computer based data acquisition system. The position of the aggregate surface was determined by slowly advancing the microelectrode towards the aggregate until, under a stereomicroscope, it visibly touched the upper surface. Measurements were carried out in a 20 ºC controlled-temperature room and the electrode was calibrated using the same seawater as that used for the experiments; this seawater was turned anoxic by bubbling N_2 to set up the zero reading of the electrode and then saturated with oxygen by air bubbling to set up the fully saturated conditions (100% oxygen). The concentration of oxygen in each solution was then calculated from the percentage of saturation as a function of temperature and salinity according to the method of Revsbech (1989).

2.9 Composition in lipids of marine aggregates

Lipids from artificial marine aggregates were analysed at the OGU in Bristol. To avoid contamination from other potential sources of OM all the glassware used for lipid extraction and consequent manipulation of samples had been previously cleaned by heating (450 °C), and all the reagents used were purchased from Fisher and Trace Analyses Grade. Glassware which can not be heated at 450 ºC such as measuring cylinders was thoroughly rinsed with dichloromethane (DCM). Lipid-free distilled water was used to process the samples and was obtained by solvent extraction of distilled water with dichloromethane (DCM), and this operation was repeated twice. All the aggregates analysed were formed as described in Section 2.2, collected with glass pipettes into glass vials and frozen at -20ºC until analysis.

Lipids were extracted and analysed from N3 and T1 (Appendix 7), the former formed from the enrichment ENR3 (Table 2.2) and the latter formed from a senescent culture of *Thalassiosira weissflogii*. Analyses from these samples were carried out to

assess the general composition of fatty acids and neutral lipids of artificial aggregates and the results are shown in Appendix 6.

The compositional changes in lipids during the decomposition of OM in marine aggregates were also investigated. Lipids were analysed from aggregates N7, N10 and TA7, which were formed and incubated as described in Section 2.3.4.

2.9.1 Extraction of lipids and separation of neutral and polar fraction

Frozen samples were thawed and centrifuged to remove most of the seawater and associated dissolved salts. The residual seawater was removed after several dilutions in lipid-free distilled water and subsequent centrifugation. Samples were finally dried using a freeze-dryer. All lipids were extracted from the dried samples by saponification (Kawamura 1995); briefly, the dried samples were sonicated for 15 minutes and then heated at 70°C for 2 hours in 10 mL of 0.1 M methanolic KOH solution with 5 % water. The samples were centrifuged for 5 minutes at 3000 rpm to separate the liquid extract from the remaining solid particles and the liquid extract was collected. The solid residue was further extracted with CH_3OH and then DCM under sonication. The extracts were combined and dried by rotary evaporation under a vacuum. 10 mL of distilled water and 10 mL of hexane:DCM mixture 9:1 were added to the extracts, and the samples agitated using a vortex mixer; subsequently the organic phase, which contained the neutral lipids, was recovered. Neutral lipids were then isolated from the vials by adding 10 mL hexane:DCM mixture 9:1, agitating the vials on a vortex mixer and removing the upper layer of the vials which contained the neutral lipids dissolved in the hexane:DCM mixture. The process was repeated three times and the solvent was removed by rotary evaporation under vacuum.

To the remaining water phase, 5M HCl was added to achieve a pH of 1, and acidic components were then extracted using 10 mL DCM. The solvent was then removed by rotary evaporation under vacuum.

The carboxylic acids present in the acid fraction were derivatised to methyl esters with 14% BF_3/CH_3OH at 70°C for 1 hour. Any trace of water was removed by running the samples through Na_2SO_4 columns, and alcohol moieties in both fractions were converted to their trimethylsilyl ethers by adding 25 µL of N,O-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane and 25 µL pyridine and heating at 70 C° for 1 hour.

2.9.2 Gas chromatography (GC) and gas chromatography-mass spectrometry (GC-MS) analysis

The samples were dissolved, according to their concentration, into 20 to 250 µL of ethyl acetate prior to analysis by gas chromatography. GC analyses were performed on a Carlo Erba GC equipped with a flame ionization detector and fitted with a chrompack fused silica capillary column (50m x 0.32mm internal diameter) coated with CP Sil5-CB stationary phase (dimethylpolysiloxane equivalent, 0.12µm film thickness). The oven temperature was programmed from 10-130°C at 20ºC/min, from 130-300ºC at 4ºC/min and held for 25 minutes. Prior to GC-MS analyses Hexadecan-2-ol and henedecane were added as internal standards for neutral and acidic lipids, respectively. GC-MS analysis was performed on a Thermoquest Finnigan Trace GC interfaced with a Thermoquest Finnigan Trace MS operating with electron ionisation at 70eV and scanning an *m/z* range of 50 to 850 Daltons. The column and temperature conditions were the same as those used for GC analyses, although He was used as the carrier gas. Fatty acids, alcohol and sterol identifications were based on fragmentation patterns and relative retention indices, and fatty acid concentrations were calculated by comparing compound peak areas to those of the standard. The position of the double bond in MUFA and PUFA was not determined as part of this work; however, multiple isomers of octadecenoic (C18:1) and tetraeicosenoic acid (C24:1) were identified on the basis of differing retention times, and the values reported for C18:1 and C24:1 reflect the summed concentrations of the two components.

2.10 Flow cytometry

Microcentrifuge tubes containing aggregates and sterile seawater (Section 2.6.4) were shuffled on a vortex mixer for ten seconds to obtain an even distribution of microorganisms in the solution. 100 µL of 250mM potassium citrate and 10 µL 1% solution (vol/vol) of the DNA stain SYBr Green® I (Marie et al. 1997) were added to both microcentrifuge tubes containing homogenised aggregates and microcentrifuge tubes containing seawater surrounding the aggregates. Samples were then incubated for one hour at 37 ºC before analyses (Marie et al. 1997).

Microbial organisms were enumerated using a FACSCalibur flow cytometer (Becton Dickinson, Oxford, UK) at NOCS: samples were injected in a flow traversing a laser at 488 nm. The intensity and wavelength of cell fluorescence was recorded and the

red autofluorescence (Chlorophyll) of phytoplankton could be easily distinguished from the green fluorescence of DNA stained heterotrophs. The intensity of the green fluorescence is related to the amount of DNA contained in the detected particle (cell), likely reflecting the size of the particles detected and allowing a distinction between viruses, bacteria and larger microorganisms.

As the volume of solution which was sipped by the flow cytometer was variable, known volumes of solutions of yellow green fluorescent beads, 0.5 μL diameter (Polysciences, USA), were added to each sample and used as an internal standard to compute the absolute concentration of particles.

2.11 Culturing bacteria present in artificial aggregates

2.11.1 Media and culturing conditions

The presence in artificial marine aggregates of fermenters as well as sulphate, Fe(III)-, Mn(IV)- and nitrate-reducing bacteria was examined at SEAS. Enrichment media were used to cultivate a variety of microbial functional groups that are able to respire anaerobically. All cultures were inoculated in triplicate with 5% (vol/vol) artificial marine aggregates that had been collected after their formation in 10 mL. Cultures were incubated at 20°C in the dark to prevent photoreduction. Abiotic controls were obtained by incubating the same medium used to cultivate the aggregates under the same conditions, but without the addition of marine particles. Abiotic controls were not incubated in triplicate. Artificial aggregates from the flasks N1 and TA1 (Table 2.3) as well as from the tanks A and B (Table 2.5) were incubated to determine the presence of anaerobic bacteria.

The base medium utilized was a modification of a published recipe (Caccavo et al. 1994) and comprised: 30 mM $NaHCO_3$, 4.7 mM NH_4Cl, 4.3 mM $NaH_2PO_4.H_2O$, 1.3 mM KCl, 10 mL L^{-1} vitamin mix (Balch et al. 1979) and 10 mL L^{-1} mineral mix (Lovley et al. 1984). The medium was amended with 0.34 M NaCl, and 15 mM $MgCl_2.6H_2O$ to emulate marine conditions (Appendix 1).

In the anaerobic cultures of aggregates for glucose-fermenting bacteria, electron acceptor and electron donors were not supplied but 20 mM of glucose were added to the medium as a carbon source. Electron donors and acceptors were instead added to the cultures for anaerobic respiration. Electron donors for the bacterial populations were supplied separately as 15 mM sodium acetate or 15 mM sodium lactate (Appendix

1). Terminal electron acceptors were added as 10 mmol L^{-1} amorphous Fe(III) oxyhydroxide (FeOOH) (Lovley and Phillips 1986), 10 mmol L^{-1} Fe(III)-nitriloacetic acid (Fe(III)-NTA) (Fredrickson et al. 2000; Lovley and Woodward 1996) for Fe(III) reducers, 1 mmol L^{-1} of synthetic vernardite (δ-MnO_2) (Villalobos et al. 2003) for Mn(IV)-reducing bacteria, 15 mmol L^{-1} KNO_3 for NO_3^- reducers and 20 mmol L^{-1} Na_2SO_4 for sulphate reducing bacteria (SRB) (Appendix 1).

Once prepared, the media were sterilised by 0.22 µm filtering or autoclaving and de-oxygenated by bubbling with an 80:20 N_2:CO_2 gas mixture for 25 minutes. The media was then poured into 10 ml, sterile, deoxygenated bottles. Samples of artificial marine aggregates were then shaken and a subsample injected into the bottles by sterile syringes. Cultures were then incubated at 20˚C in the dark for 1 to 3 months. When a significant growth was observed in the culture an aliquot was collected and inoculated into a new bottle containing sterile medium.

From the anaerobic cultures of aggregates strains were isolated aerobically in Difco™ marine broth solidified with 1.5 % agar (Difco). Difco marine broth is a medium commonly used for the isolation and cultivation of aerobic heterotrophic bacteria and comprises peptone (5 g), yeast extract (1 g), NaCl (19 g), $MgCl_2 \cdot 6H_2O$ (5.9 g), $MgSO_4$ (3.2 g), $CaCl_2$ (1.8 g), KCl (0.55 g), $NaHCO_3$ (0.16 g) and a range of trace elements including bromide, strontium, boron, phosphorus, silicon and fluoride (Appendix 1). To the solution obtained 1.5 % (w/w) agar was added, the medium was then autoclaved and poured in the sterile Petri Dishes.

2.11.2 Determination of Fe(III) reduction.

Key biotransformations in enrichment cultures were determined as indicated in Table 2.7 with comparison to abiotic control media.

Fe(III) reduction was quantified by the ferrozine colorimetric assay of Fe(II) after HCl digestion (Lovley and Phillips 1986). Briefly from each enrichment,100 µL were collected in triplicate, added to vials containing 0.9M HCl, and the solution mixed on a vortex mixer.

Table 2.7. Methods of determining positive growth in cultures.

Electron acceptor	Growth Determinant
NO_3^-	Turbidity due to cell growth, nutrient analysis
Fe(III)-NTA	Solution colour change from rust-red; ferrozine assay
FeOOH	Precipitate colour change from rust-red; ferrozine assay
δ-MnO_2	Black precipitate dissolves turning solution colour into transparent. Formaldoxime colorimetric technique
SO_4^{3-}	Black sulphide precipitate.

After 1 to 2 hours 100 µL from the solution were added to 4.9 mL of a ferrozine solution. The ferrozine solution was prepared by adding 11.9 g N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES) and 1 g of ferrozine to 1 L of MQ Water; NaOH was then added to achieve a pH of 7.0 (Lovley and Phillips 1986) and the absorbance at 562 nM was measured using a spectrophotometer provided with a 1 cm cell. During this procedure, Fe(II) was not oxidized and Fe(III) was not reduced, as determined in the standard solutions.

The concentration of Fe(III) was determined after pre-reduction of Fe(III) to Fe(II) using hydroxilammonium hydrochloride and the Fe(II) after HCl digestion using the ferrozine colorimetric assay (Lovley and Phillips 1986; Viollier et al. 2000).

Standard Fe(II) and Fe(III) solutions were prepared as described in 2.8.3.

2.11.3 Determination of Mn(IV) reduction

The reduction of amorphous manganese oxide (Section 2.10.1) was determined by measuring the dissolved Mn and the total manganese present in the enrichment cultures. The concentration of reduced Mn was then measured using the formaldehyde oxime (formaldoxime) colorimetric technique (Brewer and Spencer 1971; Tebo et al. 2007). Briefly a formaldoxime reagent was prepared by adding 37% formaldehyde solution to a 1.4 M hydroxilammonium hydrochloride solution in a ratio 1 formaldehyde to 19 hydroxilammonium. A solution of NH_4OH was then added to the above solution in a proportion to obtain pH 8.9 after addition of 7 volumes 0.5M HCl to 1 volume of formaldoxime. Under basic conditions formaldoxime forms an orange-red complex with Mn(II) (Brewer and Spencer 1971).

From the enrichment cultures an aliquot of 50 µL was collected, mixed with 950 µL of 0.5M HCl to elute any reduced Mn adsorbed onto the δ-MnO_2 particles and

filtered through 0.2 μm Millipore membrane filters (Fisher Scientific, UK). The filtered sample was then mixed with the formaldoxime solution in a ratio 7 sample to 1 formaldoxime and the absorbance at 450 nm was measured using a spectrophotometer provided with a 1cm cell.

For the total manganese an aliquot of 50 μL was collected from the enrichments and pre-reduced to Mn(II) by 950 μL of 70 mM hydroxylamine hydrochloride overnight. The solution was then filtered through 0.2 μm Millipore membranes and dissolved manganese was measured as described above.

Standard Mn(II) solutions were prepared from $MnSO_4 \cdot H_2O$ whereas standard Mn(IV) solutions were prepared from MnO_2 (both purchased from Fisher Scientific, UK).

2.11.4 Determination of NO_3^- reduction

Nitrate reduction was determined by measurements of the concentrations of NO_3^- and NO_2^-, using a Burkard autoanalyser (Hansen and Koroleff 1999) at NOCS. From the enrichments 100 μL were collected, diluted in 9.9 mL 0.7 M NaCL and frozen at -20 °C prior to analyses at NOCS as described in Section 2.8.2.

2.11.5 Isolation of anaerobic bacteria

From the anaerobic cultures of marine aggregates A and B (Section 2.6.4) eight strains of facultative anaerobic bacteria were aerobically isolated with the dilution-plating method. Strains were isolated aerobically in Difco™ marine agar or glucose enriched medium solidified with 1.5% agar (Section 2.11.1, Appendix 1).

The bacterial strains were isolated assuming that a visible circular bacterial colony growing on an agar plate, and physically separated from other colonies is generated by several divisions of one single bacterial cell. Isolations were obtained as follows: 4 dilutions of the enrichment culture (1:10, 1:100, 1:1000 and 1:10000) into sterile medium were prepared. 50 μL from each dilution were then transferred and distributed into a Petri Dish containing the solidified medium used. Plates were then incubated aerobically at 20 ºC and after one to two days microbial growth was observed. Visible colonies, where present, were collected using a sterile pipette tip and transferred into another Petri Dish containing sterile solid medium.

The strains isolated were then cultured anaerobically in marine minimal medium as described in 2.11.1. From the strains isolated an aliquot was collected, the

DNA was extracted and the 16S rRNA gene was amplified as described in Section 2.12. Each strain was cultured in triplicate with Fe(III) and in triplicate with δ-MnO_2 as electron acceptors. The Fe(III) form used as electron acceptor (Fe(III)-NTA or FeOOH) was the same as that present in the enrichments from where the strains had been isolated, or Fe(III)-NTA if the strain had been isolated from an enrichment containing δ-MnO_2 as electron acceptor. Acetate or lactate was used as electron donors and the electron donor used was the same as that present in the culture from where the strain had been isolated.

In the cultures amended with Fe(III) as electron acceptor the concentration of total HCl extractable Fe was initially measured and the concentration of HCl extractable Fe(II) was measured over time as described in 2.11.2. Similarly in the cultures amended with Mn(IV) as electron acceptor the concentration of total HCl extractable Mn was initially measured and the concentration of reduced Mn was measured over time as described in 2.11.3.

2.12 Molecular analysis of Bacterial community

2.12.1 DNA extraction

Microbial DNA was extracted from artificial aggregates collected from three samples (N1, N10 and B). Aggregates were collected from the original containers using a sterile pipette, rinsed twice in sterile seawater to remove bacterioplankton and frozen at -20 °C prior to analyses. Microbial DNA was also extracted from 2 enrichment cultures for Fe(III) reducers and 1 enrichment culture for glucose fermenters obtained from the artificial marine aggregates N1 and B; an aliquot of the enrichment cultures for Fe(III) reducers was collected for molecular analyses during the log phase of the culture growth and frozen at –20°C. The log phase of the culture was estimated after the determination of Fe(III) reduction to Fe(II). For the culture for glucose fermenters an aliquot of the enrichment was collected and frozen at -20 °C one day after the culture had been re-inoculated into a new sterile medium. Microbial DNA was also extracted from 6 strains isolated from the enrichment cultures.

Bacterial DNA was extracted using the PowerSoil DNA isolation kit (Mo Bio Laboratories), and bacterial 16S rRNA gene fragments were amplified and analysed as follows.

2.12.2 PCR amplification

16S rRNA gene amplification was performed on samples by PCR using primers 8F (5'-AGA GTTTGATCCTGGCTCAG-3') (Eden et al. 1991) and 519R (5'-TACGGYTACCTTGTTACGACTT-3') (Lane et al. 1985) using published methods (Boothman et al. 2006). From the sample N1, the 16S rRNA gene was also amplified using *Geobacter* specific primers, GEO564F (5'– AAGCGTTGTTCGGAWTTAT – 3') and GEO840R (5' – GGCACTGCAGGGGTCAATA – 3') (Cummings et al. 2003). Amplification of both sequences was performed using *Taq* DNA polymerase (Sigma Aldrich, UK) following the instructions provided by Sigma Aldrich.

PCR amplification was performed on marine aggregate samples, anaerobic cultures of marine aggregates and strains isolated from these cultures. The 16S rRNA gene fragments amplified from the isolated strains was directly purified and sequenced as described in 2.12.5, whereas the amplified fragment from mixed samples (aggregates and enrichments) were individually cloned as follows.

2.12.3 Cloning and Escherichia coli Transformation

A TA cloning® Kit (Invitrogen™) or Strataclone™ PCR cloning kit (Strategene®) were used to isolate individual PCR products from the heterogeneous amplification of 16S rRNA gene fragments. The ligation of PCR products into a TA or Strataclone™ vector preceded transformation into competent *E. coli* cells (Mead et al. 1991). Ligation was obtained following the instruction of the supplier (Invitrogen™ or Stratagene®). *Escherichia coli* competent cells, from either Invitrogen™ or Strategene® normally stored at -80˚C, were thawed on ice and a water bath was prepared for 42ºC. Vectors were afterwards inserted into competent *Escherichia coli* cells (Mead et al. 1991). Prior to transformation, LB-ampicillin-XGAL agar plates were prepared, containing 30 g L^{-1} of Luria-Broth agar media (Fisher Scientific), 50 g L^{-1} of ampicillin (Sigma Aldrich), and 40 g L^{-1} of X-Gel (Bioline). Different volumes of transformed cells were distributed on LB-Ampicilin-XGAL plates, and incubated overnight at 37ºC. Plates containing transformed cells were incubated at 37°C, and white colonies were selected for further analysis as they contained plasmids with the required inserted DNA fragment. A sterile pipette tip was used to isolate individual positive colonies and transfer them into sterile 200µL tubes. The inserted 16S rRNA gene fragments were re-amplified from white colonies using PCR with 25 pmol µl L^{-1} of SCREEN1F (5'-AGTGTGCTGGAATTCGGCTT-3') and SCREEN1R (5'-

ATATCTGCAGAATTCGGCTT-3') primers (Boothman et al. 2006)or universal primers M13F (5'-GTAAAACGACGGCCAG-3') and M13R (5'-CAGGAAACAGCTATGAC-3') primers designed against the cloning site in the vector. A PCR of the mixture was performed (as follows) and put in the thermal cycler. PCR mixture reaction was made using 25 µl of Red*Taq*Mix (Sigma Aldrich, UK), 21 µl dH_20, 1 µl each primer (10 µM). 1Kb DirectLoad^{TM} Wide Range DNA marker (Sigma Aldrich, UK) was used for band size identification.

2.12.4 Restriction Fragment Length Polymorphism (RFLP)

RFLP is a laboratory technique which uses the characteristics of endonuclease enzymes to cut DNA at precise points. Different DNA sequences may be cut by endonuclease enzymes at different points and show different patterns on an electrophoresis gel. The partial 16S rRNA gene fragments were digested with restriction enzymes and analysed by electrophoresis to sort the PCR products into different ribotypes for DNA sequence analysis. All RFLP steps were performed using a mixture of the three restriction endonucleases *Eco*RI, *Msp*I and *SAU3AI* from Roche Diagnostics, Ltd (UK) in a total mix of 10 µl, containing 8.2 µl of PCR product; 1µL 10x buffer 2 (Roche Diagnostics Ltd, UK), 0.2 µL 10 Units per µL (U/µL) *Eco*RI, 0.1 µL 20 U/µL *Msp*I and 0.5 µL 4 U/µL *SAU*3AI. Reactions were incubated at 37ºC for approximately 16 hours, and results were checked on a 3% agarose gel (Brettschneider 1998).

2.12.5 DNA purification and sequencing

The DNA sequence of each ribotype was performed using an ABI Prism BigDye Terminator Cycle Sequencing Kit in combination with an ABI Prism 877 Integrated Thermal Cycler and ABI Prism 377DNA Sequencer (Perkin Elmer Applied Biosystems, Warrington, UK). Sequencing results were analysed using CHROMAS or BIOEDIT softwares, and BLAST search using the internet tool www.ncbi.nlm.nih.gov, with the BLAST search being performed at both gene and protein level. Alignments were made using CLC free workbench (www.clcbio.com) or BIOEDIT. Phylogenetic relationships were inferred by the neighbour joining method using the internet tool www.genebee.msu.su.

2.12.6 *Phylogenetic analysis*

Sequences from GenBank were added to the 16S rRNA gene alignment to cover the known genetic diversity of the aggregate-attached bacteria sequenced in the present thesis.. The 16S rRNA gene sequences were aligned in BioEdit Sequence Alignment Editor 7.0.9 (Hall 1999) using CLUSTALW program version 1.4. The alignments were edited manually and ambiguous bases were removed. In total, 530 base pairs of the 16S rRNA gene, were used for the phylogenetic analyses. Phylogenetic trees were inferred using the Neighbor-Joining method (Saitou and Nei 1987). The optimal trees were calculated using published approaches (Felsenstein 1985). The trees were drawn to scale, with branch length in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

Chapter 3. Role of microbial populations in the release of reduced species from aggregates incubated under static conditions

3.1 Introduction and experimental setting

Elements important for cell metabolism accumulate in OM and sink with marine snow flocs leaving the upper water column and thus become unavailable for biological uptake. In pelagic waters aggregates undergo a rapid decrease in POM (Lee *et al.*, 2004), most of which is dissolved and/or remineralised before aggregates reach the seafloor. In contrast, estuarine and coastal aggregates rapidly reach the seafloor with them being less depleted in OM than pelagic particles, although resuspension processes may also contribute to significant OM degradation. In shallow waters microbial and biogeochemical processes associated with marine snow occur mainly when aggregates are already settled on the seafloor. These processes have been often investigated in the laboratory by incubating marine particles under static conditions (Azua *et al.*, 2007; Hietanen *et al.*, 2002; Turley and Mackie, 1994; Wolgast *et al.*, 1998). Here the impact of aggregates settled on the seafloor on the release of nutrients and reduced species is investigated.

Details about the experiments are given on Table 3.1 and in Chapter 2. Briefly artificial aggregates were made by rotating seawater rich in phytodetritus using a published method (Shanks and Edmondson, 1989) as described in Section 2.2. Aggregates were then incubated in the dark for 1 to 2 weeks (Sections 2.3-2.5), the concentration of chemical species in the surrounding seawater and the profile of oxygen through the aggregates was measured (Table 3.1, Section 2.8). Moreover molecular (16S rRNA, Section 2.12) and culturing (Section 2.11) techniques were applied to investigate the bacterial diversity with particular attention devoted to Fe(III)-reducing bacteria (Table 3.1).

Once formed on the roller table aggregates were incubated under static conditions. All the aggregates except N16 to N20 (Section 3.2) were transferred from the tanks in which they had been formed to smaller flasks, such that the impact of the chemical species released from aggregates was evident over the initial concentration of nutrients and trace metals (Appendix 2). Large aperture pipettes were used to transfer

aggregates into smaller flasks which were then allowed to settle to the bottom of the flasks; aggregates were prevented from piling up on top of each other.

The samples N16 to N20 were incubated in the same tanks in which aggregates were formed rather than being transferred into smaller containers. Details are given in Section 2.4. Briefly in these experiments senescent phytoplankton material was incubated in five 2L tanks. Colloidal Fe(III) was added to the tanks N17 and N19, and a mixed culture of *Shewanella* species was added to the tanks N18 and N19. Both *Shewanella* cultures and colloidal Fe(III) were added to the tanks prior to flocculation of particles into larger aggregates on the roller table. After the formation of aggregates, Tank N20 was sterilised by autoclaving and used as control. The tank N20 was autoclaved with a closed lid and contamination was not likely to occur. However the high temperature used (120 °C) might have affected the iron speciation, by promoting the decomposition of Fe-binding organics.

The release of nutrients and trace metals from aggregates to the surrounding seawater can be investigated by incubating flocs along with their surrounding seawater in parallel with seawater controls. The differences in solute concentration occurring between the flasks containing aggregates and the seawater control flasks can be attributed to the presence of aggregates in the former flasks.

In the present chapter artificial aggregates formed under different conditions have been statically incubated and the concentration of different solutes (NO_3^-, NO_2^-, PO_4^{3-}, Fe(II) and dissolved Mn) determined over time. In addition the ability of the microbial communities present in these aggregates to grow anaerobically through the reduction of several species has been investigated.

Sample	Sample type[a]	Analyses conducted	Results shown
A1	Aggregates	Nutrients	Appendix 3
A3	Aggregates	Nutrients, dissolved ferrous iron	Appendixes 3-4
A4	Filtered control[b]	Nutrients, dissolved ferrous iron	Appendixes 3-4
T3	Filtered control[b]	nutrients	Appendix 3
C4	Seawater control[c]	nutrients	Appendix 3
P1	Aggregates	Nutrients, dissolved ferrous iron	Appendixes 3-4
P2	Filtered control[b]	Nutrients, dissolved ferrous iron	Appendixes 3-4
P3	Aggregates	Nutrients, dissolved ferrous iron	Appendixes 3-4
P4	Filtered control[b]	Nutrients, dissolved ferrous iron	Appendixes 3-4
TA1	Aggregates	Nutrients, dissolved ferrous iron, anaerobic culturing	3.3.1, 3.3.2, 3.3.5, 3.4.1
TA2	Filtered control[b]	Nutrients, dissolved ferrous iron	3.3.1, 3.3.2
N1	Aggregates	Nutrients, dissolved ferrous iron, 16S rRNA, anaerobic culturing	3.3.1, 3.3.2, 3.3.5, 3.4.1, 3.5.1, 3.5.2
N2	Filtered control[b]	Nutrients, dissolved ferrous iron	3.3.1, 3.3.2
N3	Aggregates	Nutrients	Appendix 3
N4	Filtered control[b]	Nutrients	Appendix 3
N5	Fe(III) enriched[d] aggregates	Dissolved ferrous iron	Appendix 4
N6	Fe(III) enriched[d] filtered control[b]	Dissolved ferrous iron	Appendix 4
N14	Fe(III) enriched[d] aggregates	16S rRNA	Appendix 5
N16[e]	Aggregates	Dissolved ferrous iron	3.3.3
N17[e]	Fe(III) enriched[d] Aggregates	Dissolved ferrous iron	3.3.3
N18[e]	*Shewanella*[f] enriched Aggregates	Dissolved ferrous iron	3.3.3
N19[e]	*Shewanella*[f] and Fe(III)[d] enriched Aggregates	Dissolved ferrous iron	3.3.3
N20[e]	Sterile Control[g]	Dissolved ferrous iron	3.3.3
SOT1	Aggregates[h]	Dissolved manganese	3.3.4
SOL1	Aggregates[h]	Dissolved manganese	3.3.4
SOT2	Seawater control[h]	Dissolved manganese	3.3.4
SOL2	Seawater control[h]	Dissolved manganese	3.3.4

Table 3.1 Summary of the experiments carried out in the present chapter.

[a] More details about aggregate origin, formation and incubation are given in Sections 2.2-2.5.
[b] Obtained from 10-µm filtered medium where aggregates had been formed.
[c] Obtained from 10-µm filtered Southampton Water.
[d] Colloidal Fe(III) was added to the medium prior to aggregate formation.
[e] Samples N16 to N20 were incubated in the same tanks in which they had been formed rather than transferred in smaller volumes.
[f] *Shewanella* cultures were added to the medium prior to aggregate formation.
[g] Aggregates were autoclaved after their formation.

[h] Aggregates formed from natural phytodetritus, controls made from 10-µm filtered seawater, as described in Section 2.5.

3.2 Results: concentration of inorganic species

3.2.1 Nutrient release

The concentrations of NO_3^-, NO_2^- and PO_4^{3-} measured in the seawater present in the Flasks N1, N2, TA5 and TA6 are shown in Fig. 3.1. The concentrations of the same species in Flasks N3, N4, A1, A3, A4, T3, C4, P1, P2, P3, P4 are shown in Appendix 3.

NO_3^- concentrations were variable and ranged from 20 µM in TA1 to 220 µM in the seawater control N2. Even though the initial concentration of NO_3^- was similar in all samples, the concentrations of NO_3^- in both flasks containing aggregates (TA1 and N1) at the end of the experiments were lower than in the respective seawater controls (TA2 and N2). Samples prepared from Southampton Water in Flasks N1 and N2 contained more NO_3^- than those deriving from controlled phytoplankton cultures (TA1 and TA2) at the end of the experiment (Fig. 3.1). In the other flasks containing aggregates the concentration of NO_3^- decreased in A1, T3 and N3 whereas it was constant in A3, P1 and P3; in the seawater controls it increased in C4 and was constant in N4, A4, P2 and P4, over time (Appendix 3).

The concentration of PO_4^{3-} in the flasks containing aggregates increased over time whereas in the controls it was constant (Fig. 3.1). The concentration of phosphate was also variable and ranged from 0.05 µM, in TA2 on day 3 to 4.0 µM in N1 on day 7 (Fig. 3.1). Similar trends in PO_4^{3-} concentrations were also found in most of the other samples analysed: the concentrations increased over time in the flasks containing aggregates A1, T3, N3 and P3 and were constant or increased less sharply in the control flasks C4, N4, and P4 (Appendix 3).

The concentration of NO_2^- ranged from 1.5 µM in the seawater control (TA2) to 4 µM in aggregate Flask N1, and increased over time in both flasks containing aggregates (TA1 and N1) (Fig. 3.1). In N1, it increased by ~50% over 7 days. In the controls, TA2 and N2, the concentration of NO_2^- was constant or decreased slightly (Fig. 3.1). Contrasting trends in NO_2^- were found in the other samples analysed, with concentrations not always differing between the flasks containing aggregates and the related seawater controls: the concentration of NO_2^- increased in A1, T3, N3, was constant in A3, A4, P3, P4, C4 and N4 and decreased in P1 and P2, over time

(Appendix 3).

Current results highlight, in most cases a release of NO_2^- and PO_4^{3-} from aggregates (Fig. 3.1) and a potential role of aggregates in the consumpton of dissolved NO_3^-. The trend in NO_3^- and PO_4^{3-} measured in most of the aggregates agrees with a previous study (Passow *et al.*, 2003). .

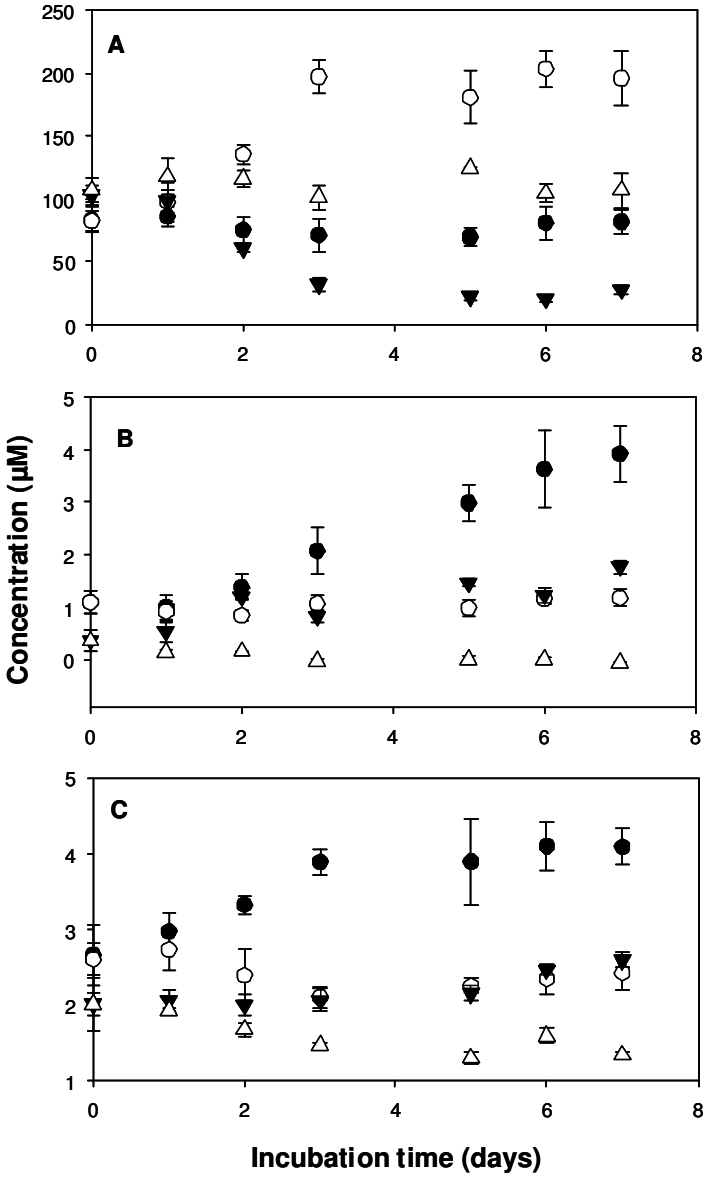


Figure 3.1. Concentration of (**A**) nitrate, (**B**) phosphate and (**C**) nitrite in the flasks containing aggregates N1 (●) and TA1 (▼) and in the seawater control N2 (○) and TA2 (Δ).

The initial nutrient content in the aggregates environment varied by almost one order of magnitude. This is likely associated with the different concentration in the

flasks, of the enrichment solution (50xF/2) originally added to SW to allow phytoplankton growth before flocculation experiments (see Section 2.2.3). Differences in the growth rates of phytoplankton in the cultures might also have affected the nutrient concentration in the water used for experiments.

3.2.2 Dissolved Ferrous iron: aggregates pre-concentrated

The concentration of dissolved Fe(II) in the aqueous environment surrounding marine aggregates ranged from 0.1 μM in N1 and TA1 at the beginning of the experiments to 0.7 μM in TA1 after 8 days and 0.55 in N1 after 11 days (Fig. 3.2). Thus, the concentration of dissolved Fe(II) increased over time in flasks TA1 and N1 by 500 and 300%, respectively. After 11 days of incubation, the concentration of Fe(II) in N1 had decreased substantially (Fig. 3.2). The concentration of dissolved Fe(II) in TA1 was not measured after day 8. The concentration of Fe(II) in the seawater controls was, on the other hand, constant (Fig. 3.2).

In samples N5 and N6, to which 100 μM of colloidal Fe(III) were added, the concentration of dissolved Fe(II) increased significantly on day 1 and then decreased, presumably reflecting the oxidation of Fe(II) to Fe(III) (Appendix 3). The concentration of dissolved Fe(II) was higher in N5 (containing aggregates) compared to N6 (seawater control) (Appendix 3). Moreover the concentration of Fe(II) over time increased in P1, P3 and A3 whereas it decreased over time in the related seawater control (P2, P4 and A4) (Appendix 3).

To the best of author's knowledge there are not any previous peer reviewed publication reports on the release of Fe(II) from aggregates. However the data reported here are in good agreement with a Master of Science (MSc) project carried out at National Oceanography Centre, Southampton (Nestoridi, 2005).

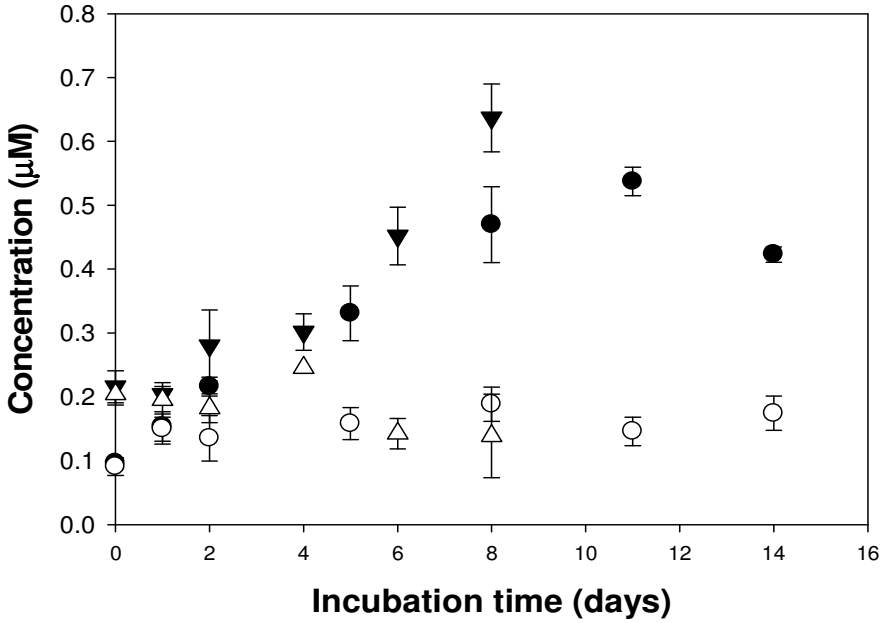


Figure 3.2. Concentration of dissolved Fe(II) in the flasks containing aggregates N1 (●) and TA1 (▼) and in the seawater control N2 (○) and TA2 (Δ).

3.2.3 Dissolved Ferrous iron: non pre-concentrated aggregates

The initial concentration of dissolved Fe(II), in the samples N16 to N20 was 110 ± 34 nM. After aggregate formation the concentration of dissolved Fe(II) decreased over time in Tanks N16 and N20. However the decline in dissolved Fe(II) was sharper in Tank N20 compared to Tank N16 (Fig. 3.3). The addition of 100 µM of colloidal Fe(III) in the tanks N17 and N19 (see Section 2.4.1 for details) involved an increase in the concentration of dissolved Fe(II) during the first two days of incubation. In N19, containing also cultures of different *Shewanella* species the concentration of dissolved Fe(II) increased by over one order of magnitude, from 110 ± 34 nM to 6300 ± 200 nM (Fig. 3.3) from day 0 to day 2. In Tank N18, the concentration of dissolved Fe(II) increased over time up to 1500 ± 200 nM on day 3. However after day 2 in the tanks N17 and N19 and after day 3 in Tank N18 the concentration of dissolved Fe(III) decreased over time towards the end of the experiments (Fig. 3.3).

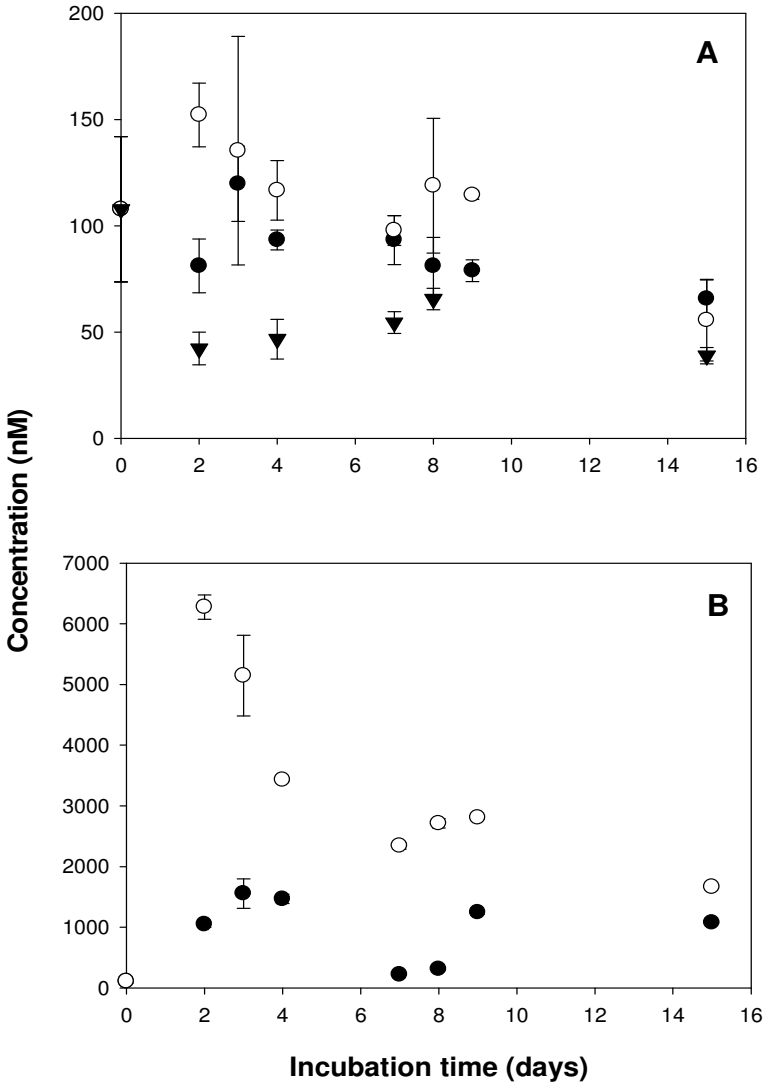


Figure 3.3. Concentration of Dissolved Fe(II) in the aggregates incubated in 2L bottles under different conditions. (**A**) Effect of the addition of 100 µmol/L of $FeCl_3$ in samples N16 (●),N17 (○) and N20 (▼). (**B**) Effect of the addition of a mixed culture of *Shewanella*: aggregates N18 (●) and N19 (○). See also Appendix 2 for details about the samples.

3.2.4 Dissolved manganese

The concentration of dissolved Mn was measured in aggregates made from natural phytodetritus as described in Section 2.5. The initial concentration of dissolved Mn present in Southampton Water was 210 ± 35 nM and was higher than that of the

Solent (130 ± 14) reflecting the decreasing concentrations associated with increasing salinities in estuaries (Fang, 1995) (Fig. 3.4). The seawater rich in phytodetritus was rotated on the roller table to form aggregates which were then transferred in 250 mL flasks. The flasks SOT1 and SOL1 contained aggregates formed from Southampton Water and the Solent, both of which were rich in phytodetritus, whereas SOT2 and SOL2 contained 10 µm-filtered Southampton and Solent Water, respectively (seawater controls).

In SOT1 the concentration of dissolved Mn increased from 210 ± 35 nM on day 0 to 500 ± 24 nM on day 8. Similarly in SOL1 it increased from 126 nM to 234 nM over the same incubation period (Fig. 3.4). In SOT2 the concentration of dissolved Mn increased from 210 ± 35 nM to 300 ± 23 nM and in SOL2 it decreased from 130 ± 14 nM to 100 ± 5 nM (Fig. 3.4, Appendix 5).

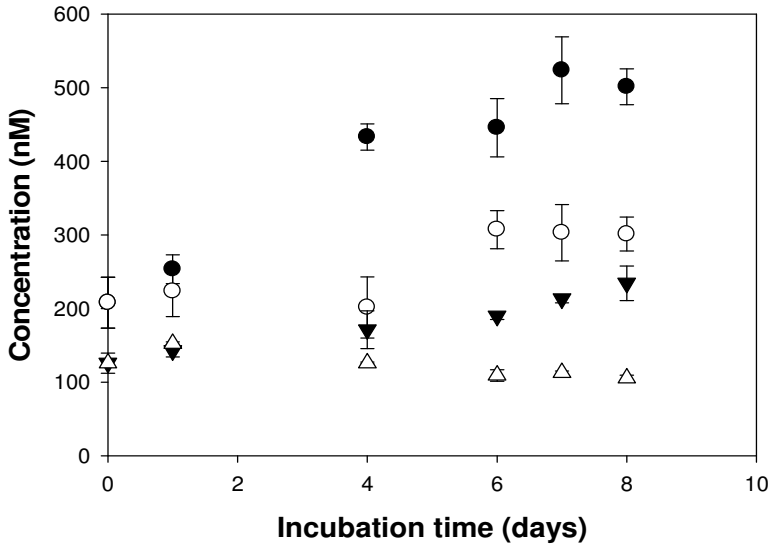


Figure 3.4. Concentration of Dissolved Mn in the flasks containing aggregates formed from natural phytodetritus. (●) SOT1; (○) SOT2; (▼) SOL1; (Δ) SOL2.

Note that the flasks SOT1 and SOL1 contained aggregates formed from Southampton and Solent Water, respectively whereas the flasks SOT2 and SOL2 contained 10-µm filtered seawater from Southampton and Solent Water, respectively.

3.2.5 Oxygen saturation

The concentration of dissolved oxygen, measured in 2 individual aggregates, was found to drop from 210 µM in the surrounding seawater to 100 µM in the centre of the aggregates, but anoxia was not detected in any aggregate (Fig. 3.5). At the base of

the particle, the oxygen concentration did not fully return to background, presumably reflecting the restricted diffusion of oxygen through the agar on which the particle was sitting. The concentration of dissolved oxygen found in the interstitial seawater of aggregates is similar to that measured in two previous studies (Alldredge and Cohen, 1987; Ploug and Jorgensen, 1999) but contrasts with a study (Ploug *et al.*, 1997) where anoxic microzones were reported to be present.

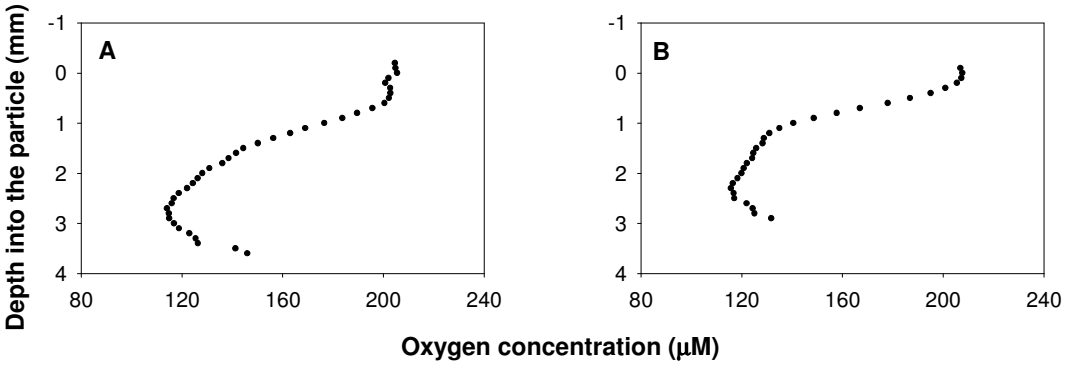


Figure 3.5 Profile of oxygen across individual aggregates: (**A**) sample N1; (**B**) sample TA1.

3.3 Results: culturing of Fe(III)-, NO_3^-- and SO_4^{2-}-reducing bacteria

Culture experiments were aimed at detecting the presence of microorganisms able to grow through the reduction of ferric iron, nitrate and sulfate and nitrate in the aggregates studied in the present work. Bacteria capable of respiring Fe(III) and NO_3^- under anaerobic conditions were found to be present in the artificial marine aggregates. Reduction of Fe(III)-NTA and FeOOH occurred, in cultures taken from aggregates collected from N1 and TA1 (Table 3.2). The presence and activity of nitrate-reducing organisms was also demonstrated for N1, by anaerobic culturing and analyses of nitrate and nitrite in the cultures (Fig. 3.6).

Reduction of sulphate occurred in media supplemented with lactate, as black precipitates, which are presumably sulfides, were observed on the bottom of the bottle. Bacterial growth was not observed in acetate/sulfate incubations. Further analyses is required to identify the SRB growing in the sulphate/lactate incubations.

3.3.1 Ferric iron reduction

In particular, 1 of the replicates of N1, incubated with lactate as the electron donor and Fe(III)-NTA as the electron acceptor, reduced most of the Fe(III) present (10 mM) as the concentration of HCl extractable Fe(II) was 9.85 mM (Table 3.2). Two months after the first incubation, an aliquot (200 μL) of this culture was inoculated into new sterile medium, and after another 2 months incubation, over 90% of 10 mM Fe(III)-NTA added were completely reduced to Fe(II) again (data not shown). However, incubations containing insoluble FeOOH as the electron acceptor and lactate as the electron donor showed a lower production of Fe(II) (Table 3.2). A lower production of Fe(II) was also measured in the anaerobic cultures of aggregates from TA1 compared to N1. The concentrations of Fe(II) in the abiotic controls were low compared to the samples and ranged from 1.1 to 1.3 mM (Table 3.2).

Table 3.2. Concentration of HCl extractable Fe(II) in the anaerobic cultures of the aggregates.

Sample	Substrates	Fe^{2+} concentration (mM)
Abiotic control	Acetate/Fe(III)-NTA	1.25 ± 0.06
Abiotic control	Acetate/FeOOH	1.15 ± 0.02
Abiotic control	Lactate/Fe(III)-NTA	1.21 ± 0.05
Abiotic control	Lactate/FeOOH	1.33 ± 0.26
TA1	Acetate/Fe(III)-NTA	3.28 ± 0.08
TA1	Acetate/FeOOH	3.10 ± 0.15
TA1	Lactate/Fe-NTA	2.97 ± 1.07
TA1	Lactate/FeOOH	1.91 ± 0.15
N1	Acetate/Fe(III)-NTA	1.52 ± 0.05
N1	Acetate/FeOOH	1.64 ± 0.06
N1	Lactate/Fe(III)-NTA	1.81 ± 0.49*
N1	Lactate/Fe(III)-NTA	9.85**
N1	Lactate/FeOOH	1.26 ± 0.27

* Only 2 replicates

** Third replicate

3.4.2 Nitrate and nitrite reduction

Analyses of NO_3^-/lactate enrichment cultures of N1 confirmed the presence and activity of NO_3^--reducing microorganisms, with a strong loss of NO_3^- but little

accumulation of the denitrification intermediate NO_2^- (Fig. 3.6). Aggregates were initially incubated with 15 mM $NaNO_3$ as electron acceptor. After 30 days of incubation, the cultures contained 0.1 mM of NO_2^- and no measurable NO_3^- (Fig. 3.6). In contrast NO_3^- reduction was not observed in NO_3^-/acetate incubations of aggregates from N1 that, after 30 days of incubation, contained 13 ± 0.7 mM of NO_3^- and no measurable NO_2^-. The concentration of NO_3^- in the abiotic controls was 14 ± 0.2 mM, whereas NO_2^- was not detected.

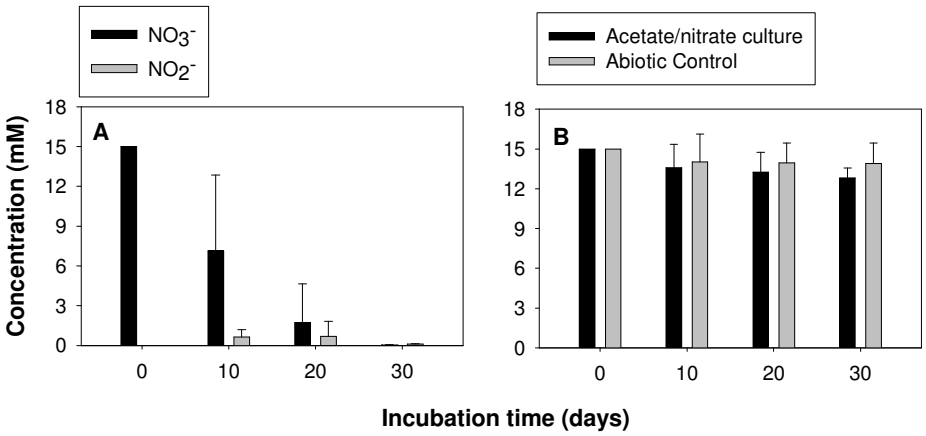


Figure 3.6 Concentration of nitrate and nitrite in the anaerobic cultures of artificial aggregates collected from sample N1 amended with nitrate as electron acceptor. (**A**) Concentration of nitrate and nitrite in the culture incubated with lactate as electron donor. (**B**) Concentration of nitrate in the culture incubated with acetate as electron donor and in the abiotic control which contained both acetate and lactate as electron donors. The concentration of nitrite measured in these enrichment cultures was lower than the detection limit of the technique applied (0.1 mM).

3.4 Results: molecular analyses

The diversity of 16S rRNA gene sequences recovered from aggregates in samples N1, N10 and N14 and 1 selected enrichment culture (N1 cultured with lactate electron donor and Fe(III)-NTA as electron acceptor) was examined by RFLP sorting followed by gene analyses.

For sample N10, 16S rRNA genes amplified using *Geobacter* specific primers Geo564F and Geo840R, allowed the distinction between three different RFLP types (Appendix 6). Comparison with the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov, NCBI) database showed these to be related to

Anaeromyxobacter dehalogenans (δ-*Proteobacterium*), uncultured clone TDNP_LSbc97_121_1_46 (δ-*Proteobacterium*) and uncultured clone PI_6G45 (unidentified bacterium). The first two RFLP types show however a very low analogy with NCBI sequences (Appendix 6).

For sample N14, to which colloidal Fe(III) was added, 16S rRNA genes amplified using 8F and 519R primers, allowed the distinction between three different RFLP types two of each were affiliated to *Planctomycetes* GMD14H10 and the other ribotype was affiliated to uncultured bacterium clone CBM02G04 (Appendix 6).

3.4.1 Bacterial composition of artificial aggregates

The diversity of 16S rRNA gene sequences recovered from one selected enrichment culture of artificial aggregates from N1 was examined by RFLP sorting of 100 different clones followed by gene analyses. Amplification of 16S rRNA gene fragments with primers 8F and 519R from N1 revealed a comparatively simple microbial community with 22 different RFLP types from 150 different clones (Table 3.3). Of the 22 different RFLP types, the most highly represented were affiliated with *Cyanobacteria* (24% of the clone library), Bacteroidetes (27%), and γ-*Proteobacteria* (13%); α-*Proteobacteria* were also present (Table 3.3). Data are in partial agreement with previous studies on microbial colonisation of aggregates (Bockelmann *et al.*, 2000; Brachvogel *et al.*, 2001; DeLong *et al.*, 1993; Selje and Simon, 2003; Simon *et al.*, 2002). The most abundant organisms identified in the clone library were related to strains SIMO-738, BT60MF2BD12, GMDsbC3, P8-GEN-18 and SIMO-110 (Table 3.3). Most of the organisms identified from molecular analysis of N1 were uncultured, except *Colwelia* sp., *Formosa* sp., *Legionella* sp., *Orientalimare eburneum*, and *Roseobacter litoralis*, which were present as low fractions of the total, ranging from 1 to 3% of the clone library (Table 3.3). No known Fe(III)-reducing bacteria were detected using these broad specificity primers.

Table 3.3. Taxonomic affiliation of the ribotypes identified in uncultured aggregates N1 after analysis of the partial 16s rRNA gene*.

Closest matching microorganism	NCBI** accession number	Group	Identities (% matches)	Percentage present
Uncultured clone SIMO-738.	AY712275	*Cyanobacteria*	432/448 (96%)	14
Uncultured clone SC1-26.	DQ289919	Bacteroidetes	50/56 (89%)	14
Uncultured clone BT60MF2BD12.	AF365593	Unknown	428/432 (99%)	10
Clone GMDsbC3.	AY162097	Bacteroidetes	470/503 (93%)	10
Uncultured clone P8-GEN-18.	AY361930	Unknown	448/472 (94%)	8
Uncultured clone PI_4z6c.	AY580687	Bacteroidetes	265/290 (91%)	5
Uncultured clone SIMO-1100.	AY710540	*Cyanobacteria*	301/329 (91%)	5
Uncultured clone CD2E6 16S.	AY038472	Unknown	435/449 (96%)	4
Uncultured clone SCF_4984.	AJ630720	Bacteroidetes	479/493 (97%)	4
Uncultured clone SC1-14.	DQ289918	γ-*Proteobacteria*	357/404 (88%)	3
Legionella sp. OUB41,	AB058918	γ-*Proteobacteria*	401/446 (89%)	3
Formosa sp. 18III/A01/079	AY612758	Bacteroidetes	474/502 (94%)	3
Uncultured clone CD13H5.	AF441870	α-*Proteobacteria*	384/446 (86%	3
Uncultured clone SBET_5159.	AJ630710	γ-*Proteobacteria*	511/526 (97%)	3
Uncultured clone Belgica2005/10-140-11.	DQ351790	γ-*Proteobacteria*	318/352 (90%)	3
Uncultured clone SIMO-1415.	AY710855	*Cyanobacteria*	440/449 (97%)	1
Uncultured clone SIMO-1334.	AY710774	*Cyanobacteria*	428/444 (96%)	1
Uncultured clone JH12_C91.	AY568926	unknown	320/356 (89%)	1
Colwellia sp. 'Egg clone D45'.	AF034927	γ-*Proteobacteria*	351/363 (96%)	1
Orientalimare eburneum clone SW-277	DQ667965	α-*Proteobacteria*	265/269 (98%)	1
Uncultured Flavobacterium clone SIMO-582	AY712119	Bacteroidetes	417/502 (83%)	1
Roseobacter litoralis Och 149 (ATCC 49566)	X78312	α-*Proteobacteria*	441/451 (97%)	1

*16S rRNA gene amplified using 8F and 519R primers.
**NCBI: National Centre for Biotechnology Information

3.4.2 Anaerobic cultures of artificial aggregates

In addition to direct molecular analyses of DNA from aggregates, Fe(III)-reducing bacteria were also enriched using appropriate growth media in separate cultures using conventional microbiological techniques. Briefly aggregates were incubated anaerobically with Fe(III) as electron acceptor and different electron donors (see Section 2.11.1 and Table 2.7 for details). Amplification of 16S rRNA gene fragments from enrichments using lactate as the electron donor and Fe(III)-NTA as the electron acceptor, performed using primers 8F and 519R, revealed the presence of 3 different RFLP types; 2 of these represented 93% of the clone library and contained genes affiliated with the genus *Marinobacter*, whereas the third was most closely related to *Desulfovibrio* sp., Clone NA302 (Table 3.4).

Closest matching microorganism	NCBI** accession number	Cluster	Identities (%matches)	Percentage present
Marinobacter aquaeolei OC-11	AY669171	*γ-Proteobacteria*	262/291 (90%)	90
Desulfovibrio sp. NA302	AJ866944	*δ-Proteobacteria*	490/493 (99.4%)	7
Marinobacter sp. CAB	U61848	*γ-Proteobacteria*	499/508 (98.2%)	2

Table 3.4. Taxonomic affiliation of the 16S rRNA ribotypes* identified in the anaerobic enrichment of aggregates collected from Flask N1 incubated with lactate as the electron donor and Fe(III)-NTA as the electron acceptor.

*16S rRNA gene amplified using 8F and 519R primers.
**NCBI: National Centre for Biotechnology Information

3.5 Discussion: element cycling in marine aggregates

The large increase of Fe(II) and PO_4^{3-} and slight increase in NO_2^- over time, measured in N1 and TA1 containing aggregates prepared from natural and defined phytoplankton cultures, respectively (Figs. 3.1, 3.2), are associated with decreasing or relatively constant NO_3^- concentrations (Fig. 3.1). This behaviour suggests the

occurrence of (1) Fe(III) reduction to Fe(II), (2) NO_2^- production due to NO_3^- reduction and/or NO_2^- release during nitrification and (3) significant consumption of OM leading to PO_4^{3-} release. As there was very little change in PO_4^{3-}, NO_2^- and Fe(II) in the seawater controls N2 and TA2 (Figs. 3.1, 3.2), the reductions occurring in N1 and TA1 appear to be related to processes inside the aggregates as oxygen became depleted due to intense bacterial respiration. The rates of reduction processes producing Fe(II) and NO_2^- must be significantly greater than their oxidation rates in the oxic regions of aggregates and in the surrounding seawater in order for measurable concentrations of these reduced species to be present. Moreover some of the dissolved Fe(II) present might have been present complexed by organic ligands and thus in a less unstable form.

3.5.1 Phosphate

The increase in PO_4^{3-} in the flasks containing artificial aggregates (Fig. 3.1, Appendix 3) indicates that a significant degradation of OM was occurring. The aerobic conditions in which aggregates were incubated and the likely lability of the OM present were likely to be the main factors promoting the significant degrading processes occurring. In contrast the OM content in the control flasks was likely to be much lower than that of the flasks containing aggregates, leading to the constant or slightly increasing PO_4^{3-} concentration registered over time in the seawater controls (Fig. 3.1, Appendix 3). Most of the labile OM present in TA1 was likely degraded during the first 5 days of dark incubation as further increases in PO_4^{3-} were not registered after day 5, whereas the OM present in N1 was likely to be continuously consumed as the concentration of phosphate in the surrounding seawater increased for all of the experiments (Fig. 3.1). A continuous degradation of OM leading to the increasing concentrations of PO_4^{3-} measured was also likely to occur in T3, N3 and P3 (Appendix 3).

3.5.2 Nitrate and nitrite

The nitrate concentrations in the two samples containing aggregates appears to be contradictory as it is constant in sample N1 and decreases in sample TA1 over time (Fig. 3.1). The NO_3^- profile in the related seawater controls, showing also a different trend in samples N2 and TA2 (Fig. 3.1), suggests different biogeochemical conditions in the two mesocosms. The amount of nitrate present in a mesocosm is the result of the balance between nitrate production, due to nitrification and nitrate removal, due to

biological uptake as well as nitrate reduction to nitrite and other reduced species.

The residual NO_3^- in aggregate incubation solutions was much lower than in the initial f/2 solutions, which indicates significant uptake into phytoplankton and bacterial biomass prior to aggregate formation. The senescent phytoplankton material used was expected to contain a range of nitrogen species including dissolved organic nitrogen (DON). As aggregates were incubated in the dark for all of the experiments, the net continued NO_3^- removal observed, especially in TA1 (Fig. 3.1), must have been caused by non-photosynthetic microbial processes. The aggregates in N1 presumably took up as much NO_3^- as they produced from oxidation of DON. The most important observation is the net production of the reduced form of nitrogen, NO_2^-, in TA1 and N1, relative to the controls. The NO_3^- production observed in the seawater control N2 (Fig. 3.1) most probably reflects release of NO_3^- from nitrification. An analogous increase in the concentration of NO_3^- was not observed in TA2 (seawater control), reflecting a different origin of biomass and balance of bacterial uptake and recycling of NO_3^-. NO_2^- production in N1 and TA1 was not stoichiometrically equivalent to the NO_3^- losses (Fig. 3.1), probably because part of the NO_2^- was further reduced and part of the NO_3^- was taken up by organisms rather than reduced by dissimilatory processes. As experiments were carried out in the dark the nitrate uptake was of non-photosynthetic origin and probably can be ascribed to bacterial processes; a number of bacteria including *Pseudomonas aeruginosa*, *Ralstonia eutropha* and *Klebsiella pneumoniae* have been shown to contain genes for nitrate assimilation (Zumft, 1997). However, the presence of NO_2^- indicates that active NO_3^- reduction or nitrification occurred. The reduction rates producing nitrite must be significantly greater than their oxidation rates in the oxic regions of aggregates as well as in the surrounding seawater for measurable concentrations to be present. The absence of anoxic microzones, as suggested by oxygen electrode profiles in N1 and TA1 (Fig. 3.5), is in agreement with some previous studies on oxygen concentrations in marine aggregates in oxic water columns (Alldredge and Cohen, 1987; Ploug and Jorgensen, 1999) and suggests that detectable anoxic microzones are unlikely to be present in natural or artificial marine snow.

3.5.3 Iron reduction

The f/2 medium made using coastal seawater contained 10 nM of EDTA and a similar concentration of Fe(III). However, these concentrations are 1 order of magnitude lower than the concentration of Fe(II) measured in the control samples, and

60-fold less than the highest values seen in the incubation experiment. Therefore, the EDTA and introduced iron should not have significantly influenced the observed results, and reduced iron in solution must have been produced by the aggregates. The Fe(II) was measured using the ferrozine method, for which any dissolved Fe(III) present does not significantly affect the measurement (Viollier *et al.*, 2000). At the high concentration of ferrozine used (0.1 mM), and given the high stability constant for Fe(II)-ferrozine complex, it is expected that any organic complexes of Fe(II) were also determined.

Significant concentrations (average 160 nM) of dissolved total iron (Fang, 1995; Head, 1971) have been observed in the water column in Southampton Water, from where the water used in the present experiments came. Measurements of particulate iron were not carried out during the present work, but artificial aggregates obtained following the same method as described here contained ~20 (range: 9 to 27) μmol of iron per gram of suspended particulate matter as measured by the author (data not shown). The containers (2L) used to form aggregates contained 10 to 50 mg L^{-1} of total suspended matter (>0.2 μm) and were expected to contain between 0.2 and 1.0 μM of particulate iron. The aggregates were transferred after their formation to smaller (0.5 l) flasks in order to increase the aggregates:seawater ratio, thus increasing the amount of particulate iron available for reduction per litre.

Iron in oxygenated seawater should be present as Fe(III) (Millero *et al.*, 1987) and the oxidation of Fe(II) is a function of the oxygen concentration as well as the seawater pH (Millero *et al.*, 1987). The pH in the samples ranged from 6.5 to 7 without showing any obvious variation over time or between aggregate samples and controls, and for samples at this pH and in contact with air, the concentration of any dissolved iron in seawater above equilibrium concentrations should decrease rapidly over time, as dissolved Fe(III) produced from Fe(II) oxidation tends to be scavenged by particles and precipitate as particulate Fe(III) (Sunda, 2001). One reason for the very high concentrations of dissolved Fe(II) in the present study could have been the presence of organic ligands that may have inhibited oxidation. Interactions of Fe(II) with DOM has been suggested to slow Fe(II) oxidation in the Southern Ocean (Croot *et al.*, 2008; Croot *et al.*, 2001; Croot *et al.*, 2007; Moffett, 2001), subarctic Pacific (Roy *et al.*, 2008), and Indian Ocean hydrothermal plumes (Statham *et al.*, 2005). It has been shown that more then 99% of dissolved iron in seawater is bound to organic chelators (Gledhill and van den Berg, 1994; Wu and Luther, 1995) including humic substances

(Laglera *et al.*, 2007). Such ligand-Fe(II) complexes would also explain the relatively high background of Fe(II) (~150 nM) in the controls in the present experiments, where high concentrations of DOM from the cultures were expected.

The increase in the concentration of dissolved Fe(II) in the samples containing aggregates indicates the net reduction of the Fe(III) present inside the flocs. No Fe(III) was added to the aggregates and so the release of Fe(II) must have been from the reduction of natural Fe(III) phases present within the aggregates, as no change in Fe(II) was observed in the seawater controls without aggregates. The relatively high concentrations of Fe(II) in the samples were 1 to 2 orders of magnitude higher than those observed in natural coastal waters (Fig. 3.2), which reflects the small volume of seawater (0.5 l) used in the experiment and the absence of the large-scale dilution that occurs in natural waters. The gradual decline in Fe(II) concentration towards the end of the experiment (Fig. 3.2) most probably reflects the predominance of oxic removal of reduced Fe(II). The predominance of Fe(II) oxidation over Fe(III) reduction is likely associated with the exhaustion of labile organic matter used by bacteria as electron donor for Fe(III) reduction. The reduction process described here can be an important natural process that adds to the pool of Fe(II) in the surrounding water and that effectively recycles the Fe(III) in the aggregates.

3.5.4 Effect of addition of Fe(III) and Shewanella cultures

Although a significant release of dissolved Fe(II) has been measured from N1, TA1 (Fig. 3.2) as well as P1 and A3 (Appendix 4), the concentration of dissolved Fe(II) in N16 did not increase over time (Fig. 3.3). However while the concentration of Fe(II) decreased over time in N16 containing aggregates, higher initial concentration were measured in N16, compared to the Tank N20 containing sterile aggregates, this supports the hypothesis that dissolved Fe(II) was likely produced inside the aggregates from the reduction of Fe(III) and that the oxidation of Fe(II) in the aerobic environment was likely retarded by organic ligands. The concentration of Fe(II) in N16 did not increase over time (Fig. 3.3) probably because the aggregate/seawater ratio was low: in contrast with the experiments shown in Fig. 3.2, in the present experiments aggregates were incubated in the same 2L tanks where they had been formed (Fig. 3.3), the aggregate/seawater ratio in Tank N16 was thus lower than in N1 and TA1 and the impact of aggregates on the cycling of iron was therefore less evident. The increase in dissolved Fe(II) measured in N17 is likely associated to the addition of colloidal Fe(III)

that occurred on day 0. The significant increase in dissolved Fe(II), measured in N18 and N19 are most probably due to the addition, in these tanks, of a mixture of *Shewanella* species. *Shewanella* species are known dissimilatory Fe(III) reducers (Lloyd, 2003; Lovley *et al.*, 2004) and their presence accounted for a significant contribution to the reduction process that occurred. The higher concentrations of dissolved Fe(II) found in Tank N19 compared to Tank N18 are likely associated to the different composition of the tanks. In addition to *Shewanella* species, 100 µmol/L of colloidal Fe(III) was added to Tank N19 prior to aggregate formation. A higher content of Fe(III) was thus present in Tank N19 compared to Tank N18. However significant concentrations of dissolved Fe(II) were also measured in Tank N18 on day 3 (1600 ± 200 nM) which did not contain any Fe(III) initially added. This likely reflects the high amounts of particulate Fe present in Southampton Water which was used to form aggregates. Although the concentration of oxygen was not measured in the present experiments, *Shewanella* species have the potential to reduce Fe(III) to Fe(II) with oxygen being present as demonstrated for *S. putrefaciens* (DiChristina, 1992; McKinzi and DiChristina, 1999).

3.5.5 Manganese reduction

The higher levels of dissolved Mn initially measured in SOT1 and SOT2 compared to SOL1 and SOL2 reflects the different concentration of dissolved Mn occurring in Southampton and Solent Water (Fang, 1995). The increase in the concentration of dissolved Mn over time occurred in SOT1 and SOL1 (Fig. 3.4) is likely associated with the reduction of particulate Mn(IV). The release of reduced manganese from marine aggregates was previously investigated during an MSc project carried out at NOCS (Turner, 2004) and higher concentrations of released dissolved manganese were found. The oxidation rate of Fe(II) in seawater is much higher than that of Mn(II) (Morgan, 2005), but the increase in dissolved Fe(II) (up to 500%, Fig. 3.2) measured in N1 and TA1 (Fig. 3.1) is more significant than that in dissolved Mn (up to 100%, Fig. 3.4) found here. However due to the different origin of the aggregates studied, the results obtained from SOT1 and SOL1 can not be compared with those related to N1 and TA1; aggregates N1 and TA1 were formed from senescent algal cultures which had been cultivated in the laboratory whereas SOT1 and SOL1 were formed from natural phytodetritus.

The freshness and biological lability of the OM present in aggregates controls

the rate of microbial respiration and therefore the redox conditions occurring inside aggregates. The oxygen consumption rate was likely to be lower in SOT1 and SOL1 compared to N1 and TA1, leading to the less likely presence of suboxic and anoxic microenvironments in these aggregates. Aggregates N1 and TA1 were obtained from algal cultures whereas SOT1 and SOL1 were formed from natural phytodetritus; algal cultures maintained in the laboratory are likely to contain more labile material compared to phytodetritus collected from the estuary.

3.6 Discussion: reducing processes in aerobic environments

3.6.1 Presence of anoxic microenvironments

The release of reduced compounds from aggregates in oxic waters, observed in the present and in previous studies (Karl and Tilbrook, 1994; Shanks and Reeder, 1993) appears to contradict the absence of detectable anoxic microzones. One possible mechanism is the reduction of the mentioned solutes inside anoxic microenvironments smaller than the sensing tip of the oxygen microelectrode used (10 μm). However, these environments would need to be strongly anoxic and abundant enough to reduce measurable amounts of all the solutes mentioned in the present study as well as those in the literature. Anoxic microzones could be generated as a consequence of an intense aerobic respiration coupled with a low porosity that would inhibit the penetration of oxygen in comparison to the rest of the aggregate. An anoxic or suboxic microenvironment can be maintained as long as the oxygen diffusion rate from the surrounding environment does not exceed the overall oxygen consumption rate in that microenvironment. The amount of oxygen diffusing into aggregates is proportional to the surface area through which it diffuses, whereas the oxygen consumption rate is proportional to the amount of OM being degraded and therefore to the volume of that microenvironment. The likelihood of a microenvironment to become anoxic is negative function of its size.

Assuming that solutes diffuse through the aggregates as quickly as they do through seawater, a microregion 10 μm thick (diameter) should consume 100 μmol O_2 mm^{-3} h^{-1} to conserve its anoxic conditions (Ploug *et al.*, 1997). To maintain this rate of consumption of oxygen from the surrounding environment, the microbial communities present in marine aggregates would need to continuously respire extremely high amounts of OM. The chemical diffusion of solutes within aggregates has been

hypothesised to be slower than in seawater (Alldredge, 2000; Shanks and Reeder, 1993) due to the presence of substances such as TEP, which are abundant in marine aggregates (Alldredge *et al.*, 1993). However, the solute diffusion rate within marine aggregates has recently been reported to range between 0.85 and 0.95 fold the value in seawater (Ploug and Passow, 2007). In that work, the sensing tip used for measuring diffusivity was rather wide (100 μm diameter), resulting in a tip section area of 7850 μm^2, 100 fold greater than the section of the oxygen microelectrode tip used in current experiments (10 μm diameter). Given the large area of the diffusivity sensor, microzones with lower diffusivity (e.g. particles surrounded by TEP) might have been present without impacting the overall diffusivity through the zones of the particles investigated. Microregions, present but not abundant in the aggregates, may thus still be present and have a diffusivity significantly lower than in the surrounding seawater.

As marine aggregates are very heterogeneous, the lability of the OM present may also change, generating different oxygen consumption rates within the same aggregate. Microenvironments rich in refractory OM may thus have lower oxygen consumption rates compared to those rich in labile OM. Low-diffusion substances such as TEP could act as gas barriers, limiting the diffusion of oxygen inside the aggregates and allowing the formation of suboxic or anoxic subregions; this process may occur especially where the OM is labile and the oxygen is therefore quickly respired by microbial communities.

3.6.2 Mechanism for iron reduction

The redox conditions in the oceans are primarily driven by microbial processes. Under dark conditions the reduction of solutes in anoxic or suboxic microenvironments is mainly associated with a direct microbial impact likely associated to dissimilatory activities.

The mechanisms of Fe(III) reduction in the environment remain heavily debated and can include direct reduction by cytochromes on the outer membrane of Gram-negative Fe(III)-reducing bacteria (Lovley *et al.*, 2004), or by secreted electron shuttles (von Canstein *et al.*, 2008) or nanowires protruding from the cell surface (Gorby *et al.*, 2006; Reguera *et al.*, 2005). However Fe(III) reduction proceeds, it is most probable that under low oxygen conditions, only the Fe(III) particles close to the microbial cell are reduced, releasing Fe(II) into the local environment, and in turn out of the aggregates by diffusion. The dissimilatory reduction of Fe(III) by *Shewanella*

putrefaciens has been recently suggested to occur via the attachment of Fe(III) colloids on the surface of the Fe(III)-reducing microorganism (Bonneville *et al.*, 2006). An anoxic microenvironment in the contact zone between the bacterial membrane and the Fe(III) oxide could exist, thus allowing the dissimilatory reduction of Fe(III) to Fe(II), and the diffusion of dissolved Fe(II) into the local environment, and in turn out of the aggregates by diffusion.

The production of reduced species has been considered to occur only under anaerobic conditions (Stumm and Morgan, 1993). However, under low oxygen conditions, *Shewanella oneidensis* was shown to simultaneously reduce Fe(III), NO_3^- and NO_2^- (DiChristina, 1992; McKinzi and DiChristina, 1999). Fe(II) has also been found under suboxic conditions in sediment porewaters (Sell and Morse, 2006) as well as in the oxygen minimum zone in the water column where NO_2^- was also found (Moffett *et al.*, 2007). The simultaneous respiration of NO_3^- and O_2 has also been shown to occur within both phytodetritus (Wolgast *et al.*, 1998) and coastal sediment (Brandes and Devol, 1995). Diatoms and *Cyanobacteria* have been shown to produce superoxides and extra-cellular enzymes that are able to reduce Fe(III) to Fe(II) in aerobic waters (Kustka *et al.*, 2005; Maldonado and Price, 1999; Maldonado and Price, 2000; Salmon *et al.*, 2006; Shaked *et al.*, 2002; Shaked *et al.*, 2004; Shaked *et al.*, 2005), although the impact of these processes on the overall iron dissolution appears to be more relevant under iron-depleted conditions. Moreover the oxygen concentration found in the aggregates studied in the present work was very high, compared to that measured in the previous studies where Fe(III) and nitrate reductions were reported to occur. The reduction of Fe(III) and NO_3^- in the presence of oxygen, even if not thermodynamically favoured, thus seems to be an important process in aggregates as well as in sediments.

3.7 Discussion: bacterial populations in marine aggregates

Aggregates analysed in the present study were formed in samples from Southampton Water whose salinity was about 30, and the bacterial community found in N1 is consistent with previous studies on bacterial colonisation of marine aggregates (Bockelmann *et al.*, 2000; Brachvogel *et al.*, 2001; DeLong *et al.*, 1993; Selje and Simon, 2003; Simon *et al.*, 2002). β-*Proteobacteria*, not detected in the present work, have been occasionally identified in marine snow (Phillips *et al.*, 1999) and shown to

dominate freshwater aggregates (Bockelmann *et al.*, 2000; Grossart and Ploug, 2000; Schweitzer *et al.*, 2001; Selje and Simon, 2003). However both particle-attached and free-living β-*Proteobacteria* appear to be adapted to freshwater rather than seawater conditions (Bouvier and del Giorgio, 2002; Simon *et al.*, 2002). Even though the samples had been stored for 3 days in the dark before the aggregation experiments and aggregates were collected for analysis 6 days after their formation, still in the dark, *Cyanobacteria* were detected. The 16S rRNA gene PCR does not distinguish between viable and inactive bacteria; however, as *Cyanobacteria* are photosynthetic microorganisms, even though they were present, they were not expected to be active in the aggregates. The bacterial community identified by PCR amplification and sequencing of 16S rRNA genes appeared to be dominated by aerobes, with obligate anaerobic microorganisms making up only a small component of the total microbial community (Table 3.3). This is consistent with the presence of oxygen throughout the aggregates in both the TA1 and N1 experiments. However, a low proportion of anaerobes does not mean that they are not active or important in biogeochemical cycling. Indeed, cultures developed from the artificial aggregates readily demonstrated the presence of viable Fe(III)-reducing microorganisms.

3.7.1 Bacteria potentially involved in Fe(III) reduction

The 3 different RFLP types found in the lactate/Fe(III)-NTA enrichment culture do not match with any well-described organisms known to respire Fe(III). As anaerobic cultures of aggregates produced much more Fe(II) than abiotic controls under Fe(III)-enriched conditions, (Table 3.2), the microbial role in the reduction of Fe(III) is evident. However, caution is required in comparing the cultured bacterial communities to those in the aggregate decomposition experiments. First, the concentration of Fe(III) used to culture the Fe(III)-reducing bacteria present in aggregates was 10 mM, 5 orders of magnitude higher than the concentration of dissolved iron measured in Southampton Water. Second, the medium used to culture the aggregates was completely deoxygenated by bubbling (20 min) a mixture of N_2 and CO_2 before sealing the bottles (the lower control concentrations of Fe(II) reflects the chemical reduction of Fe(III) under these anoxic conditions). Marine aggregates very rarely become completely anoxic. Thus, it is not clear which bacterial strains described in Table 3.4 are viable at lower substrate concentrations; nonetheless, the release of Fe(II) and consumption of NO_3^- during the experiments do indicate the presence of NO_3^- and Fe(III)-reducing

bacteria in aggregates, and these bacteria might be wholly or partially represented by the populations observed in the corresponding cultures.

The microbial role in the reduction of Fe(III) could be ascribed to *Desulfovibrio* sp.: recent work showed that 2 strains of *Desulfovibrio* sp. can also grow using Fe(III) compounds as the sole electron acceptors (Li *et al.*, 2006). The other 2 clones found in the lactate/Fe-NTA enrichment are related to *Marinobacter aquaeolei* and *Marinobacter* sp. (Table 3.4). Organisms belonging to the genus *Marinobacter* have been shown in the literature to be able to oxidise iron (Edwards *et al.*, 2003) under hypoxic or anoxic conditions, by either O_2 or NO_3^-. Recent studies have also suggested that members of the genus *Marinobacter* have the potential to respire Fe(III) with lactate supplied as the electron donor (Handley *et al.*, 2009).

3.7.2 Nitrate reducing-bacteria

The data shown in Fig. 3.6 highlight the initial presence of NO_3^- and NO_2^- reducers in aggregates that were incubated for several days. Whether the initial pool of NO_3^- in the incubations was transformed to NH_4^+ (via dissimilatory nitrate reduction to ammonia, DNRA) or gaseous compounds of nitrogen such as N_2, NO and N_2O (denitrification *sensu strictu*) (Zumft, 1997) is not clear at this stage. Cyanobacteria aggregates in the Baltic Sea were found to contain denitrification genes, but denitrification activity was found to be negligible (Tuomainen *et al.*, 2003). The results shown in Fig. 3.6 suggest that the reduction of NO_3^- in artificial marine aggregates is significant. Algal flocs are different from Cyanobacteria aggregates and do not appear to contain many nitrogen fixers (Bockelmann *et al.*, 2000; Brachvogel *et al.*, 2001; DeLong *et al.*, 1993; Selje and Simon, 2003; Simon *et al.*, 2002), so NO_3^- reduction is likely to be a significant process. The NO_3^--reducing microorganisms were cultivated in a medium containing a concentration of NO_3^- (15 mM) that was 3 orders of magnitude higher than that in coastal waters. However, the data shown in Fig. 3.6 indicate the presence of viable NO_3^- reducers in aggregates, although it is not known whether the same process would have occurred at lower substrate concentrations. Bacteria able to perform a wide variety of anaerobic or suboxic redox processes are thus present in artificial marine aggregates formed in estuarine water that contained its natural bacterial community. However, whilst bacterial reduction was shown to occur in the present study, which used enrichment media with high substrate concentrations, the bacterial process under natural conditions that leads to release of Fe(II) has yet to be fully

elucidated.

3.8 Summary

The results of the present study indicate that marine aggregates can release reduced Fe(II), produce NO_2^- and host a range of viable bacteria that can metabolise under different oxygen concentrations and appear to be involved in these processes. Whilst the amounts of NO_3^- and Fe(III) used to culture the dissimilatory NO_3^-- and Fe(III)-reducing bacteria present in aggregates are 2 to 5 orders of magnitude higher than in natural waters, the presence of these viable organisms and their capacity to reduce Fe(III) and NO_3^- were clearly shown. The release of Fe(II) indicates active reduction of Fe(III), and thus the presence of zones in the aggregates with a pE lower than –5 (Stumm and Morgan, 1993) to allow the reduction of Fe(III). This reduction could occur in anoxic microzones (not detected by the oxygen sensor because they are smaller than its tip diameter) presumably in close proximity to the cell surface of bacteria. Alternatively, some bacteria seem to be able to reduce Fe(III) even in the presence of low oxygen concentrations. In either scenario, diffusion of oxygen to the reduction site must be inhibited, or oxygen depleted, to prevent the rapid re-oxidation of any reduced iron. An important role may also be played by organic compounds, released by particles or organisms, that are able to complex Fe(II) and thus retard its oxidation to Fe(III).

In the present study, some release of NO_2^- was evident, either through NO_3^- reduction (NO_3^- reducers were present in aggregates) or through nitrification of DON. The NO_3^- depletion from the environment surrounding the aggregates was anticipated to be caused by bacterial uptake.

The present study therefore supports earlier work that showed reduced species are released to the marine water column, and adds Fe(II) to the list of thermodynamically unstable compounds that can be produced from marine aggregates. Whilst the present and previous studies have detected anaerobic microorganisms in aggregates (Bianchi *et al.*, 1992; Bockelmann *et al.*, 2000; Grossart and Ploug, 2000), their activities and mode of operation have yet to be fully investigated.

Chapter 4. Iron- and manganese-reducing bacteria in suspended aggregates

4.1 Introduction to studies on suspended aggregates

Natural and artificial aggregates have been studied extensively in incubation based experiments (Grossart & Ploug 2000, Grossart & Ploug 2001, Passow 2002, Azua et al. 2007). In the previous chapter the role of degrading static aggregates in the cycling of iron, nutrients and manganese was discussed, and the focus of a recently published paper based on this work (Balzano et al. 2009), was on release of reduced iron. However, after their formation aggregates may occasionally have densities comparable to those of seawater, such that they can be suspended for long periods of time (up to several months) before sinking to the seafloor (Najdek et al. 2002, Blazina et al. 2005). While aggregates are suspended the velocity shear occurring in the water column is likely to affect the diffusion of solutes through aggregates, promoting higher ventilation and making the formation of suboxic and anoxic microenvironments less likely (Ploug & Jorgensen 1999).

In the present chapter the role of suspended aggregates in the release of dissolved iron and manganese to the water column is investigated and the presence of aggregate-attached bacteria capable of dissimilatory Fe(III) and Mn(IV) reduction examined. To keep aggregates suspended they have been incubated while rotating on the roller table, both free-living and aggregate-attached microorganisms present in the microcosms have been enumerated by flow cytometry and the concentration of dissolved Fe(II), dissolved Fe(III) and dissolved Mn in the seawater surrounding the aggregates has been determined over time. Details of the methods applied are described in Section 2.6.

In the previous chapter the ability of aggregate-attached microorganisms to respire iron and nitrate was demonstrated and the bacteria growing anaerobically under Fe(III)-enriched conditions and presumably respiring Fe(III) were analysed (using the 16S rRNA gene as a phylogenetic marker) (Balzano et al. 2009). Although the ribotypes sequenced from the anaerobic culture for Fe(III)-reducing bacteria (Balzano et al. 2009) are affiliated to two genera (*Desulfovibrio* and *Marinobacter*) with known ability to respire Fe(III) (Li et al. 2006, Handley et al. 2009) it is not clear whether

these microorganisms are directly involved in Fe(III) respiration. Moreover, the presence of dissimilatory Mn(IV)-reducing bacteria among the aggregate-attached microorganisms has not been investigated.

In the present chapter the ability of aggregate-attached microorganisms to grow through the reduction of Fe(III), Mn(IV) and the fermentation of glucose has been assessed. Facultative anaerobes present in the enrichment cultures for Fe(III)- and Mn(IV)-reducing bacteria have been isolated and their ability to respire both Fe(III) and Mn(IV) has been determined.

4.2 Incubation of aggregates under dynamic conditions and behaviour of Fe and Mn

4.2.1 Advantages and disadvantages of dynamic systems

In previous studies aggregates were incubated under dynamic conditions to simulate their sinking behaviour on plankton wheels (Grossart & Ploug 2001) or individually on a flow jet system with an upward water flow equalising the sinking speed of aggregates (Ploug & Jorgensen 1999, Grossart et al. 2003). In addition aggregates were also incubated whilst rotated on the roller table where they were formed (Artolozaga et al. 1997, Unanue et al. 1998, Knoll et al. 2001, Ploug et al. 2002, Tuomainen et al. 2003, Grossart et al. 2006a, Grossart et al. 2006b, Tamburini et al. 2006, Azua et al. 2007, Ploug & Passow 2007), in order to mimic the physical conditions leading to the flocculation of marine particles.

In natural systems when a velocity shear occurs in the water column, aggregates are prevented from sinking and remain suspended. The movement of water around the flocs accelerates the diffusion of solutes into and out of them. The shape of aggregates is not influenced by gravity as is the case when they settle to the seafloor (or the bottom of a container in a laboratory study). The movement of aggregates on the roller table thus mimics their behaviour while suspended in the water column and not while they are sinking. Being exposed to a continuous velocity shear, both in the water column and on a roller table in the laboratory, aggregates may undergo disruption and re-aggregation events, and the size distribution of aggregates is thus variable with time.

4.2.2 Experimental setting

Aggregates were formed from phytoplankton cultures obtained from seawater collected from the Solent estuary (Solent Water). More details are given in Section 2.6.

Briefly seawater was collected, nutrients added to promote phytoplankton growth, and incubated in five 1L tanks (A, B, C, D and E) for two weeks under dark/light conditions (cultivated Solent Water). Two tanks were kept in the dark to prevent phytoplankton growth and served as control (F and G).

The Solent Water contained 170 ± 30 nM of dissolved Fe and 63 ± 10 nM of dissolved Mn (measured as described in Sections 2.8.3-2.8.4). The pH ranged from 7.5 to 8.0 at the beginning of the experiments and at the end of the experiments it increased ranging from 8.5 to 9.0. Differences in the pH were not found between one sample and another.

After 2 weeks, the tanks A to E were transferred in the dark along with the control samples F and G. Aggregates were then formed and the start of the rotation period corresponds to time 0. 100 mg of dried sediment (Section 2.6.2) were added to tanks D, E and G to act as a potential source of oxidised Fe and Mn, prior to rotation on the roller table (Section 2.6.2). The sediment contained 141 ± 18 μmol/g of iron and 2.7 ± 0.5 μmol/g of Mn. After aggregates had been formed, the tanks were incubated in the dark at constant temperature and several parameters were measured over time (Section 2.6).

4.2.3 Size of aggregates studied

Aggregate formation occurred within 4 hours from the initial rotation, their size distribution is shown in Table 4.1. Artificial aggregates ranging from 120 to 2000 μm were formed. The average size of aggregates was highly variable among the samples and in the same sample the size of the aggregates changed consistently over time. The biggest aggregates were formed in tank B (Table 4.1). Five days after flocculation started, aggregates in tanks B, D and E coagulated into one huge floc a few centimetres in diameter (Table 4.1). The big floc that formed in tank D disaggregated after one day whereas in tank B disruption occurred two days after aggregate formation and in tank E the macroaggregates were intact until the end of the experiments (Table 4.1).

4.2.4 Flow cytometry

Free-living bacteria-like particles (BLP) present in the tanks containing aggregates were enumerated on day 3 and day 7 using flow cytometry as described in Section 2.10 (Fig. 4.1). Tanks A and C contained higher concentrations of BLP compared to tanks B, D, E, F and G (Fig. 4.1). The BLP abundance ranged from

$4.8 \cdot 10^5$ BLP/mL in tank B to $3.9 \cdot 10^6$ BLP/mL in tank C (Fig. 4.1) and did not show significant differences between day 3 and day 7 in all tanks but B and E (Fig. 4.1). In contrast BLP abundance decreased in tank B whereas it increased in tank E over time (Fig. 4.1).

Free-living virus-like particles (VLP) were not detected in tanks A and G, but they were present in tanks C, E and F on day 7 and in tanks B and D on both day 3 and day 7 (Fig. 4.1). The abundance of free-living VLP was highly variable and ranged from 10^4 VLP/mL in the tank F (on day 7) to $2.8 \cdot 10^6$ VLP/mL in the tank B (on day 7) (Fig. 4.1).

The concentration of aggregate-attached BLP could only be measured for aggregates which were large enough to be successfully removed from the tanks and rinsed twice in sterile seawater before being introduced into a known volume of sterile seawater (see Section 2.10 for details). Attached BLP were enumerated in aggregates collected from tanks B, D and E and their abundance per volume of aggregate varied from $4.6 \cdot 10^7$ BLP/mL in D to $2.7 \cdot 10^9$ BLP/mL in B (Fig. 4.2). The density of BLP in aggregates exceeded that of free-living BLP by one to three orders of magnitude reflecting the higher microbial colonisation of aggregates compared to the surrounding seawater (Ploug & Grossart 2000, Simon et al. 2002).

Attached VLP were detected only on aggregates collected from tanks B and D (Fig. 4.2). Their numbers ranged from $8.4 \cdot 10^6$ per mL in aggregates D to $1.0 \cdot 10^8$ per mL in aggregates B (Fig. 4.2).

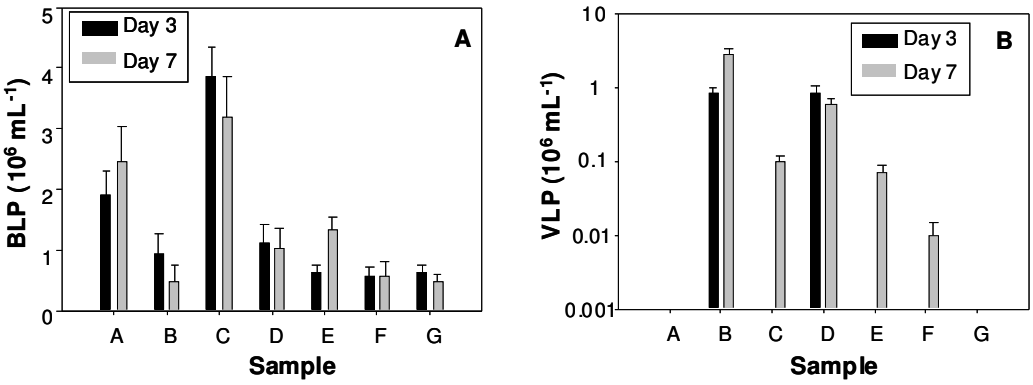


Figure 4.1. Abundance of free-living (**A**) BLP and (**B**) VLP in the tanks studied in the present experiments.

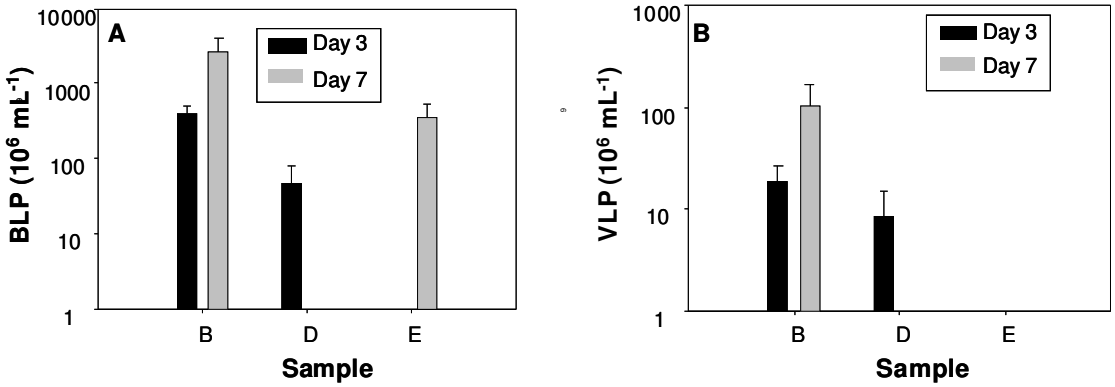


Figure 4.2. Abundance of aggregate-attached (**A**) BLP and (**B**) VLP in the present experiments. Aggregates were rinsed twice in sterile seawater before being treated for microbial enumeration.

4.2.5 Dissolved oxygen

The concentration of dissolved oxygen in the seawater present in the samples was measured using the Winkler technique and varied slightly around the value of 200 µM (Table 4.2). No obvious variations in the concentration of dissolved O_2 were found over time or between different tanks (Table 4.2).

4.2.6 Dissolved iron

Both dissolved Fe(II) and dissolved [Fe(II)+ Fe(III)] were measured in the seawater present in the experimental tanks using the ferrozine method (Viollier et al. 2000) as described in Section 2.8.3. The complete dataset is shown on Appendix 4. The concentration of dissolved ferrous iron in the samples ranged from non detectable values in the seawater controls F and G on day 8 to 140 nM in sample D on day 0 (Fig. 4.3). The control tanks usually contained less dissolved Fe(II) than the tanks containing aggregates (Fig. 4.3). Before aggregate formation (which occurred on day 0) the concentration of Fe(II) increased over time in the tanks which contained growing phytoplankton and were exposed to the light, whereas it was constant in the control samples which had been kept in the dark (Fig. 4.3). After aggregate formation, when all the tanks were transferred to the dark, the concentration of dissolved Fe(II) decreased over time in all tanks but G, where it was constant (Fig. 4.3). On day 0 the concentration of dissolved Fe(II) was lower in tank A compared to the other tanks containing aggregates B, C, D and E (Fig. 4.3).

The concentration of dissolved [Fe(II)+ Fe(III)] ranged from 30 nM in E on day 2 to 310 nM in tank C on day 0 (Fig. 4.4). The concentration of dissolved [Fe(II)+ Fe(III)] increased over time before day 0, prior to aggregation experiments and then decreased in all the samples (Fig. 4.4). The decrease was found to be sharper in the control tanks compared to the tanks containing aggregates (Fig. 4.4).

The samples D and E, did not show a significant increase in the concentrations of both dissolved ferrous and ferric iron after the addition of sieved surface sediment which occurred on the day 0 after dissolved Fe(II) and dissolved Fe(III) were determined (Figs. 4.3-4.4).

4.2.7 Dissolved manganese

The complete dataset for the concentration of dissolved Mn is shown on Appendix 5. After day 0, the concentration of dissolved manganese increased significantly over time in all the tanks containing aggregates whereas it was constant in the control samples (Fig. 4.5): the concentration of dissolved Mn ranged from 47 nM in F to 1300 nM in B (Fig. 4.5). All the samples containing aggregates showed a very sharp increase during the first two days of dark incubation; the highest concentrations of dissolved Mn were registered on day 2 for all the tanks containing aggregates but B where the concentration of dissolved Mn increased until day 4 (Fig. 4.5). Less pronounced but still significant was the release in dissolved Mn that occurred in tanks A and E (Fig. 4.5).

Also in this case, the addition of sieved surface sediment prior to flocculation of particles into larger aggregates did not enhance the release in manganese from aggregates (Fig. 4.5).

Table 4.1. Size (average ± standard deviation) expressed in µm, of the aggregates formed in the present experiments. The size was periodically measured after the initial formation of aggregates.

Tank	A		B		C		D		E	
Day	Major axis	Minor axis	Major axis	Minor axis	Major axis	Minor axis	Major axis	Minor axis	Major axis	Minor axis
1	240 ± 40	200 ± 60	1800 ± 600	1200 ± 780	580 ± 180	270 ± 170	490 ± 290	360 ± 190	160 ± 100	100 ± 68
3	220 ± 86	160 ± 75	1800 ± 500	1300 ± 490	550 ± 230	270 ± 200	480 ± 150	370 ± 230	120 ± 83	98 ± 56
5	210 ± 120	130 ± 63	34000	21000	430 ± 230	250 ± 200	350000	23000	33000	21000
7	300 ± 200	160 ± 65	34000	21000	670 ± 350	430 ± 280	2100 ± 1800	1800 ± 1100	33000	21000
11	290 ± 170	160 ± 88	1200 ± 610	710 ± 310	490 ± 300	260 ± 170	2300 ± 1700	1800 ± 1100	33000	21000

Table 4.2. Concentration of dissolved oxygen (µM) in the seawater present in the tanks studied in the present experiments.

Tank/Day	A	B	C	D	E	F	G
0	210 ± 20	185 ± 15	195 ± 20	200 ± 25	195 ± 10	205 ± 20	200 ± 15
5	190 ± 10	185 ± 15	190 ± 25	175 ± 20	170 ± 15	200 ± 15	180 ± 25
10	200 ± 20	205 ± 15	180 ± 10	190 ± 25	185 ± 15	200 ± 15	195 ± 25

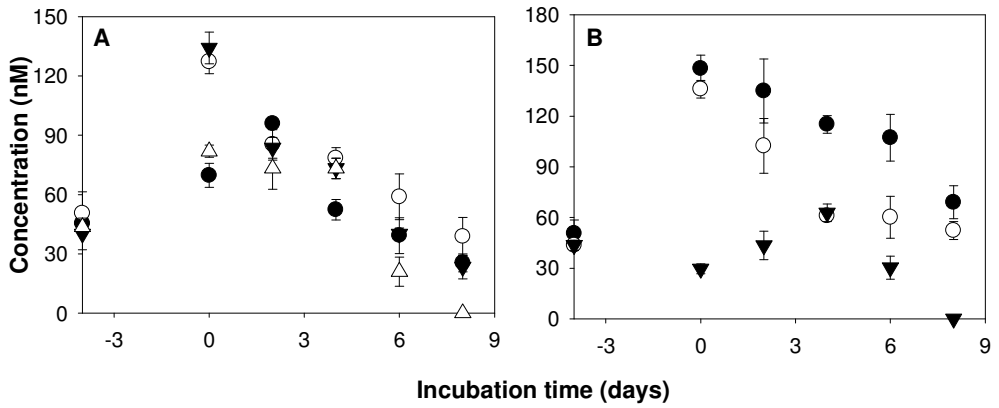


Figure 4.3. Concentration of dissolved Fe(II) over time in the aggregates studied in the present experiments. (**A**) Samples A (●), B (○), C (▼) and seawater control F (Δ). (**B**) Samples D (●), E (○) and seawater control G (▼).

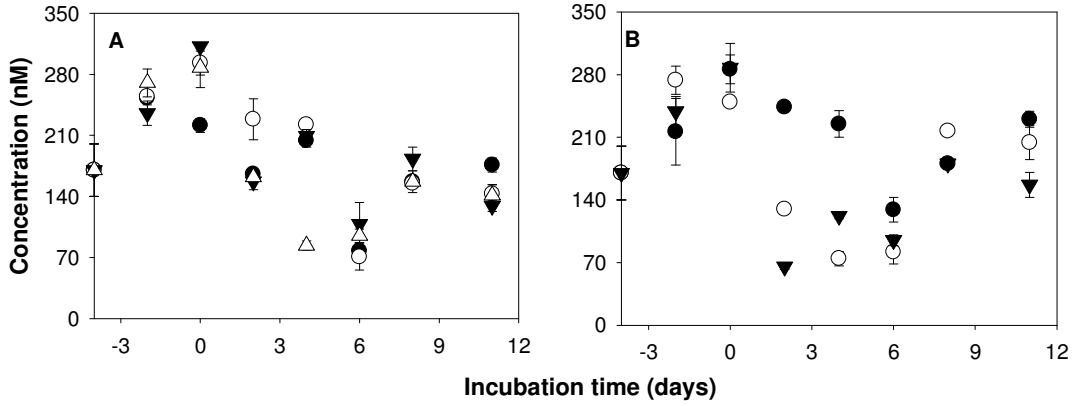


Figure 4.4. Concentration of dissolved [Fe(II) + Fe(III)] over time in the aggregates studied in the present experiments. (**A**) Samples A (●), B (○), C (▼) and seawater control F (Δ). (**B**) Samples D (●), E (○) and seawater control G (▼).

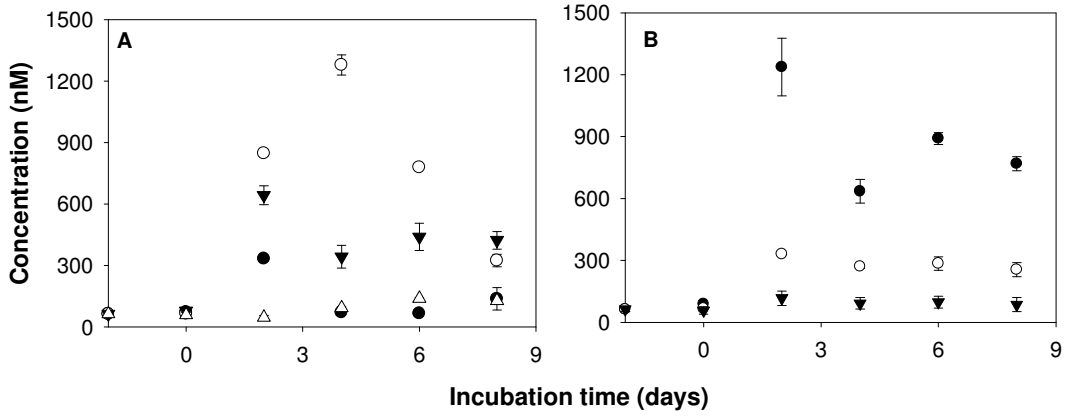


Figure 4.5 Concentration of dissolved manganese over time in the aggregates studied in the present experiments. (**A**) Samples A (●), B (○), C (▼) and seawater control F (Δ). (**B**) Samples D (●), E (○) and seawater control G (▼).

4.2.8 Iron cycling in dynamic aggregates

Significant concentrations (average 160 nM) of dissolved total iron (Head 1971, Fang 1995) have been observed in the water column in Southampton Water, from where the water used in the present experiments came. Moreover the seawater was not filtered and particulate iron and manganese were likely to be present in suspended particles.

An increase in dissolved Fe(II) over time was noted in samples A to E before the start of rotation and the flocculation experiments. As tanks were incubated under dark/light conditions, this suggests the occurrence of Fe(III) photoreduction to Fe(II) in those samples. Consistent with this hypothesis is that the concentration of dissolved Fe(II) did not increase over time in the control samples (F and G) which were not exposed to light before the experiments started (Fig. 4.3). As the initial concentration of both dissolved ferric iron and ferrous iron were not low enough to limit the growth of phytoplankton (Maldonado & Price 1996) an active role of phytoplankton in the reduction of Fe(III) as reported to occur in iron limited environments (Maldonado & Price 1996) is to be excluded. The increase in the concentration of Fe(II) in tanks A to E prior to flocculation experiments is thus likely to be caused by Fe(III) photoreduction to Fe(II) rather than other reducing processes. The reduced Fe presumably released from photo-reduction processes was stable in solution for days, or the high concentrations measured (Fig. 4.3) was maintained by a dynamic equilibrium between Fe(II) production and oxidation. A role in maintaining stable Fe(II) concentrations might have been played by Fe(II)-binding ligands, originally present in seawater or released by aggregates as discussed in Section 3.5.3.

In contrast with the results shown in the previous chapter (Balzano et al. 2009), a net release in reduced iron from aggregates while incubated in the dark was not registered over time (Fig. 4.3). If this process occurred it was not dominant over the Fe(II) oxidation to Fe(III) that likely occurred in the aerobic seawater surrounding the aggregates. The iron cycling in dynamic aggregates appears thus to be significantly different compared to that of static aggregates, as the latter were found to release reduced iron (Balzano et al. 2009). The concentration of dissolved oxygen inside aggregates was not measured in the present experiments, however the bulk concentration in the surrounding seawater was measured and did not differ much from the one found in the bulk seawater surrounding static aggregates (Balzano et al. 2009). The rotation of the tanks where the aggregates were present involved a consistent

movement of water mass promoting the diffusion of solutes, including oxygen, inside the aggregate and making more difficult the generation of anoxic or low oxygen microenvironments. Moreover, in the present experiments, aggregates were incubated in the same tanks where they were formed, whereas in the experiments shown in the previous chapter aggregates were transferred to smaller flasks (500 mL) after their formation to increase the aggregates:seawater ratio, thus increasing the amount of particulate iron available per litre for reduction. In the present experiments the aggregates:seawater ratio was likely to be too low implying that the rates of reduction processes producing Fe(II) were lower than the Fe(II) oxidation rates in the surrounding seawater.

The higher concentration of Fe(II) registered on day 6 in the tanks containing aggregates (A to E) compared to the seawater controls (F and G) (Fig. 4.3) suggests a role of aggregates in retarding the oxidation of Fe(II) to Fe(III).

The addition of sieved surface sediment on day 0, occurred before the flocculation experiments, and had no effect in tanks D and E with added sediment where an increase in Fe(II) over time was not found (Fig. 4.3).

The decreasing concentration of dissolved [Fe(II)+ Fe(III)] between day 0 and day 8, occurring in all the samples containing aggregates (Fig. 4.4) is probably due to the formation of ferric oxyhydroxides and consequent precipitation into insoluble particulate phases (Sunda 2001). However a further decrease from the day 8 to the day 11 was not observed in the samples, and may reflect the dissolved and colloidal Fe(III) being removed to particulate form at a rate equal to the rate at which dissolved iron was being added. The overall decrease in dissolved iron may also reflect adsorption of Fe to the container walls or to particulate OM.

4.2.9 Manganese dissolution in dynamic aggregates

Although photo-induced reduction of particulate to dissolved manganese has been hypothesised to occur in the coastal north Pacific (Chase et al. 2005), in the present work the concentration of dissolved (<0.2µm) manganese was not found to increase over time during the cultivation of phytoplankton under dark/light conditions (Fig. 4.5).

The dynamic conditions in which aggregates were incubated and the low aggregate:seawater ratio used did not limit the release of dissolved Mn to the surrounding seawater (Fig.4.5). The increase in dissolved Mn observed in all the

samples containing aggregates is likely due to the reductive dissolution of particulate (>0.2µm) manganese occurring inside the aggregates. The dissolution of particulate Mn usually occurs under anoxic or low oxygen conditions (Stumm & Morgan 1993). The rates of processes producing dissolved Mn inside aggregates must be significantly greater than their removal rates in the surrounding seawater in order for micromolar concentrations of dissolved Mn to be present. As the rate constant of Fe(II) oxidation is much higher than that of Mn(II) oxidation under aerobic conditions (Stumm & Morgan 1993, Morgan 2005) the concentration of dissolved Fe(II) measured in the tanks containing aggregates was not significantly higher than that found in the control tanks (Fig. 4.3). High (0.1-1 µM) concentrations of dissolved Mn in the presence of oxygen have been previously detected in the Northern Adriatic Sea (Tankere et al. 2000), Black Sea (Tankere et al. 2001) and a Scottish Fjord (Statham et al. 2005) and were suggested to derive from the action of sinking and settled aggregates.

Both release and consequent oxidation of dissolved Mn are highly variable in marine environments: for example in the Northern Adriatic Sea a net precipitation of dissolved Mn in winter and spring and a net dissolution of particulate Mn in summer and autumn, were reported (Tankere et al. 2000). Moreover oxidation rates of dissolved Mn(II), that are variable with depth and season were calculated for a Scottish Fjord (Overnell et al. 2002).

The oxidation of dissolved Mn(II) under aerobic conditions is known to be an autocatalytic process (Stumm & Morgan 1970, Sung & Morgan 1981) occurring very slowly at seawater pH (von Langen et al. 1996) due to the high activation energy required. Ligands such as siderophores (Duckworth & Sposito 2005b), and pyrophosphate and citrate (Klewicki & Morgan 1998) can accelerate the abiotic oxidation of Mn(II) and a similar behaviour was suggested for naturally occurring pyrophosphate and humic material (Klewicki & Morgan 1998). The oxidation of Mn(II) to Mn(IV) is largely catalysed by microorganisms (Tebo 1991) including bacteria and marine fungi (Tebo et al. 2005).

Traditionally particulate manganese was assumed to exist only as MnO_2 whereas dissolved manganese was assumed to be Mn(II), with Mn(III) thought to be unstable and to quickly disproportionate to Mn(II) and Mn(IV) (Kostka et al. 1995). However more recent work has highlighted that soluble Mn(III) can be stabilised by organic and inorganic chelates to prevent disproportionation (Faulkner et al. 1994, Kostka et al. 1995, Klewicki & Morgan 1998, Parker et al. 2004, Summers et al. 2005).

Dissolved Mn(III) can therefore be an important component of total dissolved Mn and is generated by oxidation of Mn(II) (Duckworth & Sposito 2005b, Trouwborst et al. 2006), non-reductive dissolution of particulate MnOOH (Duckworth & Sposito 2005a) and reductive dissolution of particulate Mn(IV) (Trouwborst et al. 2006). A recent study highlighted that dissolved Mn(III) contributed up to 100% of the total dissolved Mn in the oxic/anoxic interface of the Black Sea (Trouwborst et al. 2006). In the present work the concentration of dissolved oxygen was over 80% of air saturation in the seawater surrounding aggregates, although a strong redox gradient through the aggregates similar to that reported in previous studies (Ploug & Jorgensen 1999, Balzano et al. 2009) might have been generated. The aerobic conditions in the surrounding seawater, the likely presence of a redox gradient through aggregates, and the unlikely occurrence of fully anoxic conditions inside aggregates, suggests that an important proportion of the dissolved manganese measured in the present work (Fig. 4.5) was likely to be present as Mn(III). However aggregates B and D coagulated in one large particle on day 5 (over 10 mm in diameter, Table 4.1) likely generating internal anoxic microzones (Ploug et al. 1997). As the initial concentration of dissolved Mn was quite low, the higher concentrations measured in the later stages of incubations must have generated from the reductive dissolution of particulate Mn originally present in aggregates. The likely complexation of the intermediate Mn(III) contributed then to maintain high concentration of total dissolved Mn (Fig. 4.5).

In the presence of siderophores, metal binding ligands released by marine bacteria (Reid & Butler 1991, Reid et al. 1993, McMorran et al. 2001), the dissolution of particulate manganese yields Mn(III)-siderophore complexes which are quite stable at seawater pH (Duckworth & Sposito 2007).

The amount of released dissolved Mn measured in the present study is much higher than that reported in the previous chapter which occurred in aggregates incubated under static conditions (Chapter 3, Fig. 3.4). The occurrence of static conditions should have better promoted the formation of anoxic microenvironments compared to dynamic conditions, leading to a release in dissolved Mn even higher than that found in dynamic aggregates. However the aggregates formed in the present study were larger in size compared to those described in the previous chapter (data not shown). The release in dissolved manganese reported in the previous chapter relates to aggregates formed from natural phytodetritus whereas the present experiments use aggregates formed from natural marine phytoplankton assemblages cultivated in the

laboratory; as the aggregates studied have different origins the results from the two experiments are difficult to be directly comparable. The degradation of aggregates and the eventual formation of suboxic and anoxic microzones are controlled by the "freshness" and bioavailability of the OM present. The phytodetritus collected during the phytoplankton bloom in May 2007 was likely to be rather degraded and the OM quite refractory, being consumed at a slow rate and limiting the formation of anoxic microenvironments; only a minor component of the total Mn(IV) present was likely reduced to Mn(II). The aggregates studied in the present experiment were, on the other hand, formed from phytoplankton cultivated in the laboratory, under artificial conditions promoting exponential growth of algal cells; these aggregates probably were rich in fresh and labile OM which was rapidly consumed by microorganisms promoting the formation of low oxygen and anoxic microenvironment leading to enhanced dissolution of Mn. In addition the distribution of ligands and Mn(II)-oxidising microorganisms might have been different in the two experiments. Mn(II) requires less reducing conditions to be produced compared to Fe(II) (Stumm & Morgan 1993), therefore the reduction of Mn(IV) was more likely to occur compared to that of Fe(III). Consistent with this, the concentration of dissolved Mn exceeds by over one order of magnitude that of dissolved iron and the former has a longer residence time than the latter, at the Black Sea redoxcline (Konovalov et al. 2004).In the present incubation study the initial concentration of dissolved Mn was lower than in experiments in the previous chapter. This likely reflects the different concentrations of dissolved Mn occurring during the year across the Southampton Estuary (Fang 1995).

There is a good correlation between the size of aggregates (Table 4.1) and the release of dissolved manganese (Fig. 4.5), aggregates in tanks B and D were the largest in size and released more dissolved Mn than the other samples.

Aggregates have thus the potential to release significant amounts of dissolved Mn into the water column when they are suspended freely as well as while they are settled on the seafloor.

4.3 Anaerobic cultures of aggregates

Aggregates collected from Tanks A to E were incubated anaerobically and the production of reduced iron and manganese was periodically investigated from the anaerobic cultures (Section 2.11).

4.3.1 Fe(III) reduction

The anaerobic cultures used are listed in Table 4.3 and the complete dataset is shown on Appendix 4. Microbial reduction of Fe(III) was estimated by measuring the concentration of HCl extractable Fe(II) and Fe(III) as described in Section 2.11.2. Microbial production of Fe(II) occurred in most of the cultures.

The total HCl extractable iron in the anaerobic cultures for Fe(III)-reducing bacteria from artificial aggregates is shown in Table 4.3. The total HCl extractable iron ranged from 6.2 ± 0.4 mmol/L in AGG3(II) to 11.3 ± 0.5 mmol/L in AGG2(III) (Table 4.3).

Rapid production of Fe(II) occurred in AGG1 where Fe(II) increased significantly over 28 days (Fig. 4.6, Appendix 4). The concentration of HCl extractable Fe(II) increased sharply over time in all the replicates of AGG2 (Fig. 4.6). In the abiotic controls the amounts of Fe(II) measured increased very slightly over time rising from 0.11 ± 0.01 mM in both abiotic controls to 0.15 ± 0.02 mM and 0.36 ± 0.04 mM in BL1 and BL2, respectively (Appendix 4). The concentration of Fe(II) in the cultures AGG3 and AGG4, obtained from sample A (Table 4.3) fluctuated over time (Fig. 4.6) highlighting the less efficient reducing processes that occurred in the anaerobic cultures of aggregates from tank A when compared to those detected in the anaerobic cultures of aggregates from tank B (Fig. 4.6). A relevant production of Fe(II) was detected only in AGG4(III) (Fig. 4.6).

In the cultures AGG5 to AGG8 (Table 4.3) a production of ferrous iron over time was also detected (Fig. 4.6). The concentration of Fe(II) measured on day 0 ranged from 0.46 ± 0.08 mM to 0.92 ± 0.04 mM (Appendix 4), it was constant over time in the abiotic controls whereas it increased in all the biologically active cultures to values ranging from 1.20 ± 0.18 mM to 4.6 ± 0.4 mM. The reduction of ferric iron was more significant in AGG8 compared to AGG5, AGG6 and AGG7 (Fig. 4.6).

Table 4.3 Concentration of total HCl extractable iron in the anaerobic cultures for Fe(III)-reducing bacteria of artificial aggregates and in the abiotic controls BL1, BL2, BL3 and BL4 incubated under the same conditions as the samples.

Culture	Electron acceptor	Electron donor	Sample incubated	Concentration (mmol/L)
AGG1(I)	Fe(III)-NTA	Acetate	B	6.4 ± 0.6
AGG1(II)	Fe(III)-NTA	Acetate	B	6.5 ± 0.4
AGG1(III)	Fe(III)-NTA	Acetate	B	9.3 ± 0.3
AGG2(I)	Fe(III)-NTA	Lactate	B	6.5 ± 0.15
AGG2(II)	Fe(III)-NTA	Lactate	B	10 ± 0.8
AGG2(III)	Fe(III)-NTA	Lactate	B	11 ± 0.5
AGG3(I)	Fe(III)-NTA	Acetate	A	7.0 ± 0.4
AGG3(II)	Fe(III)-NTA	Acetate	A	6.2 ± 0.4
AGG3(III)	Fe(III)-NTA	Acetate	A	7.4 ± 0.2
AGG4(I)	Fe(III)-NTA	Lactate	A	7.3 ± 0.4
AGG4(II)	Fe(III)-NTA	Lactate	A	7.8 ± 0.3
AGG4(III)	Fe(III)-NTA	Lactate	A	8.4 ± 0.3
AGG5(I)	FeOOH	Acetate	A	8.9 ± 0.2
AGG5(II)	FeOOH	Acetate	A	7.4 ± 0.3
AGG5(III)	FeOOH	Acetate	A	9.5 ± 0.5
AGG6(I)	FeOOH	Lactate	A	9.2 ± 0.8
AGG6(II)	FeOOH	Lactate	A	9.1 ± 0.2
AGG6(III)	FeOOH	Lactate	A	7.2 ± 0.3
AGG7(I)	FeOOH	Acetate	B	8.8 ± 0.4
AGG7(II)	FeOOH	Acetate	B	11 ± 1.8
AGG7(III)	FeOOH	Acetate	B	8.7 ± 0.4
AGG8(I)	FeOOH	Lactate	B	8.9 ± 0.2
AGG8(II)	FeOOH	Lactate	B	9.1 ± 0.6
AGG8(III)	FeOOH	Lactate	B	8.2 ± 0.2
BL1	Fe(III)-NTA	Acetate	Blank	7.9 ± 0.1
BL2	Fe(III)-NTA	Lactate	Blank	8.4 ± 0.3
BL3	FeOOH	Acetate	Blank	8.7 ± 0.6
BL4	FeOOH	Lactate	Blank	8.9 ± 0.5

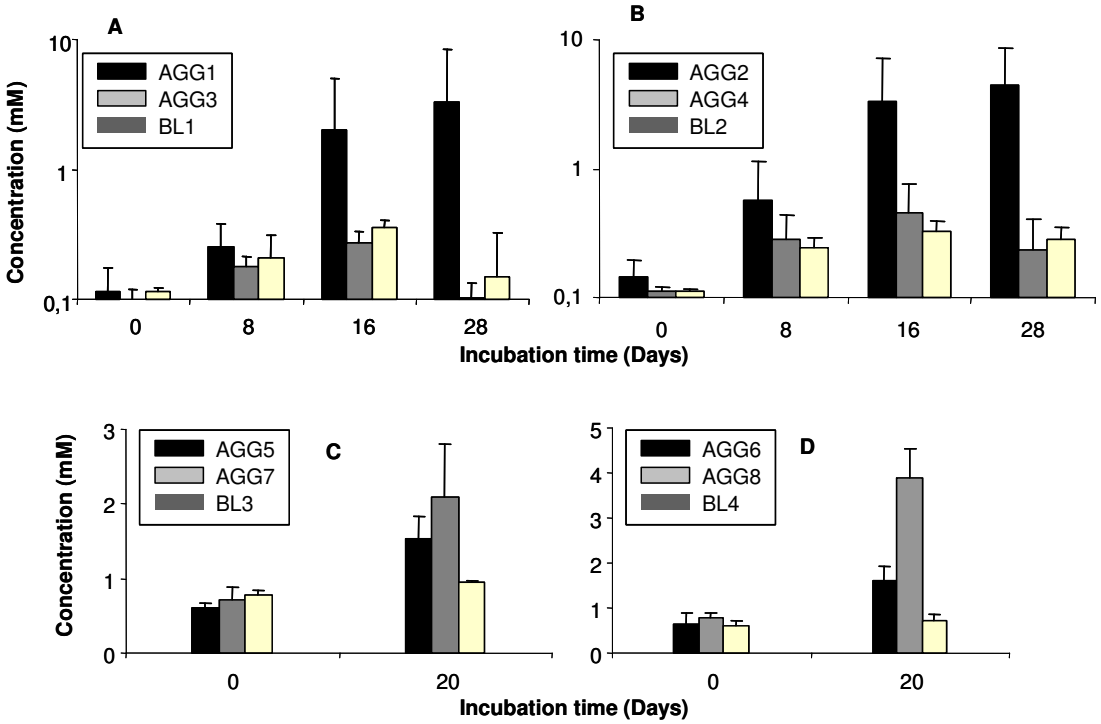


Figure 4.6 Concentration of HCl extractable Fe(II) in the anaerobic cultures of aggregates supplied with different electron acceptors and donors: (**A**) acetate/Fe(III)-NTA; (**B**) lactate/Fe(III)-NTA; (**C**) acetate/FeOOH; (**D**) lactate/FeOOH. Note that the concentration in (**A**) and (**B**) is expressed in logarithmic scale. More details about the cultures are shown in Table 4.3.

4.3.2 Manganese reduction

The complete dataset for the production of dissolved Mn from the anaerobic cultures of aggregates is shown on Appendix 5. Anaerobic cultures of artificial aggregates collected from samples A and B were originally incubated with either sodium acetate or sodium lactate as electron donors. Amorphous δ-MnO_4 (Villalobos et al. 2003) was used as a source of oxidised manganese. Microbial reduction of Mn(IV) was estimated by measuring the concentration of both dissolved and total HCl extractable manganese as described in Section 2.11.3.

The concentration of total manganese measured in the cultures is shown in Table 4.4, it ranged from 490 ± 20 µM in AGG11(II) to 1000 ± 50 µM in AGG9(III).

The concentration of dissolved Mn was constant or increased very slightly over time in the cultures of aggregates A, whereas it increased significantly over time in the cultures of aggregates B (Fig. 4.7). The initial concentration of dissolved Mn in the

cultures ranged from 110 ± 45 µM in the abiotic control BL5 to 340 ± 47 µM in the culture AGG12(III) (Appendix 5).

Significant production of dissolved Mn, most likely associated with dissimilatory Mn(IV) reduction, was observed in AGG9 and AGG10, obtained from sample B (Fig. 4.7). In contrast, the production of dissolved Mn was lower in the anaerobic cultures for Mn(IV) of aggregates A (AGG11 and AGG12) (Fig. 4.7).

The abiotic controls BL5 and BL6 contained 170 ± 40 µM and 320 ± 26 µM, respectively (Appendix 5) of dissolved Mn, values slightly higher than the initial concentrations and highlighting the occurrence of some abiotic reductive dissolution of Mn(IV).

Table 4.4. Concentration of total HCl extractable manganese in the anaerobic cultures for Mn(IV)-reducing bacteria of artificial aggregates and in the abiotic control BL5 and BL6 incubated under the same conditions as the samples.

Culture	Electron acceptor	Electron donor	Sample incubated	Concentration (µmol/L)
AGG9(I)	δ-MnO_2	Acetate	B	850 ± 10
AGG9(II)	δ-MnO_2	Acetate	B	750 ± 20
AGG9(III)	δ-MnO_2	Acetate	B	1000 ± 50
AGG10(I)	δ-MnO_2	Lactate	B	830 ± 30
AGG10 (II)	δ-MnO_2	Lactate	B	900 ± 40
AGG10 (III)	δ-MnO_2	Lactate	B	700 ± 50
AGG11 (I)	δ-MnO_2	Acetate	A	510 ± 20
AGG11 (II)	δ-MnO_2	Acetate	A	500 ± 20
AGG11 (III)	δ-MnO_2	Acetate	A	490 ± 20
AGG12(I)	δ-MnO_2	Lactate	A	650 ± 10
AGG12(II)	δ-MnO_2	Lactate	A	600 ± 30
AGG12(III)	δ-MnO_2	Lactate	A	570 ± 20
BL5	δ-MnO_2	Acetate	Blank	670 ± 10
BL6	δ-MnO_2	Lactate	Blank	700 ± 20

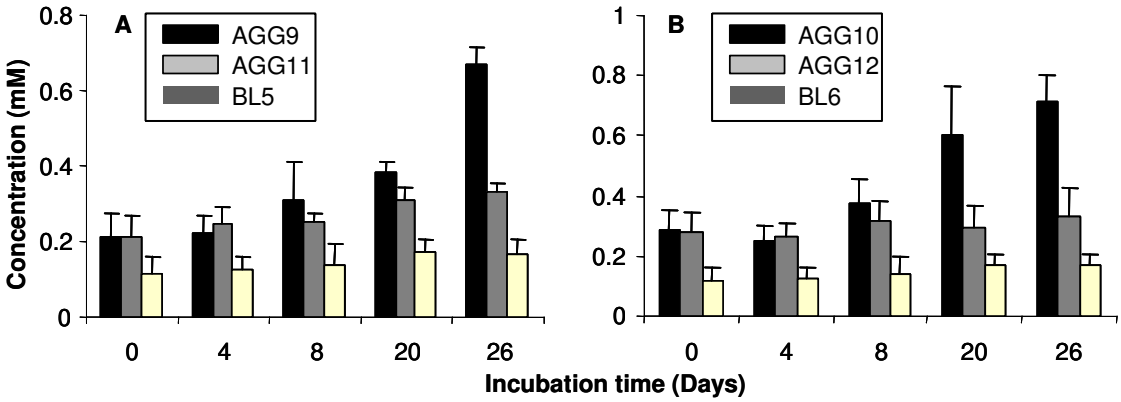


Figure 4.7. Concentration of HCl extractable Mn(II) in the anaerobic cultures of aggregates. Cultures were supplied with δ-MnO_2 as electron acceptor and (**A**) acetate or (**B**) lactate as electron donor.

4.3.3 Anaerobic microorganisms in artificial aggregates

The presence, of viable Fe(III) reducers in artificial aggregates was already demonstrated earlier in the thesis (Chapter 3 and Balzano et al. 2009), however the results in this chapter provide a more detailed evaluation of the capability of these microorganisms to grow through the reduction of Fe(III) and Mn(IV).The dissimilatory Fe(III)-reducing bacteria present in artificial aggregates can utilise both acetate or lactate as electron donors and both Fe(III)-NTA and FeOOH as electron acceptors. However the reduction process occurred at a higher rate when ferric iron was supplied in a soluble form such as Fe(III)-NTA. The microbial reduction of Fe(III)-organic complexes has been noted to occur at a higher rate compared to that of Fe(III) oxides given the relative insolubility and hence bioavailability of the latter at circumneutral pH values (Fredrickson et al. 2000). Although aggregates did not release measurable concentrations of ferrous iron when incubated aerobically in the dark (Fig. 4.3) they did contain dissimilatory Fe(III)-reducing microorganisms which can respire Fe(III) under anaerobic conditions.

Subtle differences in the physical and chemical features within the replicates of the same cultures, including the number of cells initially inoculated, might have led to the differences in Fe(II) production registered within the replicates of both AGG1 and AGG2 (Fig. 4.6). In one replicate of each of the cultures AGG1 and AGG2 near total Fe(III) reduction occurred highlighting the presence of dissimilatory Fe(III)-reducing microorganisms which reduced over 90% of Fe(III) available in 27 days. The lower reduction rates registered in the other replicates of AGG1 and AGG2 (Fig. 4.6), appear

to be contradictory, a possible contamination of some Fe(III)-reducing bacteria in the cultures AGG1(III) and AGG2(II) might be suspected. However the ratio between the concentration of HCl extractable Fe(II) and total HCl extractable iron, calculated for all the replicate cultures AGG1 and AGG2 was higher compared to the abiotic control BL1 and BL2 by the end of the experiments (Table 4.5).

In contrast with several previous studies where abiotic reduction of Fe(III)-NTA by lactate or acetate did not occur (Tebo & Obraztsova 1998, Kostka et al. 1999, Sani et al. 2002) here production of Fe(II) occurred in the abiotic controls (BL1 and BL2) (Fig. 4.6). The increase in Fe(II) concentration measured in the Fe(III)-NTA controls over 28 days, was 36% in BL1 and 150% in BL2. As the samples were incubated in the dark, the Fe(II) production measured thus can not be associated with photoreductive processes. In a previous study, abiotic Fe(III)-NTA reduction by lactate was detected, although it was found to be negligible, over three days (Kieft et al. 1999). In the present work the same reduction processes was found to occur over a longer time interval (28 days). Although a bacterial contamination of BL1 and BL2 can not be excluded, the Fe(II)/Fe-total ratio, after 28 days of incubation, was lower in BL1 and BL2 compared to the biologically active samples.

Table 4.5. Ratio between the concentrations of HCl extractable Fe(II) and total HCl extractable iron in AGG1, AGG2, BL1 and BL2.

Culture	Initial ratio	8 days	16 days	28 days
AGG1(I)	0.014 ± 0.02	0.026 ± 0.010	0.038 ± 0.019	0.061 ± 0.035
AGG1(II)	0.028 ± 0.005	0.031 ± 0.010	0.038 ± 0.020	0.075 ± 0.036
AGG1(III)	0.009 ± 0.002	0.043 ± 0.020	0.60 ± 0.14	0.970 ± 0.060
AGG2(I)	0.022 ± 0.002	0.025 ± 0.015	0.18 ± 0.07	0.360 ± 0.040
AGG2(II)	0.020 ± 0.004	0.120 ± 0.020	0.78 ± 0.09	0.920 ± 0.14
AGG2(III)	0.008 ± 0.002	0.027 ± 0.012	0.088 ± 0.03	0.170 ± 0.02
BL1	0.015 ± 0.002	0.027 ± 0.019	0.025 ± 0.009	0.019 ± 0.004
BL2	0.013 ± 0.002	0.029 ± 0.010	0.039 ± 0.010	0.034 ± 0.01

Dissimilatory Fe(III)-reducing microorganisms which can become active under anaerobic conditions were thus present in aggregates B. These data agree with results from Chapter 3. In contrast, the concentration of Fe(II) in the anaerobic cultures of aggregates A (AGG3 and AGG4) increased over time as much as it did in the abiotic controls BL1 and BL2 (Fig. 4.6), the occurrence of dissimilatory reduction of Fe(III) to Fe(II) in these cultures is thus not clear.

On the other hand, both aggregates A and B showed dissimilatory reduction of Fe(III) to Fe(II) when incubated with FeOOH as an electron acceptor (Fig. 4.6): the production of Fe(II) was higher in all the cultures compared to BL3 and BL4 (Fig. 4.6) indicating the presence of active Fe(III)-reducing microorganisms in both aggregates A and B. The stronger Fe(II) production in AGG8 (Fig. 4.6), amended with acetate as electron donor, could suggest the presence of microorganisms affiliated to *δ-Proteobacteria* which can use acetate for dissimilatory reduction of Fe(III) to Fe(II) (Lovley et al. 2004).

Phytoplankton assemblages present in Solent Water were cultivated following the same method for tanks A and B (see Section 2.6 for details), however aggregates B were larger in size (Table 4.1) being thus more likely to contain anoxic microzones, and showed higher release of dissolved Mn when incubated aerobically (Fig. 4.5). Aggregates sampled from both tanks A and B contained viable Fe(III) reducers, but higher abundances and activities of these bacteria likely occurred in the aggregates present in tank B. Anoxic microzones likely present in aggregates B promoted the growth of Fe(III)- and Mn(IV)-respiring microorganisms which readily became active when aggregates were subsequently cultured under anaerobic conditions. Most of the Mn(IV)-reducing bacteria are also able to grow through the reduction of Fe(III) (Lloyd 2003).

However under aerobic conditions the aggregates present in tank B did not release measurable amounts of Fe(II) into the surrounding seawater. In the previous chapter the occurrence of such a process under aerobic conditions was demonstrated and it is not clear whether in the present work, the aggregates produced Fe(II) which was quickly oxidised back to Fe(III) or the Fe(III) reduction did not occur or any release Fe(II) was rapidly diluted in the larger volumes of surrounding solution.

A significant production of dissolved Mn was observed in both AGG9 and AGG10 highlighting the presence of viable dissimilatory Mn(IV)-reducing microorganisms (Fig. 4.7). The production of dissolved Mn in AGG11 was less significant than that found in AGG9 and AGG10. Moreover AGG12 showed a production of dissolved Mn comparable to that occurred in BL6. Both AGG11 and AGG12 were obtained from Tank A, this observation is consistent with the hypothesis that the Fe(III)- and Mn(IV)-reducing bacteria in aggregates B were more likely to become active than the bacteria attached to aggregates A.

After 27 days aggregates were cultured anaerobically with δ-MnO_2 as the sole electron acceptor the ratio between dissolved Mn and total HCl extractable manganese increased in all the cultures but AGG12 (Table 4.6). Most of the manganese present in the cultures was present as dissolved Mn at the end of the experiments (Table 4.6). Mn(IV) needs weaker anoxic conditions to be reduced compared to Fe(III) (Stumm & Morgan 1993) and dissolved Mn is more stable in solution compared to dissolved Fe.

The release of dissolved Mn registered in aggregates incubated aerobically under seawater conditions (Fig. 4.5), was corroborated by the demonstrated ability of the microbial populations present in these aggregates to reduce nearly all the δ-MnO_2 added under anaerobic conditions (Table 4.6, Fig. 4.7), indicating the important role played by microbial populations in aggregates for the dissolution of manganese and the release of it in the water column.

Table 4.6. Ratio between the concentrations of HCl extractable dissolved Mn and total HCl extractable Mn in the cultures AGG9, AGG10, AGG11, AGG12 and the abiotic controls BL5 and BL6.

Culture	Initial ratio		8 days	20 days	26 days
AGG9(I)	0.19 ± 0.06	0.23 ± 0.04	0.50 ± 0.09	0.42 ± 0.07	0.72 ± 0.11
AGG9(II)	0.38 ± 0.09	0.37 ± 0.10	0.32 ± 0.08	0.49 ± 0.11	0.92 ± 0.17
AGG9(III)	0.19 ± 0.09	0.18 ± 0.07	0.26 ± 0.07	0.41 ± 0.10	0.69 ± 0.12
AGG10(I)	0.40 ± 0.06	0.26 ± 0.11	0.57 ± 0.09	0.91 ± 0.12	0.87 ± 0.10
AGG10(II)	0.23 ± 0.06	0.25 ± 0.07	0.35 ± 0.09	0.69 ± 0.13	0.88 ± 0.12
AGG10(III)	0.46 ± 0.12	0.45 ± 0.12	0.49 ± 0.19	0.62 ± 0.15	0.91 ± 0.17
AGG11(I)	0.32 ± 0.15	0.39 ± 0.11	0.46 ± 0.14	0.54 ± 0.17	0.61 ± 0.14
AGG11(II)	0.55 ± 0.14	0.58 ± 0.12	0.53 ± 0.12	0.68 ± 0.12	0.70 ± 0.12
AGG11(III)	0.41 ± 0.20	0.51 ± 0.16	0.55 ± 0.16	0.65 ± 0.20	0.69 ± 0.19
AGG12(I)	0.45 ± 0.09	0.37 ± 0.12	0.57 ± 0.11	0.54 ± 0.13	0.57 ± 0.11
AGG12(II)	0.36 ± 0.12	0.39 ± 0.09	0.40 ± 0.09	0.36 ± 0.13	0.38 ± 0.16
AGG12(III)	0.61 ± 0.15	0.55 ± 0.15	0.60 ± 0.18	0.57 ± 0.17	0.71 ± 0.17
BL5	0.17 ± 0.10	0.18 ± 0.08	0.20 ± 0.13	0.25 ± 0.08	0.25 ± 0.09
BL6	0.37 ± 0.13	0.39 ± 0.09	0.34 ± 0.11	0.43 ± 0.09	0.45 ± 0.07

4.4 Identification and isolation of aggregate-attached bacteria

Molecular (16S rRNA gene) analyses were carried out on aggregates collected from tank B and processed as described in Section 2.12. Molecular analyses were also carried out on AGG1(III), which is an anaerobic culture for Fe(III) reducers of

aggregates B (Table 4.3), which showed a significant production of Fe(II) (Appendix 4).

In addition different strains were isolated from the anaerobic cultures for Fe(III) and Mn(IV) reducers of aggregates B.

4.4.1. Phylogenetic analyses of non-cultured artificial aggregates from sample B

The diversity of 16S rRNA gene sequences recovered from artificial aggregates collected from tank B was examined by Restriction Fragment Length Polymorphism (RFLP) sorting of 150 different clones followed by gene analyses. Amplification of 16S rRNA gene fragments, performed using primers 8F and 519R, revealed a very simple bacterial community dominated by two clones affiliated to Planctomycete GMD14H10 and Aquatic bacterium R1-B20 (Table 4.7).

Table 4.7. Taxonomic affiliation of the bacteria identified in aggregates from sample B after amplification and analysis of the partial 16s rRNA gene fragment.

Ribotype	Closest matching microorganism (NCBI* accession number)	Group	Identities (matches)	Percentage present
sb1	Planctomycete GMD14H10 (AY162122)	*Planctomycetes*	385/405 (95%)	63
sb2	Aquatic bacterium R1-B20 (AB195752)	Unknown	485/523 (92%)	28
sb3	Uncultured Bacteroidetes 6mML2F11 (EF630174)	*Bacteroidetes*	291/313 (92%)	4
sb4	Planctomycete GMD16E07 (AY162118)	*Planctomycetes*	396/417 (94%)	2

*National Centre for Biotechnology Information.

4.4.2 Glucose fermenting bacteria

Artificial aggregates isolated from tank B and cultured anaerobically with glucose showed a vigorous growth within a few days after inoculation. Growth was evident from increasing turbidity a few days after the inoculum was added.

After three subsequent re-inoculations of the anaerobic culture for glucose-fermenting bacteria of aggregate B, 500 base pairs (bp) fragments of the 16S rRNA gene were amplified, the PCR products cloned and RFLP analyses showed the presence of only two ribotypes, both affiliated to *Vibrio lentus* Sat201 (NCBI accession

number AY292936) which shared 497 of 503 base pairs with the ribotype more abundant in the clone library and 511 of 516 base pairs with the other ribotype.

4.4.3 Acetate oxidising, Fe(III)NTA-reducing community

As the anaerobic culture AGG1(III), obtained from aggregates in sample B, reduced Fe(III) to Fe(II) efficiently (Appendix 4), the culture was subsequently re-subcultured three times in the appropriate medium to obtain a microbial community enriched for bacteria capable of reducing Fe(III) to Fe(II). An aliquot was collected during the exponential growth phase of the third subculture and copies of the 16S rRNA gene was amplified, cloned and sequenced.

The bacterial community found in the anaerobic culture of aggregates B amended with Fe(III)-NTA as electron acceptor and acetate as the electron donor included ribotypes affiliated to *γ-Proteobacteria, δ-Proteobacteria*, and *Tenericutes* (Table 4.8).

The bacterial composition of the culture is shown in Table 4.8 and the community is dominated by a strain closely related (>99% matches over 508 bp) to *Amphritea atlantica* M41, which accounted for 50% of the clone library. Two clones phylogenetically similar to *Malonomonas rubra* strain GraMal1 and *Desulfuromonas michiganensis* strain BB1 were also abundant accounting for 21% and 18% of the clone library, respectively (Table 4.8).

4.4.4 Strains isolated from anaerobic cultures of aggregates

Different bacteria were isolated aerobically from the anaerobic cultures of aggregates B and cultivated anaerobically using a range of substrates (Table 4.9). Details about the methods applied to isolate the bacteria are given in Section 2.11.5. All the isolated bacteria were affiliated to the class *γ-Proteobacteria*.

The microorganism dominating the glucose fermenting culture belonging to *Vibrio lentus* Sat201 (see Section 4.4.2 for details), was also isolated aerobically (Is-1, Table 4.9) and was found to be able to grow both aerobically and anaerobically with 20 mM of glucose as carbon substrate (data not shown).

Table 4.8. Taxonomic affiliation of the bacteria identified in the anaerobic enrichment of the aggregates B incubated with acetate as electron donor and Fe(III)-NTA as electron acceptor. Bacteria were identified after amplification and sequencing of the partial 16S rRNA gene fragment.

Ribotype	Closest matching microorganism (NCBI accession number)	Group	Identities (matches)	Percentage present
sb5	*Amphritea atlantica* M41 (AM156910)	*γ-Proteobacteria*	504/508 (99%)	50
sb6	*Malonomonas rubra* GraMal1 (Y17712)	*δ-Proteobacteria*	462/491 (94%)	21
sb7	*Desulfuromonas michiganensis* BB1 (AF357915)	*δ-Proteobacteria*	485/518 (93%)	19
sb8	*Oceanospirillaceae* bacterium ACEMC 20-5 (FM162970)	*γ-Proteobacteria*	463/502 (92%)	2
sb9	*Acholeplasma* sp. MLSB30m9D (EU517561)	*Tenericutes*	443/467 (94%)	2
sb10	Uncultured bacterium G2DCM-250 (EU037302)	Unknown	451/501 (90%)	2

The strain Is-2 was isolated aerobically from AGG1(III) (Table 4.9). The phylogenetic analysis of the partial 16S rRNA gene ascertained its affiliation to *Amphritea atlantica* with 511 base pairs from 516 matching (Table 4.9).

Two strains, Is-3 and Is-4, were isolated aerobically in Difco medium from AGG2(II) and were found to belong to *Marinobacterium* sp IC961 (AB196257) and *Marinobacter guineae* LMG 24048 (AM503093), respectively (Table 4.9). Both strains have a very good affiliation with the most closely related sequences obtained from GenBank. Is-5 was also found to be affiliated to *Marinobacter guineae*, Is-6 showed a partial 16S rRNA gene sequence weakly affiliated to *Marinobacter maritimus* and Is-7 was affiliated to *Vibrio* sp.. Is-8 was isolated from AGG8(II) and was affiliated to *Halomonas* sp. (Table 4.9). As Is-4 and Is-5 shared a nearly identical partial 16S rRNA gene sequence (Table 4.9) they have been consequently treated as a single unique strain and only one of them (Is-4) was cultivated for future studies.

Table 4.9. List of microorganisms isolated from the anaerobic cultures of aggregates and the most affiliated bacterial species.

Isolate ID	Original culture	Closest matching microorganism (NCBI accession number)	Group	Identities (matches)
Is-1	Glucose[a]	*Vibrio lentus* Sat201 (AY292936)	*γ-Proteobacteria*	511/516 (99%)
Is-2	AGG1(III)	*Amphritea atlantica* M41T(AM156910)	*γ-Proteobacteria*	498/503 (99%)
Is-3	AGG2(II)	*Marinobacterium* sp. IC961(AB196257)	*γ-Proteobacteria*	495/496 (99%)
Is-4	AGG2(II)	*Marinobacter guineae* LMG 24048(AM503093)	*γ-Proteobacteria*	476/478 (99%)
Is-5	AGG10(II)	*Marinobacter guineae* LMG 24048(AM503093)	*γ-Proteobacteria*	473/478 (99%)
Is-6	AGG10(II)	*Marinobacter maritimus* CK47T (AJ704395)	*γ-Proteobacteria*	422/505 (83%),
Is-7	AGG10(II)	*Vibrio* sp. FAL56(EU655353)	*γ-Proteobacteria*	489/492 (99%)
Is-8	AGG8(II)	*Halomonas* sp. P9 (AY800097)	*γ-Proteobacteria*	476/478 (99%)

[a] Anaerobic culture for glucose-fermenting bacteria: glucose was supplied as the sole electron source and electron acceptors and donors were not present.

4.4.5 Fe(III) reduction from bacteria isolated from anaerobic cultures of marine aggregates

The bacteria isolated from the anaerobic cultures of artificial aggregates were cultivated separately with Fe(III)-NTA or FeOOH as electron acceptors and acetate or lactate as the electron donors (see Secion 2.11.5 for details). The complete dataset is shown on Appendix 4. The total HCl extractable Fe ranged from 7.4 ± 0.3 mmol/L in Is-2 to 9.5 ± 0.4 mmol/L in Is-6 (Table 4.10). The initial concentration of HCl extractable Fe(II) (Appendix 4) was over one order of magnitude lower than the concentration of total iron (Table 4.10).

Table 4.10. Concentration of total HCl extractable iron and total extractable manganese in the anaerobic cultures for Fe(III)- and Mn(IV)-reducing bacteria.

Culture	Affiliated species	Substrates[a]	Total Fe (mM)	Substrates[b]	Total Mn (mM)
Is-2	*Amphritea atlantica*	Acetate/Fe(III)-NTA	7.4 ± 0.3	Acetate/δ-MnO_2	0.83 ± 0.02
Is-3	*Marinobacterium* sp.	Lactate/Fe(III)-NTA	8.5 ± 0.4	Lactate/δ-MnO_2	0.79 ± 0.03
Is-4	*Marinobacter guineae*	Lactate/Fe(III)-NTA	8.3 ± 0.6	Lactate/δ-MnO_2	0.96 ± 0.02
Is-6	*Marinobacter* sp.	Lactate/Fe(III)-NTA	9.5 ± 0.4	Lactate/δ-MnO_2	0.78 ± 0.03
Is-7	*Vibrio* sp.	Lactate/Fe(III)-NTA	8.8 ± 0.5	Lactate/δ-MnO_2	0.84 ± 0.02
Is-8	*Halomonas* sp.	Acetate/FeOOH	7.9 ± 0.6	Acetate/δ-MnO_2	0.91 ± 0.02

[a] Substrates used to evaluate Fe(III) respiration.
[b] Substrates used to evaluate Mn(IV) respiration.

An increase in the concentration of Fe(II) over time was registered in Is-2, Is-3, Is-4 and Is-6 (Fig. 4.8). The concentration of Fe(II) in Is-4 increased up to 3.7 mM over 60 days whereas Is-2, Is-3 and Is-6 produced between 1 and 2 mM of Fe(II) over the same time interval (Fig. 4.8). In contrast the concentration Fe(II) in Is-7 and Is-8, was constant or fluctuated over time (Fig. 4.8).

4.4.6 Mn(IV) reduction from bacteria isolated from anaerobic cultures of marine aggregates

The strains isolated from anaerobic cultures of marine aggregates were also cultivated anaerobically with δ-MnO_2 as an electron acceptor to assess the ability of the clones to grow through the reduction of Mn(IV).

The complete dataset is shown on Appendix 5. Concentration of total HCl extractable manganese measured in the strains ranged from 0.78 ± 0.03 mmol/L in Is-6 to 0.96 ± 0.02 mmol/L in Is-4 (Table 4.10). The initial concentration of HCl extractable dissolved (0.2 µm filtered) Mn ranged from 0.10 ± 0.01 to 0.15 ± 0.04 µmol/L (Appendix 5). A slight production of dissolved Mn was registered in Is-2, Is-7, Is-8 and Is-9 over 80 days (Fig. 4.8). However the production of dissolved Mn found in the cultures does not appear to be higher than that registered in the abiotic controls BL5 and BL6 (Fig. 4.8) and therefore is likely to be ascribed to chemical reduction of Mn(IV) under anoxic conditions. The abiotic reduction of Mn(IV) by lactate was shown to occur in previous studies (Kieft et al. 1999, Dollhopf et al. 2000). A sharp increase in the concentration of dissolved Mn was found in Is-3, Is-4 and Is-6 over 80

days (Fig. 4.8).

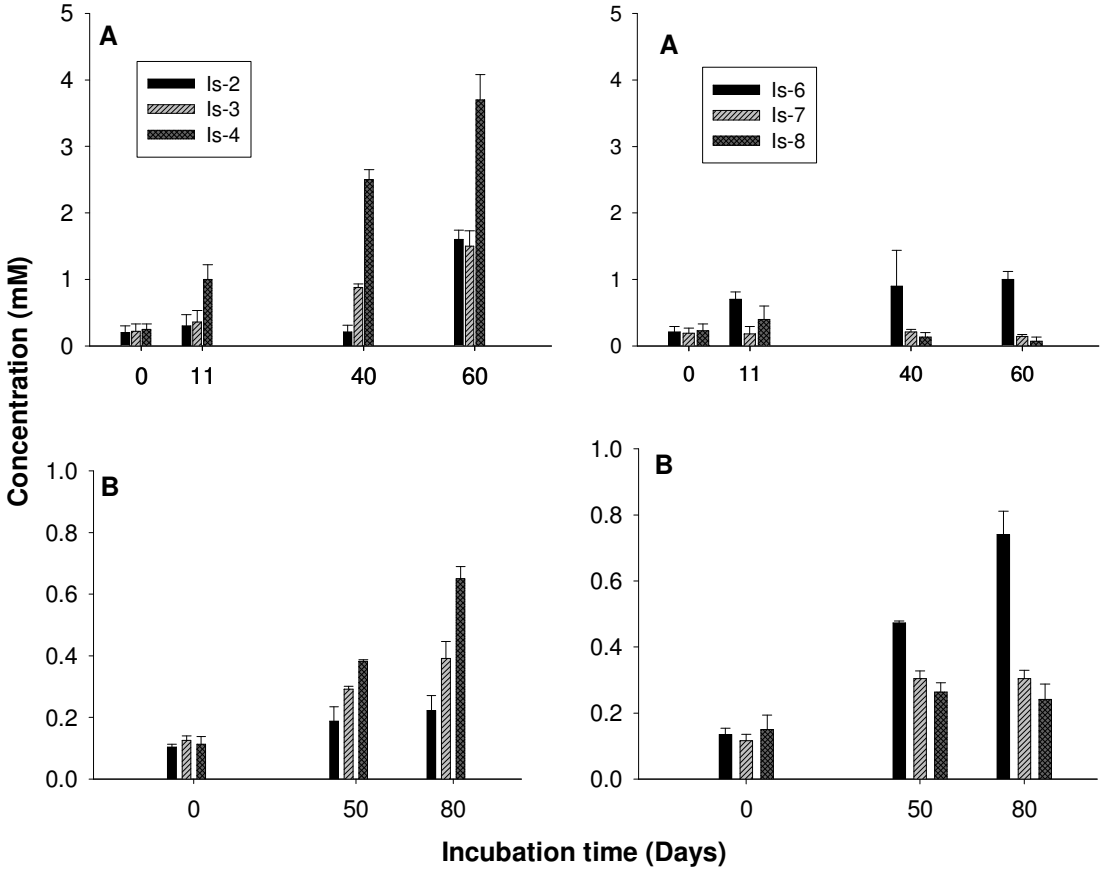


Figure 4.8. (**A**) Concentration of HCl extractable Fe(II) in the clones isolated in the present work and cultivated with Fe(III) as the sole electron acceptor. (**B**) Concentration of HCl extractable dissolved Mn in the clones isolated in the present work and cultivated with δ-MnO_2 as the sole electron acceptor. The electron acceptors and donors used to cultivate the strains are the same as those ones present in the enrichments where the clones were isolated and are listed on table 4.10.

4.5 Overview of the bacterial communities attached to aggregates

4.5.1 Uncultured aggregates

The low bacterial diversity found in aggregates from sample B (Table 4.7) does not seem to reflect the real composition in marine snow (DeLong et al. 1993, Simon et al. 2002). Marine snow without any attached bacteria has been occasionally reported in freshwater (Zimmermann 1997, Brachvogel et al. 2001) and marine (Long & Azam 1996) environments and were suggested to be associated with a higher mineral content

of the substrate (Zimmermann 1997). Artificial aggregates from sample B had a very low mineral content as they were formed from cultures of mixed phytoplankton assemblages present in Solent Water. Laboratory conditions might have strongly affected bacterial diversity leading to the dominance of two bacterial strains affiliated to *Planctomycete* GMD14H10 and Aquatic bacterium R1-B20, respectively (Table 4.7). *Planctomycete* GMD14H10 is the first cultivate representative within the order *Planctomycetales* (Zengler et al. 2002) and was isolated from the water column of Sargasso sea.

The phylum *Planctomycetes* is considered underrepresented in culture (Rappe & Giovannoni 2003) with the few cultured microorganisms available growing at a very slow sub-optimal rate in laboratory experiments. *Planctomycetes* were previously found associated with marine corals (Frias-Lopez et al. 2002), sediment particles (Jackson & Weeks 2008) as well as riverine (Bockelmann et al. 2000, Allgaier & Grossart 2006) and marine aggregates (DeLong et al. 1993, Rath et al. 1998) and were demonstrated to be adapted to attach to marine particles (Neef et al. 1998). Two ribotypes sequenced from marine snow in a previous study (DeLong et al. 1993) and affiliated to *Planctomycetes* are closely related to sb1 and sb4 (Fig. 4.9). Some *Planctomycetes* are involved in anaerobic ammonium oxidation (Strous et al. 1999) and were found attached to marine particles. They showed a wide metabolic versatility allowing them to adapt to changing redox conditions in marine snow (Kuypers et al. 2005, Woebken et al. 2007).

The ribotype sb2, accounting for 28% of the cloned partial 16S rRNA gene amplified directly from the DNA of aggregates B, shares 92% of similarity with Aquatic bacterium R1-B20 and appears to belong to the *β-Proteobacteria* (Fig. 4.9). Although occasionally found in marine particles, β-*Proteobacteria* are not common among the aggregate-attached bacteria (Simon et al. 2002). The strain sb3 shows similarities with *Bacteroidetes* previously sequenced from natural marine snow (DeLong et al. 1993).

4.5.2 Bacterial composition of the anaerobic cultures of aggregate

The presence in the culture of aggregates from sample B amended with glucose, of two single strains related to *Vibrio lentus* Sat201 and sharing over 99% of similarity to each other indicates dominance of *Vibrio* species in the glucose amended culture and suggests that *Vibrio* species have the potential to conduct fermenting processes in

marine aggregates. *Vibrio lentus* was originally isolated from Mediterranean oysters (Macian et al. 2001) whereas the *V. lentus* clone most closely related to the ribotype sequenced from the glucose fermenting culture was isolated from squids and fish organs (Nishiguchi & Nair 2003). More clones of *V. lentus* were also isolated from seawater and sediment (Maeda et al. 2003). The ability of *V. lentus* to grow through the fermentation of glucose was already ascertained when the species was initially described (Macian et al. 2001) and agrees with the current results. As discussed in Section 1.8.2, in natural systems fermentative microorganisms can break down complex OM to fermentation products, which are the primary electron donors for Fe(III) and Mn(IV) reduction (Lovley 2000) and some bacteria may also use Fe(III) to oxidise NADH produced in excess during the fermentation process (Lovley 2000). In natural marine snow, attached bacteria belonging to *Vibrio lentus* may be involved in one or both mentioned processes playing an important role in the dissolution of iron and manganese.

The bacterial community found in the anaerobic culture for Fe(III) reducers from aggregate B (Table 4.8) includes microorganisms related to previously described Fe(III)-reducing bacteria except for the strain sb5 which is affiliated to *Amphritea atlantica. A. atlantica* was isolated from a hydrothermal vent (Gartner et al. 2008) and cultivated aerobically in Marine Broth (Difco) (Gartner et al. 2008). When *A. atlantica* was isolated and described (Gartner et al. 2008), its ability to grow anaerobically was not determined. However the dominance of a ribotype affiliated to *A. atlantica*, in the anaerobic culture of Fe(III) reducers from aggregates (Table 4.8) suggests that the strain is able to survive, and is probably metabolically active, under anaerobic conditions. The production of Fe(II) monitored in the culture of *A. atlantica* isolated (Is-2) grown with Fe(III) as the sole electron acceptor (Fig. 4.8) indicates that *A. atlantica* is able to grow through the reduction of Fe(III).

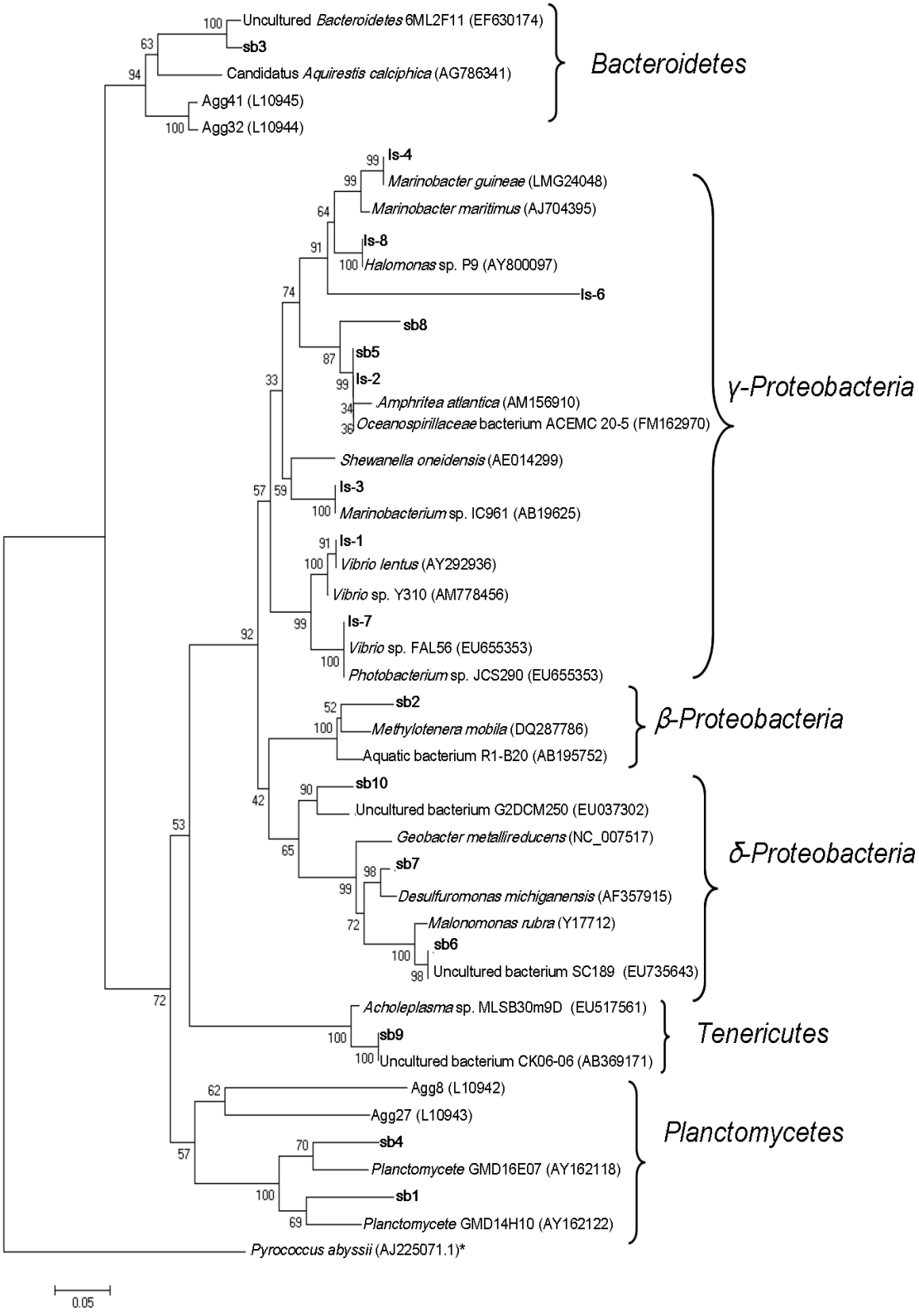


Figure 4.9. A Neighbour Joining tree based on partial 16S rRNA gene sequences (8F and 519R were used as primers) of the 8 strains isolated from anaerobic cultures of aggregates and 10 strains sequenced from uncultured and cultured aggregates. Partial 16S rRNA gene sequences of close related microorganisms were downloaded

from GenBank and included in the tree; the NCBI accession number is given in brackets. *Pyrococcus abyssii*, an Archaeon, was used as outgroup. Strains sequenced in the present work are indicated in bold. The optimal tree with the sum of branch length = 2.38977136 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches.

The full 16S rRNA genes of the *γ-Proteobacteria* known to respire ferric iron or Mn(IV) (Lovley et al. 2004, Haveman et al. 2005) have been compared with the same sequences of the microorganisms found to respire Fe(III) or Mn(IV) in the present work; all the sequences were obtained from GenBank. *Amphritea atlantica* forms a distinct clade with *Marinobacter guineae* and *Marinobacterium* sp. (Fig. 4.10). The Fe(III)-respiring microorganism more closely related to this clade are *Shewanella oneidensis* AE014299 (Lovley 1989) and *Aeromonas hydrophila* ATCC7966 (Knight & Blakemore 1998) (Fig. 4.10). *Aeromonas salmonicida* AF200329, a known Mn(IV)-respiring bacterium (Haveman et al. 2005) is also closely related to the Fe(III)-respiring bacteria isolated in the present work (Fig. 4.10). Other well known dissimilatory Fe(III)-reducing bacteria belonging to the phylum of γ-Proteobacteria such as *Ferrimonas balearica* DSM9799 (Rossellomora et al. 1995), *Acidithiobacillus ferrooxidans* AF465604 (Ohmura et al. 2002) and *Pantoea agglomerans* AF199029 (Francis et al. 2000) share lower identities with *A. atlantica*, *M. guineae* and *Marinobacterium* sp. IC961 (Fig. 4.10). *Pantoea agglomerans* AF199029 forms a distinct clade with other *γ-Proteobacteria* known to respire Mn(IV) such as *Klebsiella pneumoniae* AY043391 and *Yersinia kristensenii* AJ627595 (Haveman et al. 2005) (Fig. 4.10). The ability of *K. pneumoniae, Y. kristensenii* and *A. salmonicida* to conserve energy through the reduction of Fe(III) was not determined, however these microorganisms were isolated from a medium supplied with Fe(III) as electron acceptor (Haveman et al. 2005) and they are thus likely to be dissimilatory Fe(III) reducers. The dissimilatory Fe(III)- and Mn(IV)-reducing bacteria *A. atlantica*, *Marinobacterium* sp. IC961 and *M. guineae* may be active therefore in natural sinking and settled marine aggregates under both aerobic and anaerobic conditions and are likely to play an important role in the dissolution of trace metals.

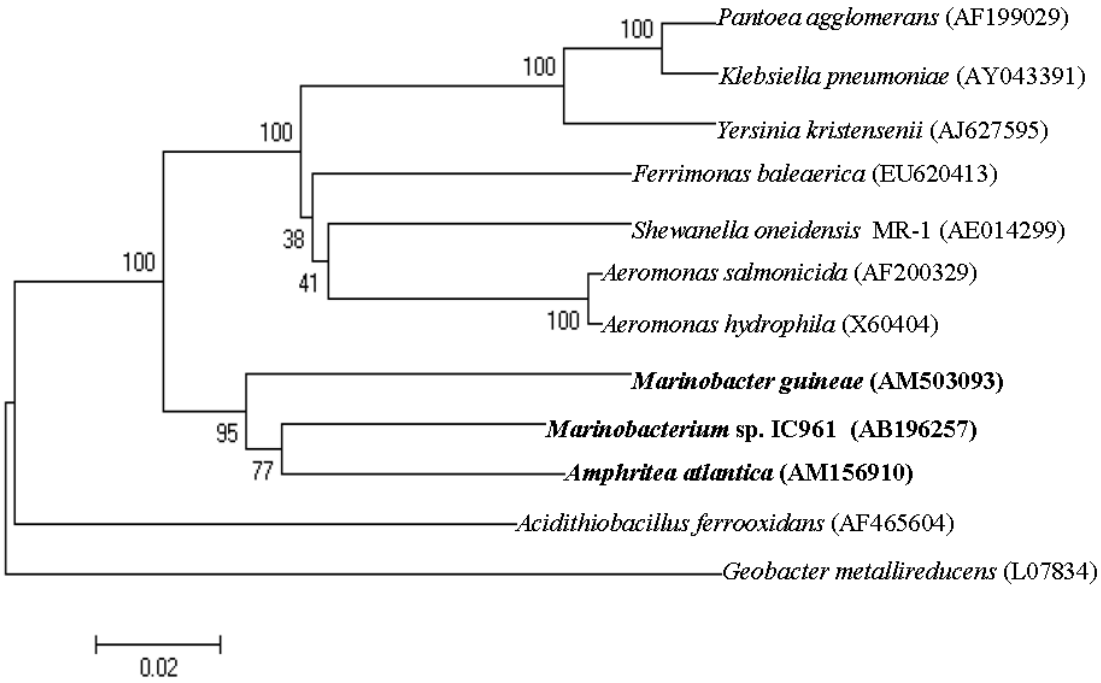


Figure 4.10. Phylogenetic tree including the full16S rRNA genes of the strains affiliated to the microorganisms found to respire Fe(III) and Mn(IV) in the present work and other γ-Proteobacteria known as dissimilatory Fe(III) or Mn(IV) reducers. All the sequences have been downloaded NCBI database, the accession number is given in brackets. *Geobacter metallireducens* a *δ-Proteobacterium* was used as outgroup. The phylogenetic tree was inferred using the Neighbor-Joining method. The optimal tree with the sum of branch length = 0.68585117 is shown. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method and are in the units of the number of base substitutions per site.

The strains sb6 and sb7 also dominating the anaerobic culture for Fe(III)-reducing microorganisms (Table 4.8) are, on the other hand affiliated to two dissimilatory Fe(III)-reducing δ-Proteobacteria: *Malonomonas rubra* (Nevin et al. 2003) and *Desulfuromonas michiganensis* (Sung et al. 2003), respectively (Fig. 4.9). As the identities in the partial 16S rRNA gene shared by sb6 and sb7 with *M. rubra* and *D. michiganensis* are lower than 97% (Table 4.8) the two ribotypes may belong to different species which have not been described yet (Stackebrandt et al. 1985, Rossello-Mora & Amann 2001). *Malonomonas rubra* was described, as an anaerobic

microaerotolerant fermenting bacterium able to grow through the decarboxylation of malonate to acetate (Dehning & Schink 1989), later it was also found to be able to grow through the reduction of Fe(III) (Knight & Blakemore 1998). The strain sb6, accounting for 21% of the clone library (Table 4.8) was probably active in the anaerobic culture and involved in the dissimilatory Fe(III) reduction. *Desulfuromonas michiganensis* was isolated for the first time from freshwater sediment, found to be able to grow through the reduction of Fe(III) coupled with oxidation of lactate and acetate (Sung et al. 2003) and to ferment fumarate and malate producing acetate and succinate (Sung et al. 2003). Both strains sb6 and sb7, dominating the anaerobic culture for Fe(III) reducers of marine aggregates (Table 4.8), appear thus to be able to carry out fermentation as well as dissimilatory Fe(III) reduction. The only carbon source supplied in the culture was acetate, which is a non-fermentative source. Strains sb6 and sb7 were probably active as dissimilatory iron reducers, but they might have been also involved in the fermentation of organic compounds deriving from other bacteria growing in the culture. In natural conditions *D. michiganensis* and *M. rubra* appear to be viable only under microaerophilic (Sung et al. 2003) or anaerobic (Dehning & Schink 1989) conditions, in sinking and settled marine aggregates they are thus probably senescent until low oxygen conditions occur. When the oxygen becomes depleted enough the strains can become active fermenting sugars and/or reducing Fe(III) and Mn(IV).

4.5.3 Bacteria isolated from the anaerobic cultures of aggregates

All the microorganism isolated aerobically from anaerobically cultured aggregates belong to the phylum *γ-Proteobacteria*, which is a phylum well represented in freshwater (Weiss et al. 1996, Grossart & Ploug 2000, Brachvogel et al. 2001) and marine snow (DeLong et al. 1993, Rath et al. 1998, Acinas et al. 1999, Grossart & Ploug 2001, Grossart et al. 2004, Abell & Bowman 2005, Blazina et al. 2005, Stevens et al. 2005). *γ-Proteobacteria* were suggested to be adapted to the marine snow environment of higher nutrient concentrations and changeable redox conditions (Nold & Zwart 1998), although they tend to be more abundant in estuarine and marine aggregates whereas *α-Proteobacteria* and *β-Proteobacteria* dominate river and lake snow (Bockelmann et al. 2000, Schweitzer et al. 2001, Simon et al. 2002). *γ-Proteobacteria* are likely to be adapted to redox changing environments because the phylum includes many opportunistic species able to survive and grow under both

aerobic and anaerobic conditions (Pinhassi & Berman 2003) thus dominating many different marine environments (Xu et al. 2005, Jiang et al. 2007).

Microorganisms affiliated to *δ-Proteobacteria*, including *D. michiganensis* and *M. rubra*, which were found in the anaerobic cultures of aggregates (Table 4.8), could not be isolated aerobically because probably they are not able to grow in the presence of oxygen. To date all the *Desulfuromonas* and *Malonomonas* species known are obligate anaerobes (Dehning & Schink 1989, Finster et al. 1994, Coates et al. 1995, Finster et al. 1997, Krumholz 1997, Sung et al. 2003, Vandieken et al. 2006). *δ-Proteobacteria* were suggested to be more adapted to anoxic environments (Nold & Zwart 1998) most of them being inactive under aerobic conditions. Under natural conditions, aggregate-attached dissimilatory Fe(III) and Mn(IV) reducers affiliated to δ-*Proteobacteria* are likely to be senescent and to become active only under highly reducing conditions which occur in the sediment and in anoxic waters such as Black Sea.

Vibrio species have been found many times associated with marine particles: two strains believed to belong to the genus *Vibrio* were sequenced from the microbial community attached to diatom aggregates (Bidle & Azam 2001) and faecal pellets (Delille & Razouls 1994). The ability of *Vibrio* species to ferment glucose and other sugars was suggested to be important in marine aggregates and other environments where transient anoxia occurs (Riemann & Azam 2002). In many previous studies *Vibrio* species were found attached to marine (Venkateswaran et al. 1990, Hsieh et al. 2007, Lyons et al. 2007, Fries et al. 2008) and freshwater aggregates (Colwell et al. 2003) and suggested to preferentially attach to marine particles (Nealson & Venter 2007). Microorganisms belonging to the order *Vibrionales* were found to be dominant in artificial marine snow (Long & Azam 2001). *Vibrio* species are thus commonly reported in marine particles and might be playing an important role in fermenting sugars.

The strain Is-2 is able to grow through the reduction of Fe(III) (Fig. 4.8) but is not capable of Mn(IV) reduction (Fig. 4.8). *A. atlantica* is able to grow under both aerobic and anaerobic conditions and is likely to be involved in the dissolution of Fe(III) described in artificial aggregates (Balzano et al. 2009). Most dissimilatory Fe(III) reducers can reduce Mn(IV) directly (Lloyd 2003, Lovley et al. 2004) or indirectly via the abiotic reduction of Mn(IV) by Fe(II) (Lovley & Phillips 1988). In natural systems Mn(IV) is reduced under anoxic conditions directly by microorganisms

or abiotically by Fe(II) produced from dissimilatory iron reduction (Lovley et al. 2004). Fe(III)-reducing microorganisms which do not have the enzymatic systems to directly reduce Mn(IV) may thus reduce it indirectly using the Fe(II) produced (Lovley et al. 2004). Even though the strain Is-2 is able to respire Fe(III), when cultured anaerobically with acetate and δ-MnO_2, the culture showed a production of dissolved Mn over time comparable to that which occurred in the abiotic control (Fig. 4.8). As a consequence it does not seem to be capable of growing through the reduction of Mn(IV).

Strains Is-3, Is-4 and Is-6 are able to grow through the reduction of both Fe(III) and Mn(IV) as demonstrated by the production of Fe(II) and dissolved Mn measured (Fig. 4.8). Both genera *Marinobacterium* and *Marinobacter* are affiliated to the order of *Alteromonadales* which contains other well described facultative anaerobic dissimilatory Fe(III) reducers including *Shewanella oneidensis*. The affiliation of Is-6 to *Marinobacter maritimus* is quite uncertain because the two partial 16S rRNA gene sequences share only 83 % over 505 bp (Table 4.9). As the percentage of shared identities is lower than 95%, Is-6 may belong to a new species or even a new genus (Rossello-Mora & Amann 2001) not previously described. When the genus *Marinobacterium* was first described, the species associated with this genus, *M. georgiense* was found to be strictly aerobic (Gonzalez et al. 1997). Six further species belonging to this genus have been described: *M. stanieri*, *M. jannaschii* (Satomi et al. 2002), *M. halophilum* (Gorby et al. 2006), *M. litorale* (Kim et al. 2007), *M. rhizophilum* (Kim et al. 2008) and all but *M. litorale* were found to be strictly aerobic. It is not clear, from the description made by Kim and co-authors (2007), which electron donors and acceptors can support the anaerobic growth of *M. litorale*. The ability of a strain affiliated to *Marinobacterium* to conserve energy for the growth through the reduction of Fe(III) and Mn(IV) has never been described before.

Marinobacter guineae was initially isolated from the Southern Ocean and described as a facultative anaerobe capable of conserving energy through the reduction of nitrate (Montes et al. 2008) but its ability to grow with iron or manganese as the sole electron acceptors has never been determined. Many other *Marinobacter* species were found to be able to grow anaerobically with nitrate (Rontani et al. 1997, Rontani et al. 2003, Takai et al. 2005, Kim et al. 2006, Antunes et al. 2007, Yoon et al. 2007) or fumarate (Takai et al. 2005, Handley et al. 2009) as the sole electron acceptors. The presence of nitrate (Allen et al. 2001) and nitrite (Oakley et al. 2007) reductase genes

was detected in some *Marinobacter* species. On the other hand the species *M. vinifirmus* and *M. lutaoensis* were found to be strictly aerobic and thus unable to reduce nitrate to nitrite (Shieh et al. 2003, Liebgott et al. 2006). Anaerobic degradation of hydrocarbons by *Marinobacter* species was also demonstrated (Rontani et al. 2002). Microorganisms belonging to the genus *Marinobacter* are able to oxidise iron (Edwards et al. 2003) and manganese (Templeton et al. 2005) under hypoxic or anoxic conditions, by either oxygen or nitrate. A recently described species, *M. santoriniensis* was found to have the potential to respire Fe(III) with lactate supplied as the electron donor (Handley et al. 2009). In the previous chapter a ferric iron reducing community enriched from marine aggregates was found to be dominated by two strains affiliated to the genus *Marinobacter* which were suggested to be directly involved in dissimilatory Fe(III) reduction. A number of new *Marinobacter* species have been isolated from a wide range of environments including the water column (Xu et al. 2008, Handley et al. 2009), sediment (Guo et al. 2007, Montes et al. 2008), sea-shore sand (Kim et al. 2006), sea-ice cores (Zhang et al. 2008), high salinity environments (Antunes et al. 2007, Yoon et al. 2007) as well as waste treatment plants (Liebgott et al. 2006) and oil contaminated sediments (Rontani et al. 1997) and soils (Gu et al. 2007). Recent studies are thus showing that *Marinobacter* species are widespread in natural and contaminated environments and can perform a wide range of metabolisms. In particular the adaptation of *Marinobacter* species to redox changing environment such as marine aggregates is suggested. The mechanism for iron reduction, in the strains affiliated to *Marinobacter* detected in the anaerobic cultures of aggregates, may be associated with siderophore production which bind to Fe(III) transporting it into the bacterial cell. Siderophore secretion to facilitate iron acquisition was detected in some *Marinobacter* strains (Barbeau et al. 2002, Gonzalez-Domenech et al. 2008b) and may be an important mechanism in iron depleted waters. However the strains affiliated to *Marinobacter*, Is-4 and Is-6 are able to reduce ferric iron and also to grow through its reduction.

As the strains Is-7 and Is-8 did not produce ferrous iron or dissolved Mn when cultured with Fe(III) and Mn(IV) as the sole electron acceptors (Fig. 4.8), they are not able to grow through the reduction of Fe(III). Strain Is-7 is affiliated to the *Vibrio* genus, as discussed above *Vibrio* species are able to grow through the fermentation of glucose and other sugars (Riemann & Azam 2002). Strain Is-8 is affiliated to *Halomonas*, a previous study demonstrated the reduction of Fe(III) to Fe(II) from an

isolate belonging to *Halomonas* (VanEngelen et al. 2008) but the reduction rate was found to be very slow as only 20% of Fe(III) present was reduced to Fe(II) after 72 days of exposure (VanEngelen et al. 2008). These data do not agree with the present study where Fe(III) reduction was not detected after 60 days of incubation. Many *Halomonas* species have been isolated from saline environments (Gonzalez-Domenech et al. 2008a, Gonzalez-Domenech et al. 2008b, Kharroub et al. 2008, Xu et al. 2008) and some have been found to be able to respire nitrate (Boltyanskaya et al. 2007, Gonzalez-Domenech et al. 2008a, Gonzalez-Domenech et al. 2008b) and nitrite (Gonzalez-Domenech et al. 2008a). *Halomonas profundus,* a microorganism isolated from a hydrothermal vent, was found to be able to ferment glucose (Simon-Colin et al. 2008). As for Is-7, the strain Is-8 isolated from the anaerobic culture of aggregates might have been actively fermenting OM produced by other bacteria directly involved in the dissimilatory Fe(III) reduction shown in Fig. 4.6.

4.6 Limitations in the present work

Seawater from the Solent collected and fertilised as described in Section 2.6.1 was transferred to replicate identical polycarbonate tanks in the expectation to have the same community grow on each. However significant differences in size, microbial abundances and release in dissolved Mn occurred among replicates (Table 4.1 and Figs 4.1, 4.2, 4.5).

As phytoplankton were cultivated in different tanks (although following the same method) very subtle differences in light exposure, and species distribution, might have led to the growth of different microbial communities in the replicate tanks. The cultivation of seawater under the same conditions in separate containers does not necessarily lead to the same ecological succession. Mixed phytoplankton assemblages from the Solent should have been cultivated in a single container and transferred to replicate tanks prior to flocculation experiments. In addition the determination of the particulate iron and manganese present at the start and at the end of the experiments would have contributed to provide a complete mass balance for the Fe and Mn cycling in aggregates.

Moreover, the isolation techniques are selective, excluding a significant proportion of marine bacteria which are unculturable (Rappe & Giovannoni 2003). The bacterial density in the anaerobic cultures of aggregates could have been measured

along with the concentration of reduced metals in order to directly relate the chemical reductions with the bacterial abundances.

4.7 Summary

Although artificial aggregates incubated aerobically under dynamic conditions did not release significant amounts of reduced iron (Fig. 4.3), the bacteria present are capable of reducing added Fe(III) when cultivated anaerobically along with an electron donor such as acetate or lactate (Fig. 4.6). Marine aggregates incubated aerobically under dynamic conditions can, on the other hand, release large amounts of reduced manganese (Fig. 4.5) and the process is probably driven by dissimilatory Mn(IV)-reducing bacteria which have been demonstrated to be present in aggregates (Fig. 4.7). A likely scenario which might help understand the significant increase in dissolved Mn measured in artificial aggregates (Fig. 4.5) not associated with any increase in dissolved iron (Figs 4.3-4.4) is hypothesised: during the aerobic incubations of aggregates, microorganisms with the capability of respiring both Fe(III) and Mn(IV) present in aggregates likely produced dissolved Mn(II) [and potentially dissolved Mn(III)] at a far higher rate than Fe(II) in the suboxic or low oxygen microenvironments of aggregates. The dissolved Mn produced and released in the oxic surrounding environment will be oxidised to Mn(IV) at a slower rate than Fe(II) oxidation to Fe(III). As a consequence while the Fe(III) reduced in the suboxic and low oxygen microenvironments of aggregates did not exceed the Fe(II) re-oxidised in the oxic surrounding environment, the reduction of Mn(IV) in the low oxygen or suboxic microenvironments of aggregates may have exceeded the re-oxidation of dissolved Mn to Mn(IV) in the oxic surrounding environments. The observations here are consistent with Mn(II) requiring less reducing conditions than Fe(II) to be produced, and the former being more stable than the latter under aerobic conditions (Morgan 2005). These results suggest that natural marine aggregates while suspended in the water column might have the potential to release significant amounts of dissolved manganese but could have little obvious effect on the iron cycling. Measurable releases of Fe(II) ascribable to the presence of aggregates were not registered for two reasons: the dynamic conditions in which aggregates were incubated involved higher oxygen diffusion from the surrounding seawater to the inner parts of the flocs, and the lower aggregate:seawater ratio rapidly diluted released Fe (II) and allowed the Fe(II)

oxidation process in the seawater predominate over the Fe(III) reduction occurring in the aggregates.

The bacteria isolated from the anaerobic cultures for Fe(III)- and Mn(IV)-reducing microorganisms of aggregates (Table 4.9) and found to be able to grow through the reduction of these metals in the present work (Fig. 4.8) belong all to the *γ* class of *Proteobacteria* and were not previously described as metal respiring microorganisms. Both *δ-Proteobacteria* and *γ-Proteobacteria* were present in the anaerobic culture of aggregates, under ferric iron enriched conditions (Table 4.8), but when the enrichment was transferred to aerobic Marine Agar (Difco), only *γ-Proteobacteria* were successfully isolated and cultivated (Table 4.9). More *γ-Proteobacteria* (e.g. *Marinobacter*) were isolated from different enrichments with ferric iron or Mn(IV) as the electron acceptor. Fe(III)- and Mn(IV)-reducing microorganisms belonging to *δ-Proteobacteria* are more likely to be inactive under aerobic conditions (Nold & Zwart 1998), whereas those affiliated to *γ-Proteobacteria* are facultative anaerobes (Lovley et al. 2004). In marine aggregates γ-Proteobacteria seem to be better adapted to the changing redox conditions occurring marine aggregates (Nold & Zwart 1998, Simon et al. 2002). The presence of different bacteria whose ability to respire using Fe(III) and Mn(IV) was not previously described indicates that, in marine systems, a number of bacteria are able to conserve energy for their growth from the reduction of Fe(III) and Mn(IV). More generally it seems very likely that a significant proportion of bacteria playing key roles in biogeochemical cycles have not been discovered and described as yet.

Chapter 5. Changes in fatty acid composition in degrading algal aggregates

5.1 Introduction

5.1.1. Linking release in solutes from aggregates to biochemistry and microbiology

The previous chapters showed the impact of marine aggregates on the release in nutrients, dissolved iron and dissolved manganese to the water column. Aggregates N1 and TA1 released significant amounts of dissolved Fe(II) (Fig. 3.2) and contained an anaerobic community including Fe(III)-reducing bacteria (Tables 3.2, 3.4, Balzano et al. 2009). In the present chapter the lipid composition of artificial aggregates formed and incubated in parallel with the aggregates N1 and TA1 was investigated.

5.1.2 Objectives

The lipid composition of natural marine snow has been investigated and the compositional changes occurring over depth elucidated (Wakeham et al. 1997a, Kiriakoulakis et al. 2001). Studies based on laboratory-incubated phytodetritus have been carried out to investigate the degradation of FA (Harvey & Macko 1997, Sun et al. 1997) phytol (Sun et al. 1998) and sterols (Sun & Wakeham 1998). Here, that previous work is complemented by comparing the compositional changes in lipids in artificial marine snow incubated in 10-µm filtered seawater to microbial assemblages and biogeochemical transformations (Balzano et al. 2009).

Within this context, this study had a number of key objectives. First, it aims to explore the biochemical differences occurring between artificial aggregates formed from cultivated phytoplankton assemblages naturally present in seawater (Southampton Water) and artificial aggregates formed from selected algal cultures (*Thalassiosira weissflogii* and *Alexandrium tamarense*); the former aggregates are likely to contain a more heterogeneous composition and a higher input of terrestrial material present in Southampton Estuary, including *n*LCFA, compared to the latter aggregates. Second, this study ascertains whether fatty acid biomarkers, which are normally present in low abundances (brFA, *n*-LCFA) in marine snow, can also be detected and quantified on

small samples that are also used in nutrient cycling studies. The study has mainly focused on FA as they undergo rapid compositional changes in marine particles (Wakeham et al. 1997a, Kiriakoulakis et al. 2001) and aims to assess whether artificial marine aggregates are depleted in labile FA (*n*SCFA and PUFA) and enriched in bacterial ones (brFA) over a short time interval (10 days). Phytol and some sterols, as tracers for phytoplankton and eukaryotic zooplankton, are also quantified for comparison.

In the present chapter FA are not indicated with their IUPAC names but using a notation commonly found in geochemical literature (e.g. Wakeham et al. 1997). The notation is defined in Table 5.3.

5.2 Experimental setting

The present study was carried out on artificial marine aggregates: similarly to the methods applied in the previous chapters artificial aggregates were obtained by rotating senescent phytoplankton cultures on a roller table and incubated in the dark. Samples N1, N7 and N10 were obtained from cultures of mixed phytoplankton populations naturally present in Southampton Water and samples TA1 and TA7 were obtained from cultures of *Thalassiosira weissflogii*, *Alexandrium tamarense* and Southampton Water (Section 2.2, Table 5.1). All aggregates were then incubated in the dark at constant temperature (Section 2.3), with nutrient concentrations measured over time (Section 2.8.2), and subsamples of N7, N10 and TA7 were periodically collected for microbial and lipid analyses as described in Section 2.3.4. Lipids were extracted and analysed as described in Section 2.9, the attached bacterial community was analysed as described in Section 2.12. The concentration of dissolved Fe(II) was measured over time in the parallel flasks N1 and TA1, the bacterial community attached to aggregates N1, with particular attention devoted to dissimilatory Fe(III) reducers, was also investigated. The results related to aggregates N1 and TA1 are shown in Chapter 3 and published in Balzano et al. 2009. The study presented in this chapter relates to fatty acid composition of aggregates N7, N10 and TA7 and the bacterial community attached to aggregates N10.

Thus, N1 and N7, as well as TA1 and TA7 are effectively replicates, whereas the enrichment N10 was prepared following the same method as N1 and N7 but using background seawater collected on a date different than N1 and N7 (10 days later, Table

5.1). The phytoplankton community collected might thus have been different in N1 and N7 compared to N10. As shown below there is significant variability in nutrient concentrations. Thus, all the current data are interpreted cautiously, especially differences between different aggregates, focussing instead on trends across all experiments.

Table 5.1. List of samples analysed in the present study.

Sample	Background material	Incubation volume	study carried out	Results shown
N1	Southampton Water phytoplankton[ab]	0.5L	chemical releases, microbiology	Chapter 3. Balzano et al. 2009
N7	Southampton Water phytoplankton[ab]	2L	Fatty acids	This chapter
N10	Southampton Water phytoplankton[ac]	2L	Fatty acids, microbiology	This chapter
TA1	90% algal cultures[d] and 10% Southampton Water[e]	0.5L	Iron release	Chapter 3. Balzano et al. 2009
TA7	90% algal cultures[d] and 10% Southampton Water[e]	2L	Fatty acids	This chapter

[a] Mixed phytoplankton assemblages harvested from Southampton Water and cultivated in the laboratory.
[b] 20-µm filtered Southampton Water collected the 10th April 2007.
[c] 20-µm filtered Southampton Water collected the 27th April 2007.
[d] Senescent cultures of *Thalassiosira weissflogii* and *Alexandrium tamarense.*
[e] 10-µm filtered Southampton Water collected the 10th May 2007.

5.3. Nutrient release and redox transformation

The concentrations of nutrients in the three bottles containing aggregates are shown in Fig. 5.1 for N7, TA7 and N10, and in Fig. 3.1 for N1 and TA1. The full dataset is shown in Appendix 3.

NO_3^- and NO_2^- concentrations were higher in N1, TA1 and N7 compared to N10 and TA7; NO_3^- ranged from 25 to 86 µM. In N1, N7 N10 and TA7 the concentration of NO_3^- was constant whereas in TA1, it decreased over time (Figs 3.1, 5.1). NO_2^- ranged from 0.9 µM in TA7 to 4.1 µM in N7 on day 8 (Fig. 5.1). The concentration of NO_2^- increased over time in N7, N1 and TA1, but was constant in N10 and TA7 (Figs 3.1, 5.1). Note that inferred NO_3^- uptake and NO_2^- production in the present study (N7, N10, TA1) were smaller than that demonstrated to occur in the previous investigation of N1

and TA1 (Balzano et al. 2009); this is because the former aggregates were incubated in larger volumes of seawater such that the impact of processes that occurred inside the aggregates had less of an effect on the bottle's chemistry.

The initial concentration of PO_4^{3-} was different in the bottles. Initial PO_4^{3-} concentrations similar to those measured in N7 were present in N1 and TA1 (Fig. 3.1). The concentration of phosphate increased sharply over time in N1, N7 and increased in TA1 whereas it was constant in N10 and TA7.

The concentrations of NO_3^- and PO_4^{3-} measured in the bottles during the dark incubation were one order of magnitude lower than the initial amounts present in the F2 medium when algal cultures were started, indicating nutrient uptake by phytoplankton. By extrapolation, the higher NO_3^- and NO_2^- concentrations in N1, TA1 (Balzano et al. 2009) and N7 (Fig. 5.1) likely reflect a lower nutrient uptake. However, greater oxidation of organic nitrogen in N1, TA1 and N7 cannot be discounted, and the increase in PO_4^{3-} over time in N7 (Fig. 5.1), N1 and TA1 are consistent with aerobic remineralisation of OM. Thus, nutrient uptake in the N10 and N7 enrichments was higher than that N1, TA1 (Balzano et al. 2009) and N7.

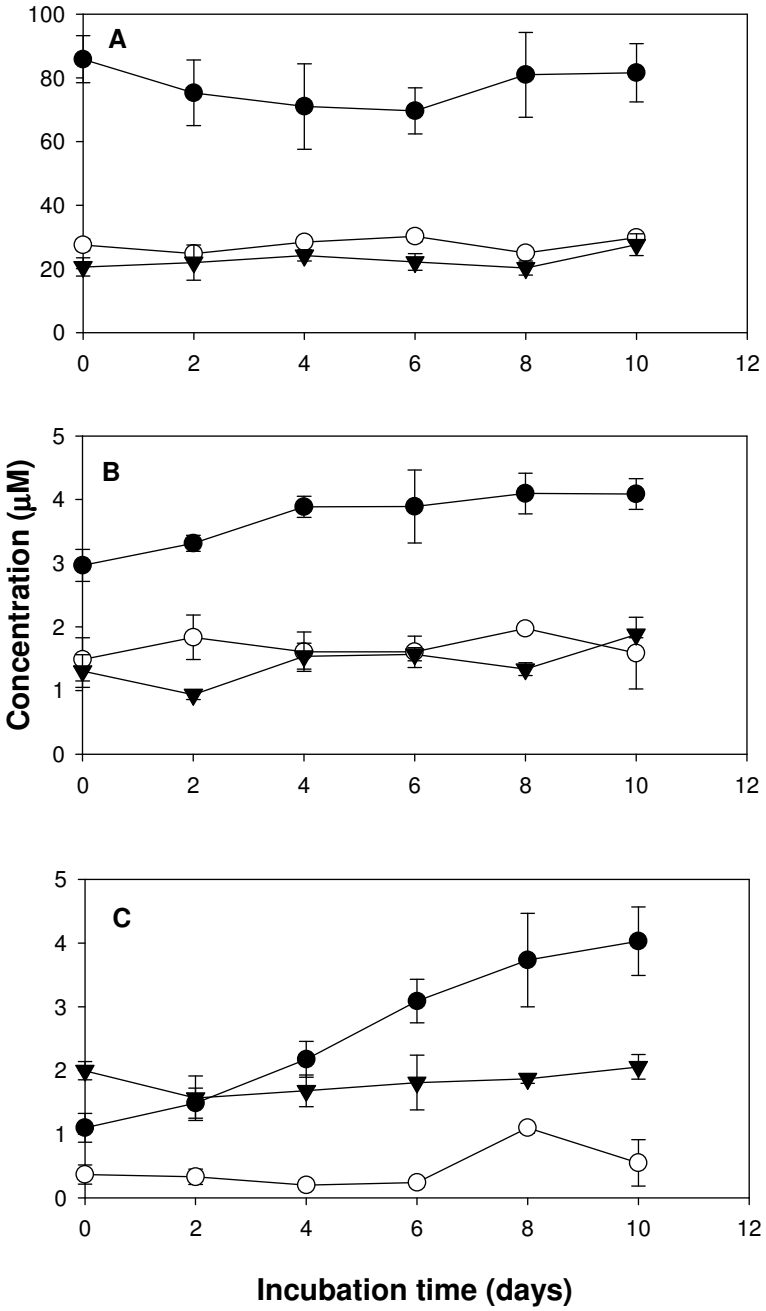


Figure 5.1. Concentrations of dissolved (**A**) nitrate, (**B**) nitrite and (**C**) phosphate in the seawater enrichments where artificial aggregates were incubated in the present work. (○) N7; (●) N10; (▼) TA7.

5.4 Bacterial populations

Molecular analyses were carried out on S2N10, which is a subsample of aggregates N10 collected on day 5 of dark incubation. The diversity of 16S rRNA gene sequences recovered from artificial aggregates isolated from sample S2N10 was examined by RFLP sorting of 150 different clones and followed by gene sequence analyses. Amplification of 16S rRNA gene fragments, performed using primers 8F (Eden et al. 1991) and 519R (Lane et al. 1985), revealed a simple bacterial community constituted by 12 ribotypes. The most abundant classes were the α-*Proteobacteria* and *γ-Proteobacteria* accounting for 38% and 36% of the clone library, respectively (Table 5.2), *Planctomycetacia* and *Sphingobacteria* (Table 5.2) were also present and accounted for 21% and 5% respectively. The most abundant ribotypes were N10-1, N10-2, N10-3 and N10-4 affiliated to *Planctomycete* GMD14H10, uncultured *γ-Proteobacterium* 29 T9d, uncultured *γ-Proteobacterium* 3m04AISH9R and uncultured bacterium SIMO-1790, respectively. Other microorganisms accounting for lower percentages were also present (Table 5.2). Only sequences N10-7, N10-8 and N10-9 are closely related to well characterised species (Table 5.2) whereas all the other ribotypes are affiliated to uncultured microorganisms. However, the ribotypes N10-3, N10-5, N10-6 and N10-10 do show low similarities with *Pseudomonas* sp. (EF657880, 92% matches over 492 bp*)*, *Stappia* sp. DG1611(EU052769, 89% matches over 447 bp), *Sphyngopsis* sp. (FJ889532, 88% matches over 443 bp) and *Maricaulis parjimensis* (NR_025323, 94% matches over 435 bp), respectively.

The bacterial community present in aggregates S2N10 (Table 5.2) is similar to that found in N1 in the parallel study (Balzano et al. 2009), and all have similarities to those reported for natural sinking particles (DeLong et al. 1993, Rath et al. 1998, Simon et al. 2002). The most abundant ribotype, N10-1 is affiliated to *Planctomyctes* and the species in culture that are most closely related to it are *Blastopirellula marina* and *Planctomyces limnophilus* (Zengler et al. 2002). *Planctomycetes* were demonstrated to be adapted to attach to marine particles (Neef et al. 1998) and are present in both marine and riverine aggregates (DeLong et al. 1993, Bockelmann et al. 2000, Allgaier & Grossart 2006). Artificial aggregates obtained following the same method used here were also found to be dominated by a strain affiliated to *Planctomycete* GMD14H10 (Table 4.7).

The strain N10-2, affiliated to the class γ-*Proteobacteria* is not related to any well described bacterial species whereas N10-3 is poorly affiliated to the genus *Pseudomonas* (EF657880). Although not dominant γ-*Proteobacteria* were also abundant in N1, as the cloned 16S rRNA fragments affiliated to γ-*Proteobacteria* accounted for 13% of the total clone library (Balzano et al. 2009). Moreover γ-*Proteobacteria* are often dominant in natural marine snow particles (Rath et al. 1998, Bidle & Azam 2001, Simon et al. 2002, Selje & Simon 2003, Abell & Bowman 2005).

The ribotypes N10-4, N10-5, N10-6, N10-8, N10-9 and N10-10 are affiliated to α-*Proteobacteria* which also constitutes an important class of microorganisms attached to natural marine snow (DeLong et al. 1993, Simon et al. 2002). The ribotype N10-4 is not related to any described species, but only to uncultured α-*Proteobacteria*. On the other hand, the ribotypes N10-5, N10-6, N10-8, N10-9 and N10-10 are affiliated, albeit with lower percentages, to different α-*Proteobacteria* species.

Table 5.2. Taxonomic affiliation of ribotypes identified in aggregates collected from sample S2N10 after amplification and analysis of the partial 16S rRNA gene fragment*.

Ribotype	Closest matching microorganism (NCBI† accession number)	Cluster	Identities (%matches)	Percentage present
N10-1	*Planctomycete* GMD14H10 (AY162122)	*Planctomycetes*	410/446 (91%)	21
N10-2	Uncultured *γ-Proteobacterium* 29 T9d (FM242299)	*γ-Proteobacteria*	363/388 (93%)	19
N10-3	Uncultured *γ-Proteobacterium* 3m04AISH9R (EF629797)	*γ-Proteobacteria*	480/485 (98%)	17
N10-4	Uncultured bacterium clone SIMO-1790 (AY711156)	*α-Proteobacteria*	381/419 (90%)	14
N10-5	Uncultured *α-Proteobacterium* RAN-88 (AY499450)	*α-Proteobacteria*	(422/443 (95%)	8
N10-6	Uncultured *α-Proteobacterium* 1m04AMLC6R (EF630049)	*α-Proteobacteria*	438/439 (99%)	6
N10-7	*Lewinella nigricans* (AB301615)	*Sphingobacteria*	468/496 (94%)	5
N10-8	*Labrenzia alba (*EF512127)	*α-Proteobacteria*	485/490 (99%)	5
N10-9	Uncultured *Hyphomonas* sp. clone MD3.38 (FJ425626)	*α-Proteobacteria*	436/441 (98%)	3
N10-10	Uncultured *α-Proteobacterium* pltb-HW-94 (AB294921)	*α-Proteobacteria*	429/433 (99%)	2

* 16S rRNA gene amplified using 8F and 519R primers.
† National Centre for biotechnology information.

5.5. Lipids and likely sources

The aggregates studied in the present chapter were incubated as described in Secion 2.3.4, lipids were extracted and analysed as described in Section 2.9. The lipids identified in the artificial aggregates include FA, alcohols and sterols and are listed in Table 5.3. The main compounds detected are described below. The concentration of total FA extracted and identified ranged from 3890 µg g^{-1} dry weight in sample S1N7 to 76 µg g^{-1} dry weight in sample S3N7 (Table 5.3).

5.5.1. Monocarboxylic Fatty Acids

*n*SCFA, brFA, MUFA and PUFA were identified (Fig. 5.2), and the number of carbon atoms ranged from 14 to 28. Subsample S3N7 exhibited a weak fatty acid profile, allowing the identification of only a few compounds at low concentration, and caution is necessary when interpreting data related to that sample. Fatty acids were grouped into five different classes according to their number of carbon atoms, degree of saturation and branching characteristics (Table 5.3).

5.5.1.1. Short-chain (C_{14} to C_{20}) n-alkanoic acids (nSCFA)

The most abundant class of FA was the *n*SCFAs, the total concentration of which ranged from 19 µg g^{-1} dry weight in S3N7 to 2200 µg g^{-1} dry weight in S1N7 (Table 5.3). Even numbered *n*SCFA such as C14:0, C16:0, C18:0 and C20:0 dominated over the odd numbered *n*SCFAs such as C15:0, C17:0 and C19:0 (Fig. 5.2). C16:0 was the most abundant fatty acid in all of the samples except for S3N7 which was instead dominated by C18:0 (Table 5.3). C14:0 was detected in all samples except S3N7, and its concentration, where present, varied between 0.4 µg g^{-1} dry weight in S2N10 to 170 µg g^{-1} dry weight in S1N7; C16:0 ranged from 4.5 µg g^{-1} dry weight in S3N7 to 1900 µg g^{-1} dry weight in S1N7; and C18:0 varied from 11 µg g^{-1} dry weight in S3N7 to 100 µg g^{-1} dry weight in S1TA7. C20:0 was found in all the samples, and its concentration ranged from 2.7 µg g^{-1} dry weight in S3N7 to 31 µg g^{-1} dry weight in S2N7. Odd numbered *n*SCFA, such as C15:0 and C17:0 acids, were present in all samples but S3N7, and low amounts of C19:0 were also measured in S2N7, S3N7, S2N10 and S3N10.

Even numbered *n*SCFA are quite widespread in nature and can derive from phytoplankton, zooplankton as well as bacteria and higher plants (Cranwell et al. 1987,

Meyers 1997, Wakeham et al. 1997a, Dijkman & Kromkamp 2006). They are the main FA in phytoplankton (Dijkman & Kromkamp 2006) and are also dominant in both suspended and sinking marine particles (Wakeham et al. 1997a, Kiriakoulakis et al. 2001, Brinis et al. 2004, Tolosa et al. 2004, Blazina et al. 2005). Odd numbered *n*SCFA such as C15:0 and C17:0 are also present in marine particles (Brinis et al. 2004) and can be produced by bacteria (Mayzaud et al. 1989, Najdek et al. 2002); diatoms can also contain low concentrations of these FA (Viso & Marty 1993, Dijkman & Kromkamp 2006).

5.5.1.2 Long-chain (>C_{20}) n-alkanoic acids (nLCFA)

*n*LCFA (21-28 carbon atoms) were present in all aggregates (Table 5.3), with total concentrations ranging from 29 µg g^{-1} of dry weight in S3N7 to 180 µg g^{-1} of dry weight in S2N7. C24:0 was the dominant *n*LCFA with its concentration varying between 20 to 110 µg g^{-1} dry weight (in S3N7 and S2N7, respectively; Table 5.3). C22:0 and C26:0 were also present in all samples (Table 5.3), with concentrations of the former ranging from 5.9 µg g^{-1} dry weight in S1N10 to 51 µg g^{-1} dry weight in S2N7, and the latter ranging from 1.7 µg g^{-1} dry weight in S3N7 to 37 µg g^{-1} dry weight in S3TA7 (Table 5.3). Low concentrations of C28:0 were present in S2N10, S3N10 and S3TA7; S2N10 and S3N10 also contained C23:0 which was the only odd numbered *n*LCFA detected in the present study.

The presence of *n*LCFA in marine particles and sediment has been often attributed to terrigenous inputs, and especially the epicuticular waxes of terrestrial plants (Grimalt & Albaigés 1990, Rielley et al. 1991, Meyers 1997). Several studies indicate that *n*LCFA are absent, or present in very low concentrations, in marine POM or aggregates collected from different environments such as the surface of the Adriatic sea (Najdek 1996, Blazina et al. 2005), the upper water column of the central Pacific Ocean and Black Sea (Wakeham 1995), the northwest shelf of Australia (Burns et al. 2003), the coastal north Atlantic (Galois et al. 1996) and estuarine environments in east and west coast of United States (Canuel & Zimmerman 1999, Mannino & Harvey 1999, Canuel 2001). In other studies, *n*LCFA occurred at higher concentrations in aggregates collected from sediment traps in the North Atlantic (Kiriakoulakis et al. 2001), the deep central Pacific (Wakeham et al. 1997b), and shallow (200m) waters of the north-western Mediterranean Sea (Marchand et al. 2005) and attributed to terrestrial source.

High molecular weight *n*-alcohols (≥ 20 carbon atoms) in marine particles are also attributed to higher plant leaf wax input (Cranwell 1982, Yoshinaga et al. 2008). Previous studies on marine particles and sediment show *n*LCFA concentrations to be correlated with those of long chain n-alkanols (Kiriakoulakis et al. 2001, Yoshinaga et al. 2008), and the presence of both compound classes was used to infer a terrestrial origin to the OM examined. However high molecular weight *n*-alcohols were not detected in any of the samples analysed, apparently contradictory with a higher plant origin of *n*LCFA. In addition higher concentrations of *n*LCFA are present in TA7 aggregates compared to N7 and N10. Because the TA7 aggregates are algal-derived, they are expected to have a lower rather than higher concentration of terrestrially-derived long-chain FA than the seawater-derived aggregates N7 and N10. *n*LCFA could have been produced during the phytoplankton cultivation or the consequent dark incubation. The typical fatty acid composition of *T. weissflogii* and *A. tamarense* (Viso & Marty 1993, Ishida et al. 2000, Dijkman & Kromkamp 2006) does not include *n*LCFA, but other *Thalassiosira* species such as *T. pseudonana* and *T. stellaris* have been reported to contain trace amounts of C22:0 and C24:0 (Volkman et al. 1989, Dunstan et al. 1993).

5.5.1.3 Alternative sources for nLCFAs in marine environment

The production of *n*LCFA from cells of *T. weissflogii* seems a likely source, and the lower contributions from such organisms to N7 and N10 aggregates could explain their low *n*LCFA concentrations. In addition a range of microorganisms including bacteria, microalgae and marine yeasts can also produce *n*LCFA (Nichols et al. 1984, Rezanka & Podojil 1984, Rezanka et al. 1987, Volkman et al. 1989, Yokoyama et al. 2001, Bertoldi et al. 2006).

The synthesis of *n*LCFA has been demonstrated in a range of fungi including *Saccharomyces cerevisiae, Schizosaccharomyces pombe, Cryptococcus albidus* and *Rhodotorula glutinis* (Welch & Burlingame 1973, Rezanka et al. 1987, Dittrich et al. 1998, Yokoyama et al. 2001), several yeasts belonging to this genera have been isolated from marine and estuarine environments (Fell 1961, Yamasato et al. 1974, Cheng & Lin 1977, Kutty & Philip 2008). Mixotrophic microalgae, able to grow heterotrophically under dark conditions, might have been present in artificial aggregates and involved in *n*LCFA production. *Scenedemus quadricauda*, *Euglena gracilis*, *Chlorella kessleri* and *Chlorella vulgaris* are able to incorporate organic carbon for growth and can produce

*n*LCFA (Rosenberg 1963, Rezanka et al. 1983, Rezanka & Podojil 1984, Furusato et al. 2004, Bertoldi et al. 2006). In addition other microalgae, including, *Scenedesmus acuminatus* (Rezanka et al. 1983), *Stauroneis amphioxys* (Gillan et al. 1981), *Botryococcus braunii* (Douglas 1969) can produce *n*LCFA. Some microalgae initially present in artificial aggregates might thus have contributed to *n*LCFA.

The presence of *n*LCFA in the incubated artificial aggregates may also be associated with bacterial growth. Prokaryotes were suggested to produce part of the *n*LCFA found in marine sediment (Ratledge & Wilkinson 1988, Volkman et al. 1988). *Spirulina platensis*, a mixotrophic cyanobacterium (Chojnacka & Noworyta 2004) was reported to contain *n*LCFA (Rezanka et al. 1983). In addition *n*LCFA were found to be present in a range of bacteria including *Francisella tularensis* (Jantzen et al. 1979, Nichols et al. 1985), *Lactobacillus heterohiochii* (Uchida 1974), *Mycobacterium smegmatis* (Bloch & Vance 1977) *Streptomyces cinnamonensis* (Rezanka et al. 1984), *Vibrio* sp. (Morita et al. 1992) and *Vibrio vulnificus* (Linder & Oliver 1989) A *Vibrio* species with the potential for glucose fermentation, *V. lentus* was present in artificial aggregates formed following the same method as in the present work (Chapter 4). Natural marine aggregates were also occasionally shown to be colonised by *Vibrio* species (Delille & Razouls 1994, Bidle & Azam 2001).

Consistent with this, the carbon isotopic composition of FA extracted from marine aggregates from the shallow (100-300m) Northwest Mediterranean Sea revealed a marine rather than terrestrial origin of the *n*LCFA (Tolosa et al. 2004). Similar isotopic studies have suggested a partial marine origin for the *n*LCFA found in some marine sediments (Gong & Hollander 1997, Naraoka & Ishiwatari 2000, Ratnayake et al. 2005, Niggemann & Schubert 2006). Thus, the saturated long chain FA in artificial aggregates could derive from microalgae present in the samples before aggregate formation, leaf wax inputs associated with the seawater used in aggregate formation or possibly even bacteria or other heterotrophs growing during the experiment.

5.5.1.4. Monounsaturated Fatty Acids (MUFAs)

MUFAs with 16 to 26 carbon atoms were present and abundant in all three aggregates subsampled on day 0 (Fig. 5.2, Appendix 7). The total concentrations varied between 15 and 1400 µg g^{-1} dry weight, in S3N7 and S1N7, respectively. Most of the detected MUFA were even numbered with C16:1, C18:1 and C24:1 being the most abundant. C16:1 was dominant in S1N7, S2N7, S1N10 and S1TA7, whereas C18:1 was

dominant in S3N7, S2N10, S3N10, S2TA7 and S3TA7. C16:1 was not found in S3N7, but in the other samples its concentration ranged from 15 µg g^{-1} dry weight in S2N10 to 970 µg g^{-1} dry weight in S1N7. The C18:1 concentration varied between 9.1 µg g^{-1} dry weight in S3N7 and 370 µg g^{-1} dry weight in S1N7. C17:1 was only detected in S3N10 and the three TA samples, but in very low concentrations (Table 5.3). Higher molecular weight unsaturated FA were common, with: C24:1 fatty acid detected in all of the samples (with concentrations ranging from 4.2 to 25 µg g^{-1} dry weight); C20:1 (0.7 to 19 µg g^{-1} dry weight) and C22:1 (1 to 17 µg g^{-1} dry weight) detected in S1N7, S2N7, S2N10 and all three TA samples; and C26:1 was only found in S2N7 and S2N10 at very low concentrations.

MUFAs have diverse sources and most of them are not specific for a single taxonomic group. C16:1 and C18:1 can derive from algae (Killops & Killops 2005), zooplankton (Lee et al. 1971) and bacteria (Gillan & Johns 1986). The most common C16:1 components in the marine environment are C16:1ω7 and C16:1ω10, the former being abundant in diatoms (Volkman et al. 1989) but also present in bacteria (Gillan & Hogg 1984) and the latter being mainly attributed to bacteria (Sicre et al. 1988). Two major C18:1 components are C18:1ω9 and C18:1ω7, the former is widespread in marine environments and mainly derives from zooplankton (Sargent 1976), whereas the latter is more abundant in bacteria than eukaryotic microorganisms (Harwood & Russell 1984). C20:1 and C22:1 are not common in marine phytoplankton, although they have been previously detected in marine aggregates (Wakeham 1995, Wakeham et al. 1997a, Kiriakoulakis et al. 2001, Burns et al. 2003).

5.5.1.5 11-methyloctadecenoic acid

A branched nonadecenoic acid was also detected among the MUFAs in all of the aggregates, with concentrations ranging from 1.3 (S2N10) to 61 (S1TA7) µg g^{-1} dry weight. Based on its mass spectrum (Fig. 5.3) and relative retention time with respect to that of the internal standard used, the compound was tentatively identified as 11-methyloctadecenoic acid (Me11,C18:1) (Rontani JF, personal communication). Although the presence of Me11,C18:1 is poorly recorded in the literature, it has been detected in bacterial cultures, including, in marine organisms, *Mycobacteriaceae* (Couderc 1995), *Shewanella putrefaciens* (Shirasaka et al. 1997), *Thiobacillus* (Kerger et al. 1986) and aerobic anoxygenic phototrophs belonging to the genera *Erythrobacter*, *Roseobacter* and *Citomicrobium* (Rontani et al. 2005, Rontani & Koblizek 2008). Its

presence in artificial aggregates could be associated with the *α*- and *γ-Proteobacteria* (Rontani et al. 2005, Rontani & Koblizek 2008), which are an important component of natural (DeLong et al. 1993, Grossart & Ploug 2001, Simon et al. 2002) and artificial aggregates (Balzano et al. 2009) and account to an important proportion of the bacteria detected in the present study. Among the α-*Proteobacteria,* Me11,C18:1 was found to be present in several *Roseobacter* strains (Rontani et al. 2005), and artificial aggregates prepared identically to N7 did contain a clone affiliated to *R. litoralis* (Balzano et al. 2009).

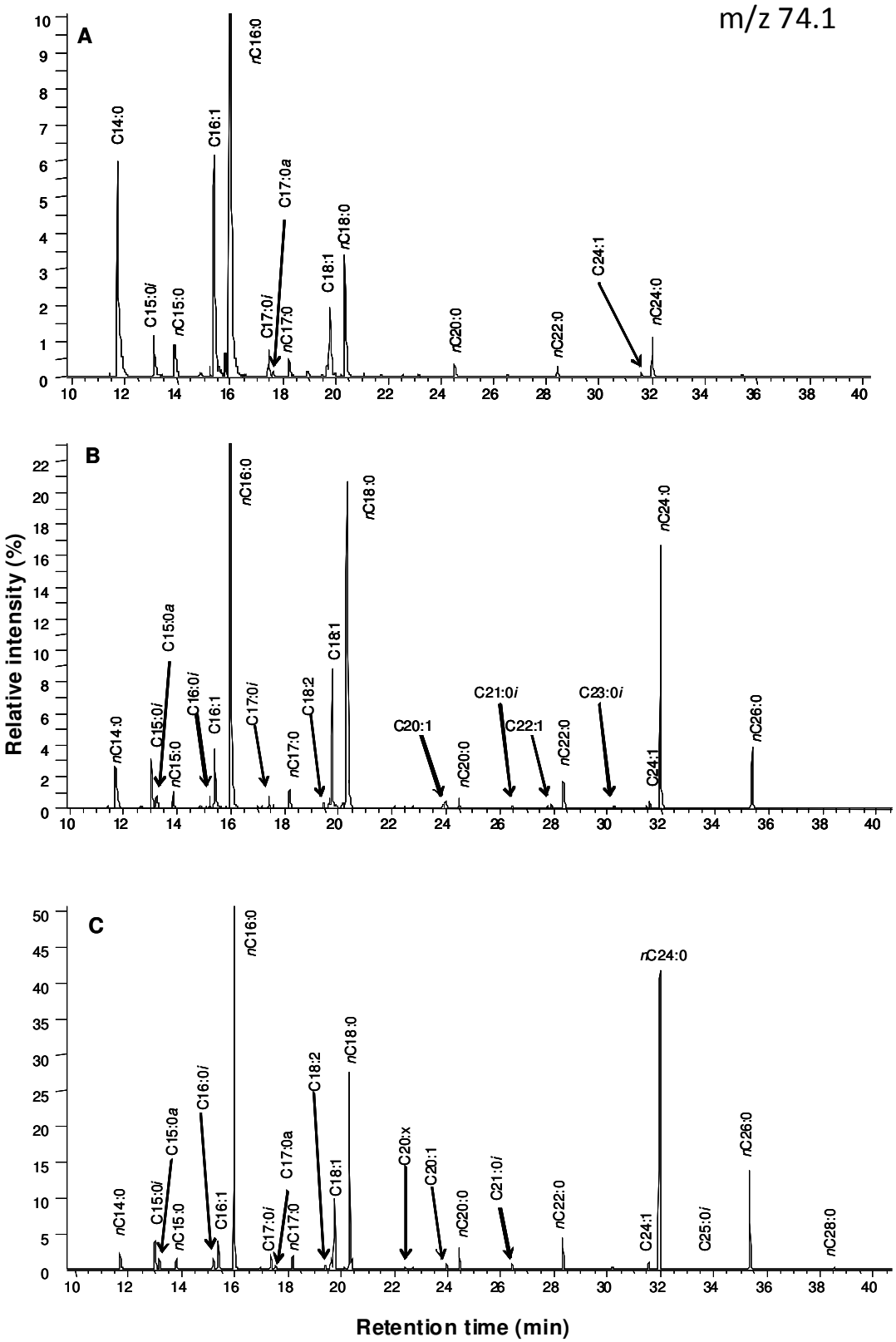


Figure 5.2. Partial *m/z* 74 mass chromatograms of the acid fraction of samples (**A**) S1N10, (**B**) S1TA7 and (**C**) S3TA7 showing fatty acid methyl esters. The peaks of MUFA and PUFA are not well represented in *m/z* 74 mass chromatograms compared to saturated ones. Note that the chromatograms are plotted in different scales and the peaks are normalized to C16:0 (=100%) which is off scale in all the figures.

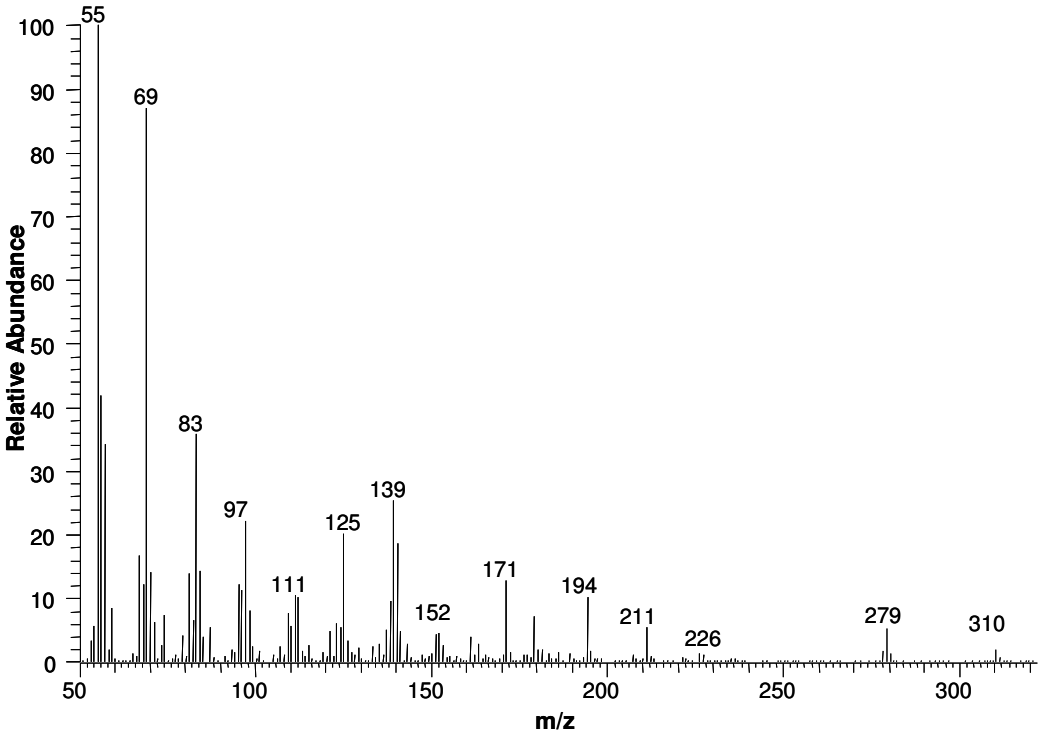


Figure 5.3. Mass spectrum of 11-methyloctadecenoic acid (Me11,C18:1).

5.5.1.6. Polyunsaturated Fatty Acids (PUFA)

Only 5 PUFAs were detected in the aggregates and the total number of double bonds could not be determined for two of them (C16:x and C20:x) because the molecular ion was not visible on their mass spectrum. The concentration of total PUFAs ranged from 4.2 to 110 µg g^{-1} dry weight (Table 5.3). C18:2 was present in all of the aggregates with a concentration ranging from 1.5 µg g^{-1} dry weight in S3N7 to 52 µg g^{-1} dry weight in S1N7, and C18:3 was detected only in S2N10 and S3N10 (3.2 and 1.9 µg g^{-1} dry weight, respectively). C16:x was present in all samples but S3N7, S1N10 and S2N10, and C20:2 was found only in S1N7 and S2N7 (Table 5.3). Overall, these are low PUFA concentrations compared to sinking particles analysed in previous studies (Galois et al. 1996, Wakeham et al. 1997a, Tolosa et al. 2004).

Most PUFAs derive from phytoplankton (Volkman et al. 1989, Viso & Marty 1993, Wakeham et al. 1997a, Dijkman & Kromkamp 2006) and are used as indicators of OM lability (Zimmerman & Canuel 2001). As aggregates had been incubated in the dark, phytoplankton growth was prevented and only a small amount of PUFA could have remained from degradation (Hama 1999). The unidentified C16 PUFA detected in the present work (Table 5.3) probably derives from diatoms (Volkman et al. 1989), whereas C18:2 and C18:3 appear to be more diagnostic for Chlorophyceae,

Prasinophyceae or higher plants (Galois et al. 1996, Volkman et al. 1998, Dijkman & Kromkamp 2006). C20:x found in the present work might be C20:5, often reported to be abundant in marine particles (Galois et al. 1996, Tolosa et al. 2004, Blazina et al. 2005) or one of its degradation products. The lack of C22:6, which can be abundant in marine particles (Galois et al. 1996, Wakeham et al. 1997a, Tolosa et al. 2004) and derives from dinoflagellates (Nichols et al. 1984, Mansour et al. 1999) which used to generate the TA7 aggregates, is surprising.

5.5.1.7 Branched saturated Fatty Acids (brFA)

A wide range of brFAs occurred in all of the artificial aggregates (Table 5.3). The total concentration of brFAs ranged from 7.3 to 89 µg g^{-1} dry weight, in S3N7 and S2N7, respectively. Most brFAs contained an odd number of carbon atoms except for the C16:0*iso* which occurred in all the samples but S3N7 with concentrations ranging from 0.3 µg g^{-1} dry weight to 5.2 µg g^{-1} dry weight (Table 5.3). The most abundant brFAs were C15:0*iso* (1 to 18 µg g^{-1} dry weight) and C17:0*iso* (2.2 to 14 µg g^{-1} dry weight). C15:0*anteiso* and C17:0*anteiso* were also present in most of the samples, whereas a branched C17:0 with a tentatively identified 10-Me branching point (C17:0br) was present in S3N10. Higher molecular weight brFAs, including C19:0*anteiso*, C19:0*iso*, unidentified branched enacosanoic acid (C21:0br), C21:0*iso*, C23:0*anteiso*, C23:0*iso* and C25:0*iso* were also present in the samples (Table 5.3).

5.5.1.8 Sources for Short chain branched saturated fatty acids (SbrFA)

Short chain brFA (≤17 carbon atoms, SbrFA) are quite common in bacteria (Parkes & Taylor 1983, Taylor & Parkes 1983, Kaneda 1991), and branched C15:0 and C17:0 FA have often been detected in marine particles (Wakeham et al. 1997a, Tolosa et al. 2008) and ascribed to bacteria (Zimmerman & Canuel 2001, Yoshinaga et al. 2008). In addition, *Planctomycetes,* one of the main bacterial classes sequenced from artificial aggregates (Table 5.2), can contain significant amounts of C16:0*iso* (Schlesner et al. 2004).

When detected in anoxic environments such as sediments or the deep Black Sea, branched C15:0 and C17:0 have been attributed to sulfate reducing bacteria (SRB) (e.g. Wakeham 1995). Aggregates can contain anoxic microenvironments (Ploug et al. 1997), and marine snow can contain sulfide (Shanks & Reeder 1993) likely derived from dissimilatory sulfate reduction; consistent with this, SRB have been detected

among bacteria attached to aggregates (Bockelmann et al. 2000, Grossart & Ploug 2000). Artificial aggregates N1 and TA1 both release reduced iron, likely formed in suboxic/anoxic microenvironments, and N1 contained anaerobic bacteria including sulfate reducers (Balzano et al. 2009). Thus, SRB might have contributed to the branched C15:0 and C17:0 FA found in aggregates.

5.5.1.9 Sources for long chain branched saturated fatty acids (LbrFAs)

Longer chain brFA (>17 carbon atoms, LbrFA) were also present in artificial aggregates and accounted for a significant proportion of the total brFA (Fig. 5.6). While SbrFA are widely reported as components of bacterial membranes (Parkes & Taylor 1983, Kaneda 1991), the production of LbrFA from bacteria is poorly understood. C23:0*anteiso* and C25:0*anteiso* FA were found in the phospholipid fatty acid (PLFA) fraction extracted from POM (Brinis et al. 2004). Because PLFAs are generally thought to be degraded quickly to free FA after the death of cells (White et al. 1979), the C23:0*anteiso* and C25:0*anteiso* FA found in Mediterranean POM (Brinis et al. 2004) were likely to be associated with living microorganisms. Branched C19:0, C21:0, C22:0, C23:0 and C25:0 FA were detected in suspended particles from western Mediterranean Sea and found to have isotopic ratios typical of OM formed in marine environments (Tolosa et al. 2004). brFA with 21 to 25 carbon atoms were detected in the sediment of a Swiss lake and attributed to autochthonous bacteria (Daher & Gulacar 2005). Long chain *anteiso* FA were found associated with fine particles of an acidic Japanese lake and attributed to planktonic microbes (Fukushima et al. 2005). Long chain brFA have also been found in hyperthermophilic communities at Octopus Spring, Yellowstone National Park (Jahnke et al. 2001), New Zealand geothermal springs (Pancost et al. 2006) and in salt lake sediments (Wang et al. 1998).

5.5.2. β-OH Fatty Acids

Very low amounts of β-hydroxy FA (β-OH-FA) were detected in aggregates S2N7, S1TA7 and S2TA7 with total concentrations always ≤ 3.2 µg g^{-1} dry weight. Because quantification of β–OH-FAs associated with lipopolysaccharide requires acid hydrolysis (Wakeham 1999), concentrations measured here might not reflect the total β-OH-FA concentration. The β-OH-FAs identified are all straight chain and saturated with 12 to 18 carbon atoms (Table 5.3). C12:0-β-OH was not found in S2N7, whereas C_{14} to C_{18} β-OH-FA were present in very low concentrations in S2N7, S1TA7 and S2TA7

(Table 5.3). The presence of β-hydroxy FA is generally attributed to the lipopolysaccharide cell wall component of gram negative bacteria (Wollenweber et al. 1984, Wakeham et al. 2003) including SRB (Edlund et al. 1985). However β-OH-FA could also be intermediates of beta oxidation of FA and can be present in microorganisms other than bacteria (Wakeham 1999). The lack of other OH-FA such as α-OH-FA, ω-OH-FA and (ω-1)-OH-FA is consistent with previous studies on POM where such OH-FA were absent or present in lower concentrations compared to β-OH-FA (Wakeham 1999).

5.5.3. Hopanoids

Bishomohopanoic acid (C32HA) was present in all of the samples, with concentrations ranging from trace amounts in both S1N10 and S3N10 to 4.6 μg g^{-1} in S3TA7. The hopanoids are membrane components of a wide range of bacteria (Ourisson et al. 1987, Rohmer et al. 1992, Pancost & Sinninghe Damsté 2003). Bishomohopanoic acid is probably formed via the oxidative vicinal cleavage of the side-chain in bacteriohopanetetrol or other tetra-functionalised hopanoids (Rohmer et al. 1984, Rohmer et al. 1992). Thus, their concentrations track both the size and nature of the bacterial community, but also work-up of bacterial biomass. Although hopanoic acids are ubiquitous components in sediments (Killops & Killops 2005) they have not yet been reported for marine particles.

5.5.4. Phytol and n-alkanols

Alcohols, including low amounts of *n*-alkanols with 12 to 20 carbon atoms, trace alkenols (data not shown) and phytol were identified in the neutral fraction of the extracted lipids. *n*-alkanols were found in all the samples but S3TA7, and the total *n*-alkanol concentration ranged from trace amounts in S2N10 to 24 μg g^{-1} dry weight in S1N7 (Table 5.3); phytol concentrations ranged from 6.7 μg g^{-1} dry weight in S3TA7 to 490 μg g^{-1} dry weight in S1N10. Given the use of saponification in the extraction protocol used, fatty alcohols with fewer than 20 carbon atoms could derive from the hydrolysis of wax esters which occur in most algae, bacteria, and zooplankton (Sargent et al. 1981, Wakeham et al. 1997a), and phytol could derive from either hydrolysis of various algal chlorophylls during incubation (Baker & Louda 1983) or during extraction.

Most of the studies on marine particles have focused on FA or sterols, and there are few investigations of *n*-alkanols; high amounts of *n*-alkanols with 14 to 28 carbon atoms were present in sinking aggregates collected from north Atlantic but concentrations rapidly decreased with depth as alcohols are quite labile and can be easily degraded (Kiriakoulakis et al. 2001). However when the activity of metazoan grazers is not significant phytol can accumulate on the seafloor (Harvey & Johnston 1995). Phytol associated to eukaryotic microalgae is a relatively labile component and tends to be quickly removed from aquatic environments (Harvey & Macko 1997), but the samples were saponified prior to analysis, such that quantified phytol could derive from either degraded or intact chlorophylls. Low concentrations of phytol have been previously detected in natural aggregates (Wakeham et al. 1997a) although it may occasionally account for substantial portion of neutral lipids in POM (Loh et al. 2006)

5.5.5. Sterols

A wide range of sterols, ranging in carbon number from C_{26} to C_{30}, occur in the neutral fraction of the extracted lipids; cholesta-5,22-dien-3β-ol, cholest-5-en-3β-ol (cholesterol), 24-methylcholesta-5,22-dien-3β-ol and 24-methylcholesta-5,24-dien-3β-ol are typically dominant. Two unidentified C_{28} and C_{30} sterols are also present (data not shown). Cholesterol concentrations ranged from trace amounts in S1N7 to 200 μg g^{-1} dry weight in S3TA7 (Table 5.3). The summed concentration of the other sterols ranged from trace in S1N7 to 850 μg g^{-1} dry weight in S3TA7. The presence of cholesta-5,22-dien-3β-ol and cholesterol in marine samples is commonly attributed to input from zooplankton (Volkman 1986, Killops & Killops 2005), whereas 24-methylcholesta-5,22-dien-3β-ol and 24-methylcholesta-5,24-dien-3β-ol are dominant in different phytoplankton classes including diatoms (Volkman 1986). C_{30} sterols, specifically 4-methylsterols, are thought to generally derive from dinoflagellates (Nichols et al. 1984).

5.5.6. Other lipids

Archaeal lipids, such as archaeol, hydroxyarchaeaol and pentamethylicosene (PMI) and associated saturated analogues, were not detected in any of the samples analyzed, suggesting minimal archaeal, and specifically methanogen, contributons. In contrast archaeol was detected in marine particles in both oxic and anoxic environments (Turich et al. 2007). In addition glycerol dialkyl glycerol tetraethers (GDGTs), which

are also archaeal membrane lipids (DeLong & Karl 2006) were also found in marine particles (Hoefs et al. 1997, Turich et al. 2007). Archaea are now known to account for a significant proportion of microbial biomass in the marine water column (Karner et al. 2001) and play key roles in biogeochemical processes such as nitrification (Konneke et al. 2005) and methanogenesis.

5.5.7 Summary of likely sources for lipids in artificial aggregates

The dominant FA in all three aggregates at all three time intervals were the C16:0, C16:1 and C18:1 components. This likely reflects the phytoplankton origin of the aggregates (Wakeham 1995, Canuel 2001). In a simulated phytoplankton bloom in the coastal North Pacific a dominance of C14:0, C16:0, C16:1 and C20:5 FA was associated with the initial diatom bloom, whereas the successive dominance of C18:1, C18:2, C18:3 and C22:6 were associated with a second bloom driven by dinoflagellates (Hayakawa et al. 1996b). Diatoms do contain higher amounts of C14:0 and C16:1 compared to other algal groups including dinoflagellates, whereas the proportion of C18:1 is variable among dinoflagellates (Volkman et al. 1989, Viso & Marty 1993, Dijkman & Kromkamp 2006). The high proportion of C16:1 in aggregates S1TA7 is consistent with the abundance of diatoms in that sample, and the high proportions of C14:0 and C16:1 in aggregates S1N7 and S1N10 suggest that diatoms might also be abundant in these aggregates. However C16:1, C16:0 and C18:1 are also the dominant FA in bacteria isolated from mucilaginous aggregates and morphologically identified as α- and γ-*Proteobacteria* (Blazina et al. 2005); the same FA are also abundant in *Planctomycetes* (Schlesner et al. 2004) which are also present in marine aggregates (DeLong et al. 1993, Allgaier & Grossart 2006). *Planctomycetes*, α- and γ-*Proteobacteria* have been detected in the artificial aggregates studied for the present thesis (Tables 3.3, 4.7, 5.2, Balzano et al. 2009), are thus, additional sources of the C16:1, C16:0 and C18:1 measured.

Table 5.3. Concentration (µg/g dry weight) of individual FA, alcohols and sterols in artificial aggregates.

Sample *	S1N7	S2N7	S3N7	S1N10	S2N10	S3N10	S1TA7	S2TA7	S3TA7
Total FA **	**3900**	**2100**	**76**	**2100**	**370**	**410**	**1200**	**420**	**560**
C14:0	170	75		110	0.4	3.5	16	1.7	3.0
C15:0	27	14		22	0.9	2.9	8.2	2.1	2.4
C16:0	1900	1000	4.5	1100	62.0	120	380	130	130
C17:0	5.9	10.0		4.6	3.4	7.4	5.4	2.2	3.1
C18:0	99	83	11	58	32	35	100	33	43
C19:0		tr	0.6		1.5	1.2			
C20:0	30	31	2.7	7.8	6.8	5.4	4.4	5.8	4.7
nSCFA	**2200**	**1200**	**19**	**1300**	**100**	**180**	**520**	**180**	**180**
C22:0	32	51	8.1	5.9	13	15	12	7.1	8.7
C23:0					2.0	2.7			
C24:0	73	110	20	26	28	20	130	69	94
C26:0	2.4	9.2	1.7	1.8	3.1	3.4	32	19	37
C28:0					tr	0.8			1.7
nLCFA	**110**	**180**	**30**	**34**	**46**	**42**	**170**	**95**	**140**
C15:0*i*	18	19		19	1.0	9.2	16	4.1	7.2
C15:0*a*		3.9				1.1	5.4	1.2	3.0
C16:0i	4.1	4.2		1.3	0.3	5.2	3.9	2.0	2.9
C17:0br						2.5			
C17:0*i*	7.5	7.8		14	4.5	12	3.2	2.2	2.6
C17:0*a*		10		4.5	4.9	8.6	0.7	0.8	1.6
C19:0*i*	tr	tr	0.2		tr	0.9			0.6
C19:0*a*		tr			1.0	0.4			

C21:0*br*					1.5	0.4			
C21:0*i*	3.4	8.9	2.8		7.0	2.6	1.8	0.8	2.9
C23:0*i*	15	26.0	4.3		1.2	1.3	tr	1.1	0.9
C23:0*a*						0.3			
C25:0*iso*		9.1			1.9	0.4		tr	0.9
Me11,C18:1 ǂ	9.5	21	2.0	2.3	1.3	1.4	60	33	28
brFA	**47**	**89**	**7.3**	**38**	**23**	**45**	**31**	**12**	**23**
C16:1	970	280		490	15	24	100	21	40
C17:1						0.6	tr	tr	0.5
C18:1	370	190	9.1	190	100	100	250	60	95
C19:1	9.5	21	2.0	2.3	1.3	1.4	61	33	28
C20:1	19	19			0.7		9.7	2.8	5.2
C22:1		17			6.2	3.8	7.3	1.0	7.6
C24:1	7.0	10	4.2	5.0	8.5	9.1	25	9.5	14
C26:1		Tr			0.4				
MUFA	**1400**	**550**	**15**	**690**	**130**	**140**	**450**	**130**	**190**
C16:x	5.6	4.6				1.0	6.0	0.3	1.7
C18:3					3.2	1.9			
C18:2	52	18	1.5	6.1	8.9	6.2	6.2	2.4	4.0
C20:x	14	15	2.7	tr	9.8	1.6	5.7	1.4	12
C20:2	36	27							
PUFA	**110**	**64**	**4.2**	**6.1**	**22**	**11**	**18**	**4.2**	**18**
C12:0-β-OH							0.3	0.2	
C14:0-β-OH		1.5					0.7	0.3	
C15:0-β-OH		0.1					0.1	0.4	
C16:0-β-OH		0.5					0.2	0.5	
C17:0-β-OH		0.8					0.3	0.2	
C18:0-β-OH		0.2					0.4	0.5	
β-OH-FA		**3.2**					**1.9**	**2.1**	
C32HA	2.4	0.2	0.3	tr	0.4	tr	0.8	1.6	4.6
Phytol	17	24	98	490	22	160	70	10	6.7
n-alkanols	24	3.8	2.6	13	tr	4.2	12	0.3	
Cholesterol	tr	43	68	150	38	24	77	140	200
Other sterols		41	48	380	36	28	160	530	850

* Note that S1, S2 and S3 correspond to the time point at which aggregates were subsampled from the mesocosms and analysed. S1 = day 0; S2 = day 5; S3 = day 10. S1 time points (S1N7, S1N10 and S1TA7*)* represent aggregates collected on day 0 but already pre-degraded prior to the start of the incubation.

** Key for the compounds abbreviations: Cx:y where x denotes the carbon number and y the degree of unsaturation of the FA. *i = iso* branched FA. *a = anteiso* branched FA. br = branched FA with unidentified branching point. *n*SCFA, straight short-chain saturated FA (C_{12} to C_{20}); *n*LCFA, long-chain saturated FA (> C_{20}); MUFA, monounsaturated FA; PUFA, polyunsaturated FA; brFA, branched saturated FA; Cx:0-β-OH, β-hydroxi FA , where x denotes the number of carbons; C32HA, bishomohopanoic acid;

$^{+}$ 11-methyloctadecenoic acid

5.6. Variations among aggregates at start of incubation

The seawater-derived aggregates, S1N7 and S1N10, had higher total fatty acid concentrations, represented primarily by higher abundances of short-chain FA, including C14:0 and MUFA (Table 5.3). Concentrations of branched FA (brFA) were similar in all of the initial samples (Fig. 5.4).

5.6.1. Short-chain and Monounsaturated fatty acids

The high concentrations of the C14:0 and C16:1 FA in S1N7 and S1N10 might reflect the dominance of diatoms (Volkman et al. 1989, Dunstan et al. 1993, Dijkman & Kromkamp 2006), also observed by light microscopy for both S1N7 and S1N10 prior to aggregation. The lower concentrations of C14:0 and C16:1 for S1TA7 compared to S1N7 and S1N10 might be due to either a lower contribution of diatoms (due to mixing of diatoms and dinoflagellates) or a higher degradation of OM. However, the very low concentration of C14:0 found in S1TA7 (Fig. 5.5) is also unexpected, given the typical fatty acid composition of both *Thalassiosita* and *Alexandrium* species (Volkman et al. 1989, Dunstan et al. 1993, Viso & Marty 1993, Cho et al. 2001, Dijkman & Kromkamp 2006); these previously reported fatty acid compositions typically refer to strains harvested during the exponential phase of their growth, whereas in the present work cells were harvested from senescent cultures. In contrast the C18:1 content of S1TA7 is far higher than that typically reported in both *T. weissflogii* and *A. tamarense* (Volkman et al. 1989, Dunstan et al. 1993, Viso & Marty 1993, Cho et al. 2001, Dijkman & Kromkamp 2006), and it might derive from bacteria and/or zooplankton (Lee et al. 1971, Sargent 1976, Parkes & Taylor 1983, Morris et al. 1985). All of these observations, as well as the low concentrations of PUFAs, suggest that significant

degradation of algal materials had occurred prior to aggregation experiments, particularly in TA7. The presence of cholesterol in S1TA7 and S1N10 (Fig. 5.5) further suggests an input from eukaryotic zooplankton in the pre-aggregation materials for both experiments (Volkman 1986, Killops & Killops 2005). In contrast S1N7 did not contain any cholesterol (Table 5.3) and also had the highest concentrations of *n*SCFAs, MUFAs and PUFAs, suggesting the least degradation prior to aggregate formation.

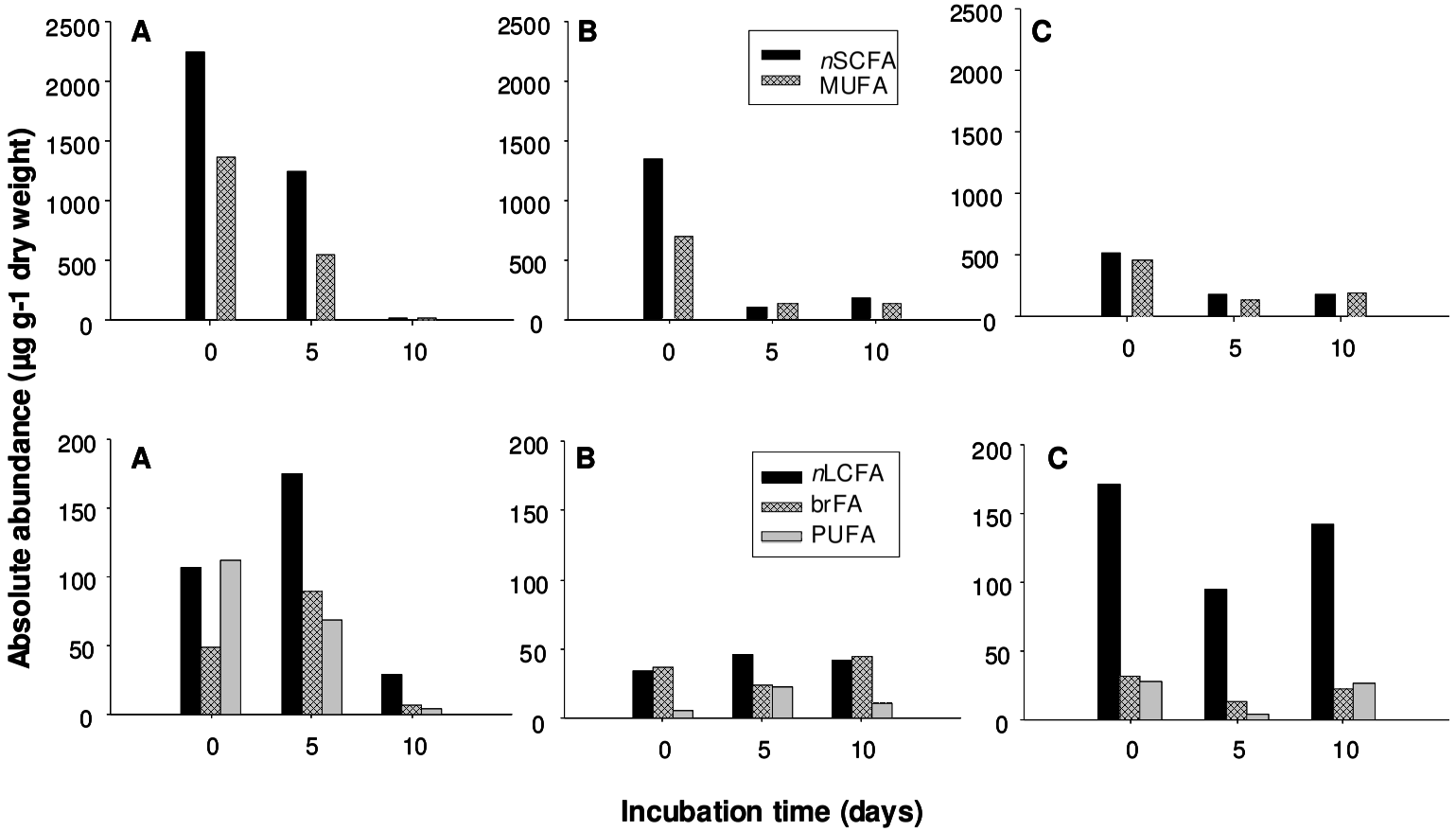


Figure 5.4. Distribution of *n*SCFA, MUFA, *n*LCFA), brFA and PUFA in aggregates N7 (**A**), N10 (**B**) and TA7 (**C**). S1 time points (S1N7, S1N10 and S1TA7) represent aggregates collected at the start of the incubations which were already pre-degraded prior to aggregation experiment.

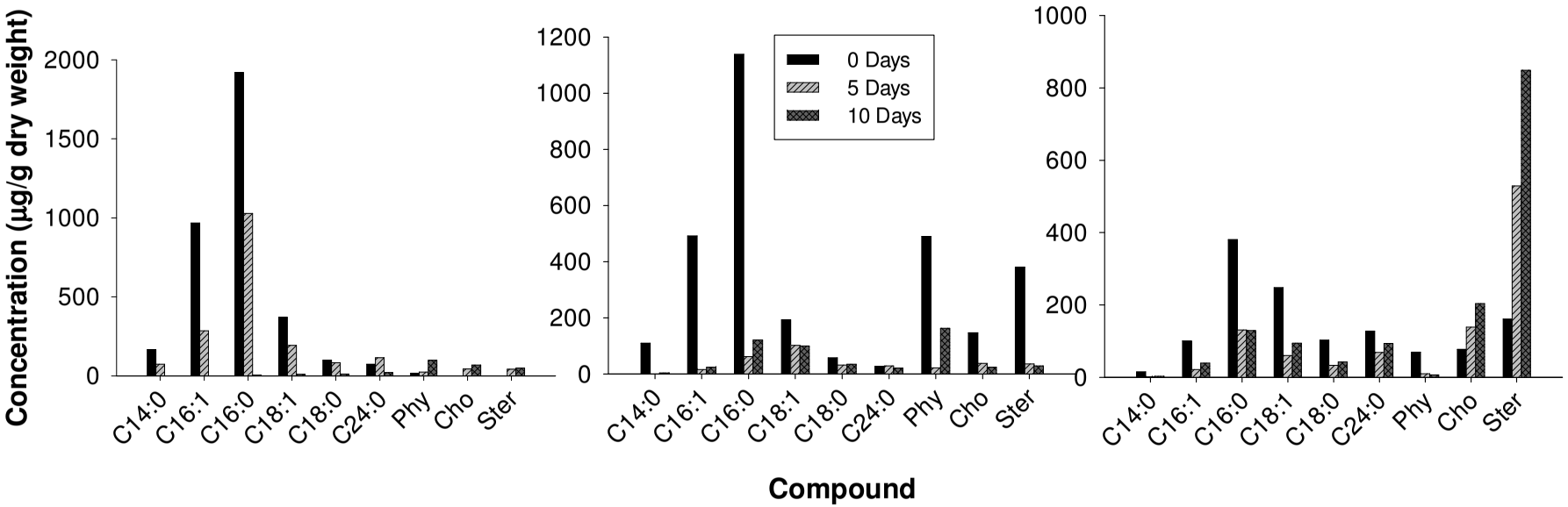


Figure 5.5. Changes in the concentration of major lipids present in the artificial aggregates (**A**) N7, (**B**) N10 and (**C**) TA7 during the degradation experiments. Phy = phytol; Cho = cholesterol; Ster =other sterols.

5.6.2 Algal fatty acid ratios

Various fatty acid ratios, representing the freshness and relative diatom abundance of marine particles (Claustre et al. 1989, Mayzaud et al. 1989, Najdek 1996, Najdek et al. 2002, Blazina et al. 2005), have been calculated for the fresh aggregates studied in the present chapter (shown and defined in Table 5.4). All except the C16:1/C18:1 ratio were similar in the initial aggregates (Table 5.4); the C16:1/C18:1 ratio was much lower in S1TA7 compared to S1N7 and S1N10, due to the higher concentration of C18:1 likely derived from zooplankton and/or bacteria (Table 5.3). The diatom ratio [(C14:0+C16:1+C16:2+C16:3)/C16:0], indicates the relative proportion of diatom biomass in respect to other phytoplankton classes (Najdek et al. 2002). The diatom ratios obtained here (Table 5.4) are similar to those calculated for many natural aggregates (Wakeham et al. 1997a, Kiriakoulakis et al. 2001, Marchand et al. 2005). However, higher diatom ratios have been reported for mucilaginous flocs in the Adriatic Sea (Blazina et al. 2005) and sinking aggregates collected from surface waters (Hayakawa et al. 1996a, Tolosa et al. 2004). Diatom ratios higher than one indicate the prevalence of diatoms over other algae (Leveille et al. 1997), however this ratio may be strongly affected by the proportion of unsaturated over saturated components. The low values of the diatom ratio found in artificial aggregates are likely to be associated with the scarcity of unsaturated over saturated components rather than the lack of a diatom dominance.

The ratio of unsaturated to saturated FA (UNS/SAT) is thought to reflect the degree of OM degradation (Reemtsma et al. 1990); the UNS/SAT ratios calculated for S1N7, S1N10 and S1TA7 are similar to those calculated for natural aggregates (Hayakawa et al. 1996a, Wakeham et al. 1997a, Kiriakoulakis et al. 2001, Blazina et al. 2005) (Table 5.4). Higher ratios have been observed in particles collected from upper waters, including sinking particles collected in winter from the surface of the Southwest Mediterranean Sea (Tolosa et al. 2004). In all cases the particles analysed were reported to be labile, in contrast refractory sinking particles collected in summer from Northwest Mediterranean Sea showed a lower UNS/SAT ratio (Marchand et al. 2005) (Table 5.4).

The ratios C16:1/C16:0 and C16:1/C18:1 have been suggested to reflect the growth stage and conditions of the diatoms in aggregates (Claustre et al. 1989, Mayzaud et al. 1989, Najdek 1996). The C16:1/C16:0 ratio in artificial aggregates is also similar to that calculated for most natural aggregates (Table 5.4). Higher values have been calculated for particles collected from the surface of Southern ocean (Table 5.4) which

are likely to have a high diatom content (consistent with higher diatom ratios). The ratios are also higher (>1) in healthy diatoms harvested from exponentially growing cultures (Volkman et al. 1989, Dunstan et al. 1993, Najdek 1996, Dijkman & Kromkamp 2006), such that the values (<1) observed here and for natural particles probably indicate formation of aggregates from senescent and/or degrading diatoms.

The C16:1/C18:1 ratio is highly variable in artificial as well as natural aggregates (Table 5.4) (Najdek 1996). Values higher than those found for S1N7 and S1N10 (2.6) occurred in Southern Ocean particles (Hayakawa et al. 1996a). In contrast, equatorial Pacific aggregates (Wakeham et al. 1997a) and summer Mediterranean aggregates (Marchand et al. 2005) have low C16:1/C18:1 ratios similar to those found for S1TA7 (Table 5.4). The variability of this ratio among natural aggregates is likely to be associated with the variability of C18:1 sources, with zooplankton and bacteria considered important contributors (Lee et al. 1971, Sargent 1976, Parkes & Taylor 1983).

Table 5.4. Significant fatty acid ratios calculated for the aggregates analyzed in the present work at the start of incubations and in natural aggregates investigated in previous studies.

Sample	Type of aggregates	Depth and Provenance	Diatom ratio [a]	UNS/SAT [b]	C16:1/C16:0 [c]	C16:1/C18:1 [c]	Bacterial growth [d]	Odd/Even [e]	Reference
S1N7	Artificial aggregates	Southampton Water	0.6	0.6	0.5	2.6	0.7	0.02	This study
S1N10	Artificial aggregates	Southampton Water	0.5	0.5	0.4	2.6	0.8	0.03	Thisstudy
S1TA7	Artificial aggregates	Algal cultures	0.3	0.7	0.3	0.4	2.7	0.09	This study
BI1	Manually collected fresh mucilaginous aggregates	Surface Adriatic Sea	1.5 ± 0.8	1.2 ± 0.9	0.8 ± 0.8	1.1 ± 0.5	3.1 ± 2.5	0.04 ± 0.02	Blazina et al. 2006
BI2	Manually collected aging mucilaginous aggregates	Surface Adriatic Sea	1.4 ± 0.2	0.8 ± 0.1	0.7 ± 0.1	2.1 ± 1.2	3.1 ± 1.1	0.09 ± 0.03	Blazina et al. 2006
BI3	Manually collected aged mucilaginous aggregates	Surface Adriatic Sea	1.9 ± 0.6	0.8 ± 0.2	0.9 ± 0.3	1.8 ± 0.4	6.5 ± 2.8	0.22 ± 0.02	Blazina et al. 2006
Wak1	26 µm filtered material	Surface Equatorial Pacific	0.5	1.2	0.2	0.4	1.0	0.08	Wakeham et al. 1997
Wak2	Sediment trap material	105m Equatorial Pacific	0.4	0.8	0.2	0.5	0.8	0.09	Wakeham et al. 1997

Wak3	Sediment trap material	1000m Equatorial Pacific	0.4	0.6	0.1	0.2	1.0	0.11	Wakeham et al. 1997
Wak4	Sediment trap material	3500m Equatorial Pacific	0.6	1.3	0.3	0.2	1.0	0.08	Wakeham et al. 1997
Wak5	Seafloor settled phytodetritus	4350m Equatorial Pacific	0.9	0.6	0.6	0.6	4.5	0.26	Wakeham et al. 1997
Kir1	Sediment trap material	1000m North Atlantic	0.5	2.1	0.3	0.1	n.a.	0.0	Kiriakoulakis et al. 2001
Kir2	Sediment trap material	3000m North Atlantic	0.6	1.0	0.4	0.3	0.5	0.1	Kiriakoulakis et al. 2001
Kir3	Sediment trap material	4700m North Atlantic	0.6	0.5	0.4	0.8	1.2	0.2	Kiriakoulakis et al. 2001
Tol	Sediment trap material	100-300 m South West Mediterranean Sea	1.1 ± 0.2	1.2 ± 0.1	0.6 ± 0.1	1.0 ± 0.2	0.5 ± 0.1	0.09 ± 0.03	Tolosa *et al.* 2004
Mar1	Sediment trap material	200m North West Mediterranean Sea	0.7 ± 0.4	0.3 ± 0.1	0.2 ± 0.1	0.5 ± 0.3	0.7 ± 0.1	0.06 ± 0.01	Marchand *et al.* 2005
Mar2	Sediment trap material	1000m North West Mediterranean Sea	0.5 ± 0.2	0.2 ± 0.02	0.2 ± 0.1	0.7 ± 0.4	0.7 ± 0.2	0.09 ± 0.03	Marchand *et al.* 2005
Hay	Sediment trap material	300m Southern Ocean	1.4	0.7	0.9	3.3	0.6	0.1	Hayakawa *et al.* 1996

[a] (C14:0+C16:1+C16:2+C16:3)/C16:0. The diatom ratio indicates the relative proportion of diatom biomass in respect to other phytoplankton classes, ratios higher than one indicate the prevalence of diatoms over other algae (Leveille et al. 1997; Najdek et al. 2002)

[b] ratio of unsaturated to saturated FA. It indicates the degree of OM degradation (Reemtsma et al. 1990)

[c] Both the ratios C16:1/C16:0 and C16:1/C18:1 reflect the growth stage and conditions of the diatoms in aggregates (Claustre et al. 1989, Mayzaud et al. 1989, Najdek 1996).

[d] Ratio between the sum of all branched C15:0 over *n*C15:0. Under favourable environmental conditions, bacteria yield higher proportions of branched-chain over straight-chain pentadecanoic fatty acid (Najdek et al. 2002; White et al. 1980).

[e] Ratio of the sum of all odd over all even FA.

5.6.3. Bacterial Lipids

Concentrations of SbrFA were similar for all three aggregates at the start of incubations (Fig. 5.6), suggesting comparable bacterial abundances (Parkes & Taylor 1983, Taylor & Parkes 1983, Kaneda 1991). In contrast to the SbrFAs, initial concentrations of hopanoic acids, LbrFA, mainly related to C21:0*iso* and C23:0*iso*, and Me11,C18:1 differed amongst the aggregates. LbrFA and hopanoic acid concentrations were higher in S1N7 compared to S1N10 and S1TA7 (Fig. 5.6), whereas Me11,C18:1 was more abundant in S1TA7 compared to S1N7 and S1N10 (Fig. 5.7). All are of likely bacterial origin (see above) such that these variations likely reflect differing initial bacterial assemblages.

5.6.4. Phytol and Sterols

Phytol was very abundant in S1N10, present in very low concentration in S1TA7 and nearly absent in S1N7 (Fig. 5.5). Cholesterol and other sterols were also more abundant in S1N10 compared to S1TA7 and S1N7 (Fig. 5.5). Because cholesterol is mainly produced by eukaryotic zooplankton (Killops & Killops 2005), the variable levels initially found in aggregates might reflect different abundances of heterotrophic eukaryotes in the samples prior to aggregation experiments. However, the cause of variations in algal-derived C_{28} and C_{29} sterol (Volkman 1986, 2003) concentrations is less clear, as they are essential components of eukaryotic cells and sterols in decaying phytodetritus do not seem to be completely depleted in few days (Harvey & Macko 1997). Thus, the cause of the variations in phytol and sterols appears to be related to different degrees of degradation; although the magnitude of the differences is surprising, it is consistent with the fact that the aggregate with lowest concentrations (S1N7) also has the highest C32HA concentrations.

5.7. Degradation of algal-derived lipids

The degradation of lipids as well as other organic compounds has been previously described for marine particles in natural systems (Wakeham et al. 1997b, Kiriakoulakis et al. 2001, Lee et al. 2004) and is mainly associated with the activity of heterotrophic microorganisms which consume and solubilise OM from particles (Kiorboe 2001, Beaulieu 2002). The present work confirms the occurrence of this process under laboratory controlled conditions, in the dark and in the absence of

microzooplankton and mesozooplankton (prior to phytoplankton cultivation Southampton Water was 10-µm filtered).

5.7.1. Degradation of nSCFA and MUFA

During the first 5 days of experiments, *n*SCFA and MUFA concentrations in all aggregates significantly decreased (Fig. 5.4); in N7 aggregates, concentrations continued to decrease over the next five days, whereas concentrations were relatively low and stable between days 5 and 10 in TA7 and N10. The decrease in *n*SCFA mainly reflected decreases in C14:0, C15:0 and C16:0 concentrations, whereas the loss of C18:0 was less dramatic (Fig. 5.5) and C20:0 concentrations did not change (Fig. 5.7). The decrease in MUFA concentrations over time also represents loss of low molecular weight components such as C16:1 and C18:1, with C16:1 concentrations decreasing more than those of C18:1 (Fig. 5.5). C20:1 concentrations varied but did not systematically decrease during the incubations (Fig. 5.7).

The temporal depletion in C16:0, C18:0, C16:1 and C18:1 has been observed by previous studies on natural sinking marine snow (Wakeham 1995, Wakeham et al. 1997a, Kiriakoulakis et al. 2001). However, those same studies revealed more complex behaviour for the C20 to C24 homologues: C20:0 and C22:1 concentrations decreased, whereas C20:1 and C24:1 concentrations exhibited a range of behaviour with depth (Wakeham 1995, Wakeham et al. 1997a, Kiriakoulakis et al. 2001), suggesting that the latter fatty acid might have been partially produced *in situ.* Consistent with this, in the current experiments, concentrations of long chain MUFAs such as C22:1 and C24:1 fluctuated over time (Fig. 5.7), suggesting that some MUFAs were produced *in situ* by heterotrophic microorganisms. All of these observations are consistent with previous work, showing that low molecular weight FA are more labile relative to higher molecular weight homologues in the natural environment (Wakeham et al. 1997a, Kiriakoulakis et al. 2001) as well as in the laboratory under oxic conditions (Harvey & Macko 1997, Sun & Wakeham 1999).

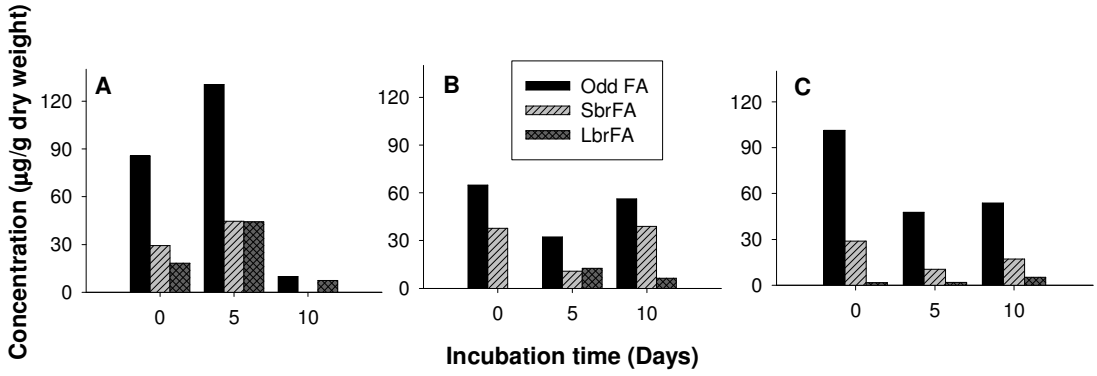


Figure 5.6. Concentration of total odd-carbon-numbered FA (Odd FA) and total brFA, including both short chain (C_{14-18}; SbrFA), and long chain (LbrFA, $>C_{18}$) homologues, in aggregates (**A**) N7, (**B**) N10 and (**C**) TA7.

5.7.2. Degradation of PUFAs

The concentration of total PUFA declined over time in N7 whereas it fluctuated in N10 and TA7, in N10 (Fig. 5.4). C16:x decreased over time in all the samples, C18:2 decreased over time in N7 and fluctuated in N10 and TA7, and C20:x exhibited a variable trend over time without being systematically depleted (Fig. 5.7). The variations found are not significant and the experiments were not carried out in triplicate, caution is thus needed when interpreting the results found. However the present results are in contrast with the quick depletion of PUFA with depth reported in marine particles (Wakeham & Lee 1993, Wakeham 1995, Wakeham et al. 1997a, Fileman et al. 1998, Kiriakoulakis et al. 2001). PUFA are consumed faster than MUFA and *n*SCFA (Lee et al. 2004) and can be also produced *in situ* by heterotrophic microorganisms. An important bacterial contribution in the PUFA present in marine environment has been recently suggested after different species were demonstrated to produce these FA (Nichols 2003). PUFA producing bacteria include several *Colwellia* species (Nichols 2003); one strain affiliated to *Colwellia* sp. is present in aggregates N1 (Balzano et al. 2009). However prokaryotic PUFA production in marine environments appears to be limited to extreme environments such as deep sea and sea ice (Nichols 2003) and was thus unlikely to occur in incubated artificial aggregates. In addition PUFA can also be produced by heterotrophic eukaryotes (Bowles et al. 1998).

5.7.3. Degradation of sterols and phytol

The concentration of phytol increased in N7 from 17 to 98 µg g^{-1} dry weight over 10 days whereas it decreased from 490 to 160 µg g^{-1} dry weight in N10 and from 70 to 7 µg g^{-1} dry weight in TA7 over the same time interval (Fig. 5.5). Sterols were not detected in S1N7 but were present in both S2N7 and S3N7 were an increase of both cholesterol and the summed concentration of the other sterols was registered (Fig. 5.5). Similarly cholesterol and the other sterols accumulated over time in aggregates TA7, whereas aggregates N10 were strongly depleted in all the sterols over time (Fig. 5.5).

The temporal increase in phytol found in N7 is contradictory with the depletion of phytol registered for N10 and TA7 and also described in previous studies for decaying phytodetritus (Harvey & Macko 1997, Sun et al. 2002). Phytol is a component of chlorophylls and is not produced under dark conditions where aggregates had been incubated. Sterols, being important component of cellular membranes can also be produced under dark conditions and its presence is mainly ascribable to eukaryotic microorganisms.

5.7.4 Long-chain fatty acids

The absolute abundance of *n*LCFA increased in N7 (excluding S3N7) and fluctuated in N10 and TA7 over time (Fig. 5.4). These variations are mainly related to C22:0, C24:0 and C26:0 (Figs 5.5, 5.7). The lack of high molecular weight *n*-alkanols suggests *n*LCFA were likely to be partially produced *in situ* during the dark incubation by small (<20µm) heterotrophic microorganisms associated with artificial aggregates. Long chain FA account for a low proportion (0.5-3%) of FA in eukaryotic membranes (Schweizer & Hofmann 2004). The nanoplankton community of Southampton Estuary appears to be dominated by Chrisomonads, Choanoflagellates and heterotrophic dinoflagellates with diatoms accounting for a smaller proportion (Tong 1997b, a, Brandt & Sleigh 2000), many of these microorganisms might have been present in the incubated aggregates and likely contributed to a proportion of the *n*LCFA found.

5.8 Changes in Bacterial Fatty Acids

5.8.1 Absolute abundances

In contrast to the sharp temporal decrease of total FA (Table 5.3), concentrations of putative bacterial FA were much less variable. Concentrations of odd-carbon-numbered FA, which have been invoked as predominantly of bacterial origin (Budge et al. 2001), increased in N7 (excluding S3N7), were relatively stable in N10 and slightly decreased in TA7 over time. This concentration reflected mainly odd SbrFA, odd LbrFA, Me11,C18:1 which are bacterial FA as well as *n*C15:0 and *n*C17:0 which may derive by diatoms (Viso & Marty 1993, Dijkman & Kromkamp 2006) and bacteria (Mayzaud et al. 1989, Najdek et al. 2002). The same trend was registered for SbrFA, reflecting mainly by C15:0*iso*, C16:0*iso* and C17:0*iso.* In contrast the concentration of LbrFA (reflecting mainly C21:0*iso* and C23:0*iso*) increased in N7 (excluding S3N7) and to a lesser degree in N10 and were constant in TA7 over time (Fig. 5.6). Concentrations of the bacterial marker Me11,C18:1 fluctuated in N7, whereas it decreased over time in N10 and TA7 (Fig. 5.7).

These changes record variable abundances and/or activities of aggregate-attached bacteria (Parkes & Taylor 1983, Taylor & Parkes 1983, Harwood & Russell 1984, Kaneda 1991). In particular, *Bacteroidetes,* an important class of bacteria attached to artificial (Balzano et al. 2009) and natural aggregates (Simon et al. 2002) contain high amounts of C15:0*iso*, C15:0*anteiso*, C16:0*iso*, C17:0*iso* and C17:0*anteiso* (Blazina et al. 2005), and Me11,C18:1 in artificial aggregates likely derives from α- and γ-*Proteobacteria* (Shirasaka et al. 1997, Rontani et al. 2005). However, the changes are relatively minor and could reflect either temporal processes or simply experimental variability.

5.8.2 Significant ratios

Clearer trends are revealed when putative bacterial fatty acid concentrations are normalised against FA of predominantly algal origin. Four ratios, SbrFA/(C16:0+C18:0), LbrFA/(C16:0+C18:0), the ratio of odd over even numbered FA (odd/even) and the bacterial growth ratio, were calculated and are shown an defined on Table 5.4 and Figs 5.8-5.9.

Under favourable environmental conditions, bacteria yield higher proportions of branched-chain over straight-chain C15:0, and the ratio between C15:0br/nC15:0 is

thus considered an indicator of bacterial growth (White et al. 1980, Najdek et al. 2002). This ratio was highest in TA7 aggregates when incubation started (Fig. 5.8) but increased over time in all aggregates (Fig. 5.8). The ratio of SbrFA to (nC16:0 + nC18:0) increased over time in N7 and was constant in N10 and TA7 (Fig. 5.8) whereas LbrFA to (nC16:0 + nC18:0) is constant over time (Fig. 5.9). As in most cases the ratios calculated were constants or slightly increased in TA7 and increased more significantly in N7 and N10 (Fig. 5.9), reflecting a relative increase (with respect with total FA) of bacterial fatty acid in N7 and N10, these differences likely reflect changes in bacterial abundance and activity being more constant in TA7 compared to N7 and N10. However the experiments were not carried out with replicates and these differences may simply reflect experimental variability.

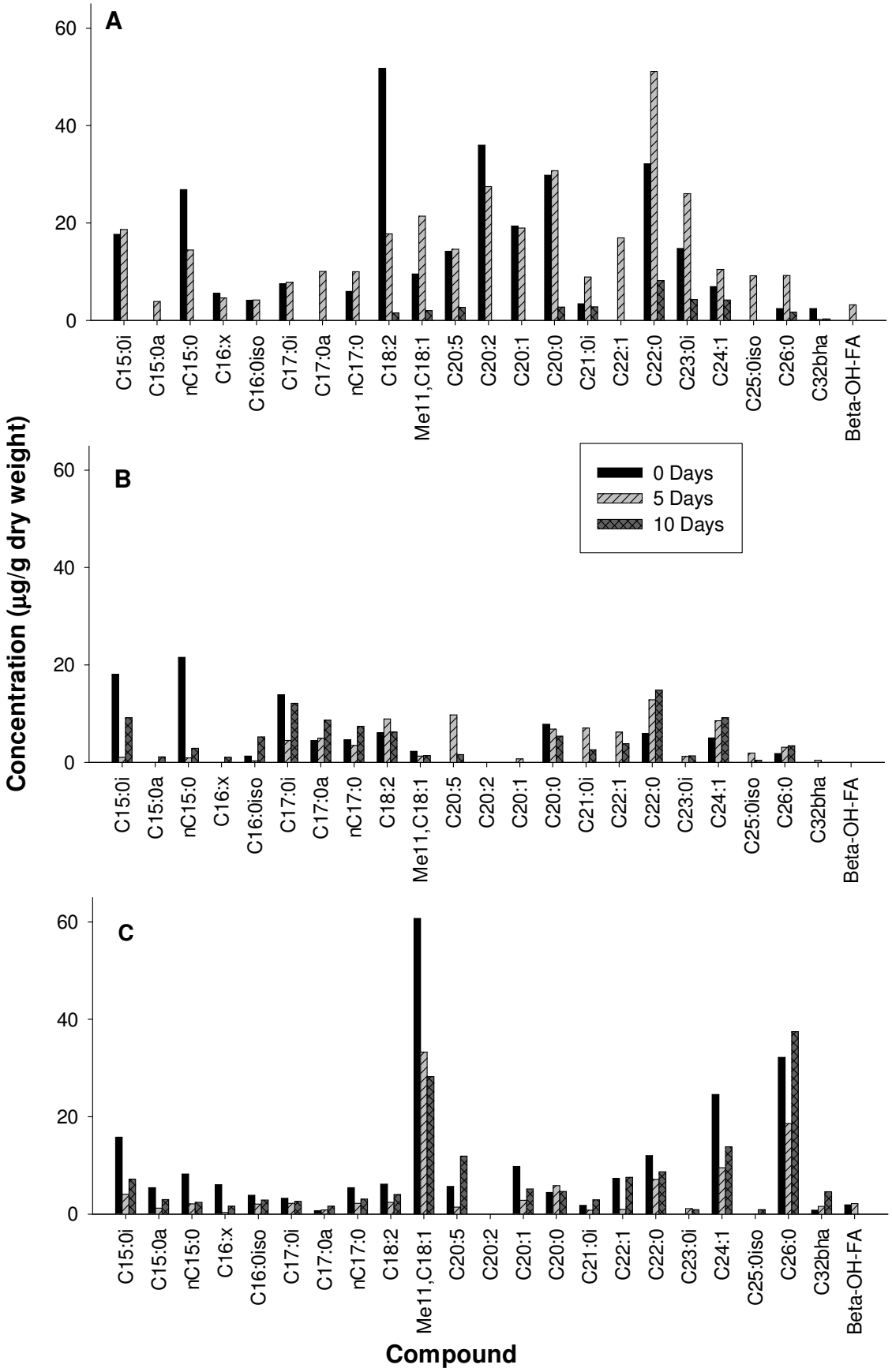


Figure 5.7. Changes in the concentration of selected minor lipids present in the artificial aggregates (**A**) N7, (**B**) N10 and (**C**) TA7 during the degradation experiments.

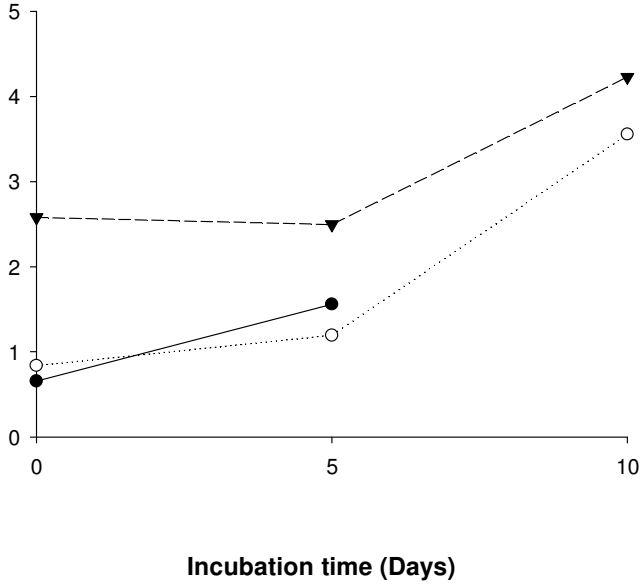


Figure 5.8. Trend of the bacterial growth ratio [(C15:0*iso* + C15:0*anteiso*)/nC15:0] calculated in aggregates during the dark incubation. S3N7 has been excluded because all odd-numbered FA were found in trace amounts. Note that sample S3N7 has been excluded as it exhibited a weak fatty acid profile.

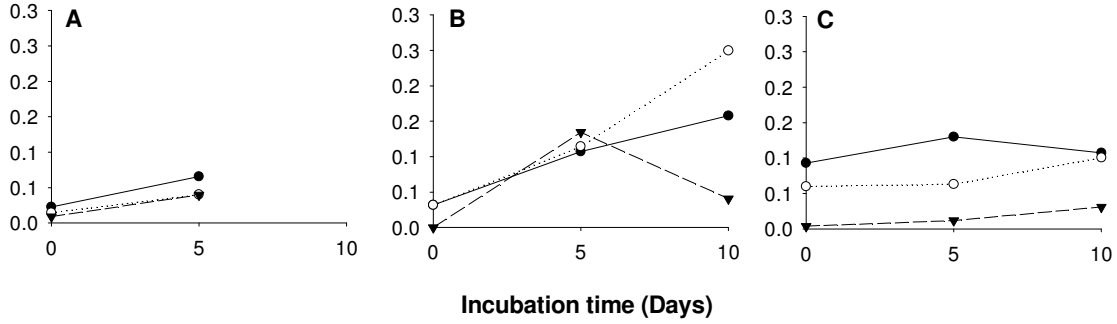


Figure 5.9. Ratios of (●)SbrFA/(nC16:0+nC18:0), (○),LbrFA/(nC16:0 + nC18:0) and (▼) sum of all odd over all even numbered FA in aggregates (**A**) N7, (**B**) N10 and (**C**) TA7. The three ratios were calculated in the present work to infer the proportion of each class of putative bacterial FA with respect with the two most abundant FA in marine OM (nC16:0 and nC18:0). S3N7 has been excluded because all odd-numbered FA were found in trace components.

5.9. Linking redox state of aggregates with lipid and bacterial composition

Consistent with previous studies on natural aggregates (DeLong et al. 1993, Rath et al. 1998, Grossart & Ploug 2000, Bidle & Azam 2001, Moeseneder et al. 2001, Simon et al. 2002, Selje & Simon 2003, Abell & Bowman 2005) the N1 and N10 aggregates were mainly colonised by oxic heterotrophs such as *Planctomycetes*, *α-Proteobacteria*, *γ-Proteobacteria, Bacteroidetes* and *Cyanobacteria* (Tables 3.2, 4.7, 5.2, Balzano et al. 2009). The TA1 and N1 aggregates released significant amounts of reduced iron, showed some nitrate uptake and were found to contain bacteria able to respire both NO_3^- and Fe(III), including *Desulfovibrio* and *Marinobacter* species (Balzano et al. 2009). A more recent study (Chapter 4) confirms that *Planctomycetes* and *Bacteroidetes* may dominate artificial aggregates (Table 4.7), highlights the presence of several dissimilatory Fe(III) and Mn(IV) reducers affiliated to γ- and δ-*Proteobacteria* (Table 4.8) and indicates that γ-*Proteobacteria* are likely to be active under redox changing conditions in controlling fermentation as well as Fe(III) and Mn(IV) reduction (Table 4.9). In particular facultative anaerobes belonging to the genera *Amphritea*, *Marinobacter* and *Marinobacterium* are present in artificial aggregates and are able to grow through Fe(III) and/or Mn(IV) reduction (Fig. 4.8).

The compositional changes in lipids observed in aggregates incubated in parallel with N1 and TA1, with *n*SCFA and MUFA being rapidly depleted and brFA accounting for a minimal proportion of total lipids (Fig. 5.4) suggest that most of OM degradation occurred under oxic rather than anoxic conditions (Wakeham 1995). Low molecular weight FA are consumed quicker than higher molecular weight homologues (Figs 5.5, 5.7). These results agree with previous studies on degradation of aggregates in the oxic water column (Wakeham et al. 1997a, Kiriakoulakis et al. 2001). Bacteria and picoeukaryotes were likely to be involved in the consumption of OM in aggregates. In contrast with previous studies (Wakeham et al. 1997a, Kiriakoulakis et al. 2001), artificial aggregates were not always depleted in PUFA which might have been partially synthesised by heterotrophic prokaryotes (Nichols 2003) or eukaryotes (Bowles et al. 1998) active during dark incubations.

The degradation of OM is controlled by its availability to microorganisms (labile vs. refractory) and strongly affects the oxygen consumption, the redox conditions of the environment and the cycling of nutrients and trace metals. In the present work phytodetritus was already significantly degraded prior to aggregation

experiments, and a consistent further consumption of labile components occurred during the first 5 days of incubation. Before aggregation experiments, and during the early stages of incubation, high amounts of labile OM were probably present and OM was likely to be rapidly consumed, preventing O_2 from being diffused back in aggregates and driving some internal microenvironments to anoxia. Under these conditions most of the dissolved Fe(II) measured (Balzano et al. 2009) was probably released. This is supported by the lack of anoxic microenvironments in aggregates N1 and TA1 and the dominance of aerobic heterotrophic bacteria in N1 at a middle stage (5 days) of incubation (Balzano et al. 2009). The competing rates of O_2 consumption by OM degradation over O_2 diffusion in artificial aggregates were thus dominated by the former, during the earlier stages of the experiments, and by the latter after day 5 of incubations. Proxies for methanogenesis were not found suggesting that although some circumscribed suboxic or anoxic microenvironments were likely to be present allowing Fe(III) reduction (Balzano et al. 2009), the highly reducing conditions required for methanogenesis did not occur in such microenvironments (Stumm & Morgan 1993).

5.10 Limitations in the present work

In the present study, different algal material was present in the three tanks (N7, N10 and TA7) which can not be considered replicates. Seawater collected in different days was used to form the enrichments N7 and N10, and although the same culturing conditions were applied, different microbial communities were likely to be present. Higher amounts of biomass were probably necessary to ascertain a complete study of OM in aggregates. Phytodetritus material prepared in a single large (10-20L) container should have been transferred to three replicate tanks prior to flocculation experiments.

The lipid composition of aggregates should have been assessed also during the cultivation of phytoplankton, before flocculation experiments started to understand the typical distribution of FA under non-senescent conditions. A precise microscopy-based study on the species distribution in the microcosm was also necessary to understand the phytoplankton distribution and succession during the simulated flocculation event and the species present in aggregates.

Lipids were extracted from aggregates (Section 2.9.1) using a method different from those applied in previous studies on marine particles. Frozen aggregates were thawed and then rinsed in lipid-free distilled water and centrifuged twice (to remove

salts). Although this methodology differs from that one applied in literature (e.g. Wakeham et al. 1997) it should not lead to ambiguous results. Although distilled water promoted the disruption of some of the cells present, FA are not hydro-soluble at circumneutral pH and a loss from the sample was unlikely to occur. However this technique likely underestimated the dry weight of aggregates because hydro-soluble components were lost. PLFA analyses would have allowed to promptly associate the FA found to microorganisms alive and active.

As different MUFA and PUFA isomers may have different origin, after lipid extraction samples should have been dimethyl disulfide adducted (Francis 1981), to locate the double bonds and distinguish the different isomers. For example C18:1ω9 derives mainly from eukaryotes (with zooplankton being an important contributor) whereas C18:1ω7 may also be produced by bacteria (Mayzaud et al. 1989, Tolosa et al. 2004).

5.11 Summary

Biochemical analysis permitted the assessment of compositional changes in lipids in degrading aggregates which were investigated for biogeochemical studies on iron dissolution (Balzano et al. 2009). Artificial aggregates incubated in the dark were rapidly depleted in *n*SCFA and MUFA (Fig. 5.4) including C16:1, C16:0, C18:1 and C18:0 (Fig. 5.5) similar compositional changes occur in natural sinking particles (Wakeham et al. 1997a, Kiriakoulakis et al. 2001). In contrast the abundances *n*LCFA, brFA and PUFA fluctuated over time suggesting that they were produced by heterotrophic microorganisms attached to the aggregates. The present work contributes to the increasing evidence in the literature that *n*LCFA in marine particles although often deriving from higher plants may also be produced by marine microorganisms. In addition LbrFAs which are poorly reported in the geochemical literature were also likely to be produced by heterotrophic microorganisms while aggregates were incubated.

Among the *n*LCFA producing bacteria, a *Vibrio* species was present in artificial aggregates formed following the same method as in the present work (Balzano et al. unpublished) has been also sequenced from natural aggregates (Bidle & Azam 2001). Moreover Me11,C18:1, detected in marine aggregates for the first time, can be produced by a range of bacteria including α- and *γ-Proteobacteria* which are common

in natural (Simon et al. 2002) and artificial marine aggregates (Balzano et al. 2009). In addition *Roseobacter litoralis*, a Me11,C18:1 containing α-*Proteobacteria* (Rontani et al. 2005) was sequenced from artificial aggregates analysed in the parallel study (Balzano et al. 2009). Further work, based on phytodetritus incubations with ^{13}C and/or PLFA analyses, is necessary for elucidating the production of *n*LCFA and LbrFA in decaying phytodetritus and assessing which heterotrophic microorganisms are directly involved with the processes.

The combination of molecular (16S rRNA) and lipid (FA) analysis is a good approach for the assessment of microbial diversity and the related biogeochemical processes. However most of the microorganisms present in seawater are uncultured (Rappe & Giovannoni 2003) and their fatty acid composition is not described in the literature. Although the dominant bacteria attached to artificial aggregates have been identified (Table 5.2, Balzano et al. 2009), the microorganisms affecting important biogeochemical processes and producing measurable specific lipid biomarkers are not necessarily dominant in a given environment being not detectable with a standard molecular analysis (PCR, clone library, sequencing). More detailed investigations including metagenomic techniques along with screening of larger libraries and the use of functional gene probes are necessary to help in associating specific microbes with biogeochemical processes.

Chapter 6. Effect of phytodetritus addition to sediment on release of reduced Mn and Fe

6.1 Introduction

6.1.1 Phytodetritus deposition on the seafloor

During the decay of a phytoplankton bloom, marine aggregates can sink and accumulate on the sea floor forming a phytodetritus layer. Phytodetritus accumulation has been observed in shallow and deep waters of all the world oceans (Beaulieu 2002). During phytodetritus deposition events, the OM consumption by aggregate-attached microorganisms and benthic communities, may drive the seafloor to anoxia (Beaulieu 2002).

The oxygen diffusion rate in phytodetritus was shown to be similar to that occurring in seawater (Ploug & Passow 2007), although the presence of oxygen diffusion barriers and small (<10 μm) suboxic or anoxic microenvironments have been recently suggested to exist in marine aggregates (Balzano et al. 2009). The macrofaunal activities and the granulometric composition are the main factors controlling the diffusion of oxygen into the sediment (Glud 2008), whereas the amount and lability of the OM present along with the microbial community composition affect the consumption of oxygen.

6.1.2 Sediment core studies

Several studies have been carried out using sediment cores collected *in situ* or prepared in the laboratory to investigate the impact of phytodetritus deposition on the seafloor. Under laboratory conditions sediment can be sieved and grains of a given size selected, to obtain a uniform layer. Moreover shells and benthic animals including polychaetes, which may introduce some experimental variability, can be removed. Sediment core microcosm studies were mainly focused on nutrient recycling (Hansen & Blackburn 1992, Conley & Johnstone 1995, Holmer 1996, Hansen & Kristensen 1998, Grenz et al. 2000). Similar studies have been also applied to investigate the solubility of iron and manganese in the sediment (Canfield 1989, Canfield et al. 1993).

6.1.3 Objectives

In the present chapter the impact of phytodetritus deposition onto the sediment surface on the dissolution of Fe and Mn in seawater was investigated. The study has two objectives: first to evaluate if the addition of phytodetritus to the sediment cores affects significantly the redox conditions of the surface sediments and therefore the dissolution of iron and manganese, second to evaluate the heterogeneity of releases of dissolved Fe and Mn occurring in replicate sediment cores amended with phytodetritus.

6.2 Experimental setting

The methods applied in the present chapter are given in detail in Section 2.7, and the experiments were carried out in collaboration with WB Homoky. The overall strategy was to monitor any metal release under oxic and then sub-oxic conditions. Briefly near shore sediment was collected from Southampton Estuary and homogenised by sieving. 4 cylindrical plastic tubes (6 cm in diameter) were then filled to a depth of 16 cm by sieved sediment and 300 mL Southampton Water was carefully added. Atmospheric air was sparged into the seawater overlying the sediment cores throughout the experiments, using an aquarium pump. Cores were pre-incubated for two weeks and then phytodetritus, obtained as described in Section 2.7.3, was added to cores CO1, CO2 and CO3, whereas the fourth core, CO4 served as control. Both the sediment and the phytodetritus were not disturbed by the active aeration. The cores were incubated at 20º C in the dark for 1 week (Fig. 6.1). The pH was measured regularly 1 cm below the water surface and 1 cm above the water-sediment interface. The concentrations of dissolved Fe(II), dissolved [Fe(II) + Fe(III)], dissolved Mn and the vertical oxygen profile across the sediment interface, were measured over time.

These experiments were repeated in October with the addition of phytodetritus: four sediment cores were prepared and pre-incubated as described above and in Section 2.7. At the start of the experiments aeration was stopped and phytodetritus added to CO5, CO6 and CO7, whereas CO8 was unamended and was used as control (Fig. 6.1).

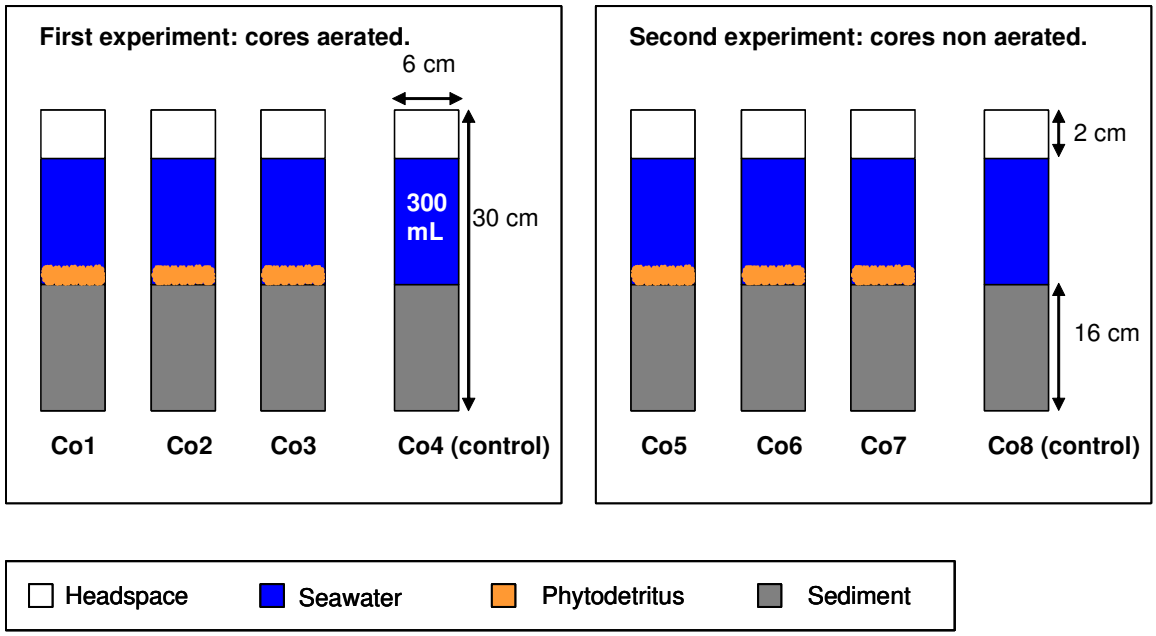


Figure 6.1. Experimental design of the sediment core microcosms used in the present study.

6.3 Results: first sediment core experiment

The first sediment core based experiments were carried out in July 2007. The cores were actively aerated to prevent the oxygen consumption from driving the system to anoxia. The pH ranged from 7.1 to 8.3 (Appendix 8), the pH measured 1 cm below the seawater surface was often slightly higher than that measured 1 cm above the sediment surface (Appendix 8). The lowest pH values were measured on day 3 in all the cores, whereas the highest pH values occurred on days 4 and 5 (Appendix 8).

6.3.1 Dissolved ferrous and ferric iron

The concentration of dissolved Fe(II) ranged from 61 ± 23 nM in CO3 to 480 ± 10 nM in CO1 (Fig. 6.2). The initial concentration of dissolved Fe(II), measured on day 0 before phytodetritus addition, was higher in CO4 than in the other cores (Fig. 6.2). The concentration of dissolved Fe(II) increased in CO1 and CO3, slightly increased in CO2 and fluctuated in CO4 over time (Fig. 6.2).

The concentration of dissolved [Fe(II) + Fe(III)] in the seawater overlying the

sediment cores ranged from 160 ± 20 nM in CO3 to 1100 ± 10 nM in CO1 (Fig. 6.2). The initial concentration of dissolved [Fe(II) + Fe(III)] was higher in CO4 compared to the other cores and it increased over time in CO1, CO2 and CO3 whereas it fluctuated in CO4 over time (Fig. 6.2), both for total Fe and for the dissolved Fe(II).

6.3.2 Dissolved manganese

The initial concentration of dissolved Mn was higher in CO4 than in the other cores throughout the experiments (Fig. 6.2) and ranged from 0.15 ± 0.01 µM in CO3 to 6.91 ± 0.03 µM in CO4 (Fig. 6.2). The concentration of dissolved manganese decreased over time in CO1, CO2 and CO3, whereas it decreased and then increased again in CO4 (Fig. 6.2).

6.3.3 Oxygen gradient

The concentration of dissolved oxygen was measured in the seawater overlying the sediment, in the phytodetritus layer and in the sediment using a Unisense microelectrode as described in Section 2.8.5. The conditions were deemed to be anoxic when the value measured was lower than the detection limit (~1µM) of the instrument.

The concentration of dissolved oxygen decreased from 200-250 µM in the seawater to 0 µM below the sediment surface, with most of the sediment being anoxic and most of the overlying seawater being well oxygenated (≥ 200 µM) throughout the experiments (Fig. 6.3). Oxygen was always present at the water-sediment interface with a concentration ranging from 10 µM on day 1 in CO1 to 200 µM on day 6 in CO2. The oxygen concentration at the water-sediment interface reached lowest values on day 1 (CO1, CO3 and CO4) or day 3 (CO2) (Fig. 6.3).

6.3.4 Diffusive boundary layer (DBL) and oxic/anoxic interface

The oxygen concentration initially varied across a DBL ranging from 3 to 6 mm (Fig. 6.6). The DBL became then 3 mm thick and did not change over time or between one core and another (Fig. 6.6).

The oxic/anoxic interface was found to be stable, at a few mm below the sediment surface, and its position did not change substantially among the cores and over time (Fig. 6.6).

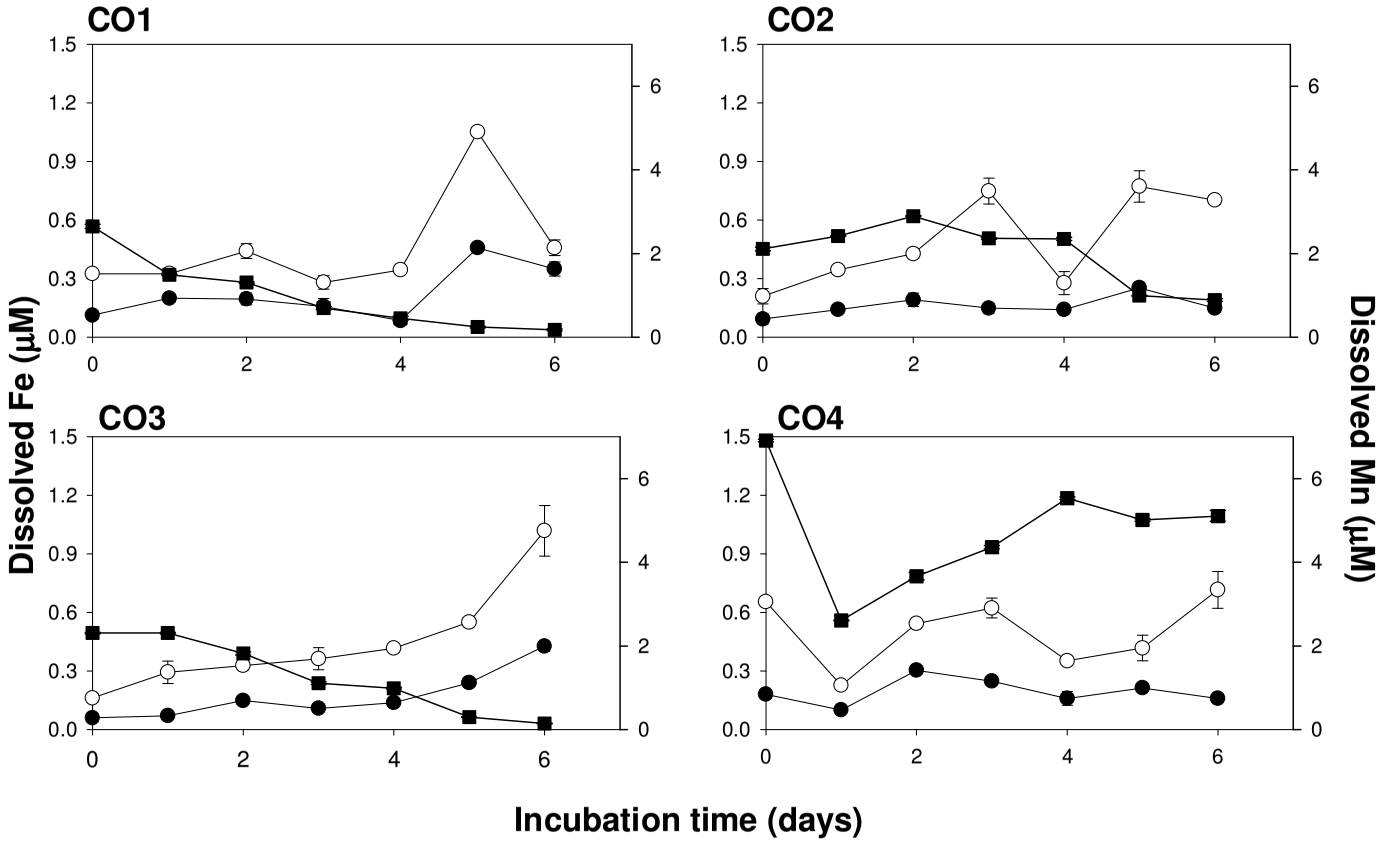


Figure 6.2. Concentration of (●) dissolved Fe(II), (○) dissolved [Fe(II) + Fe(III)] and (■) dissolved Mn in the seawater overlying the sediment cores during the first sediment core based experiment. Error bars show the standard deviations of triplicate measurements carried out on the same sediment core. If no bar is shown the standard deviation lay within the symbol size.

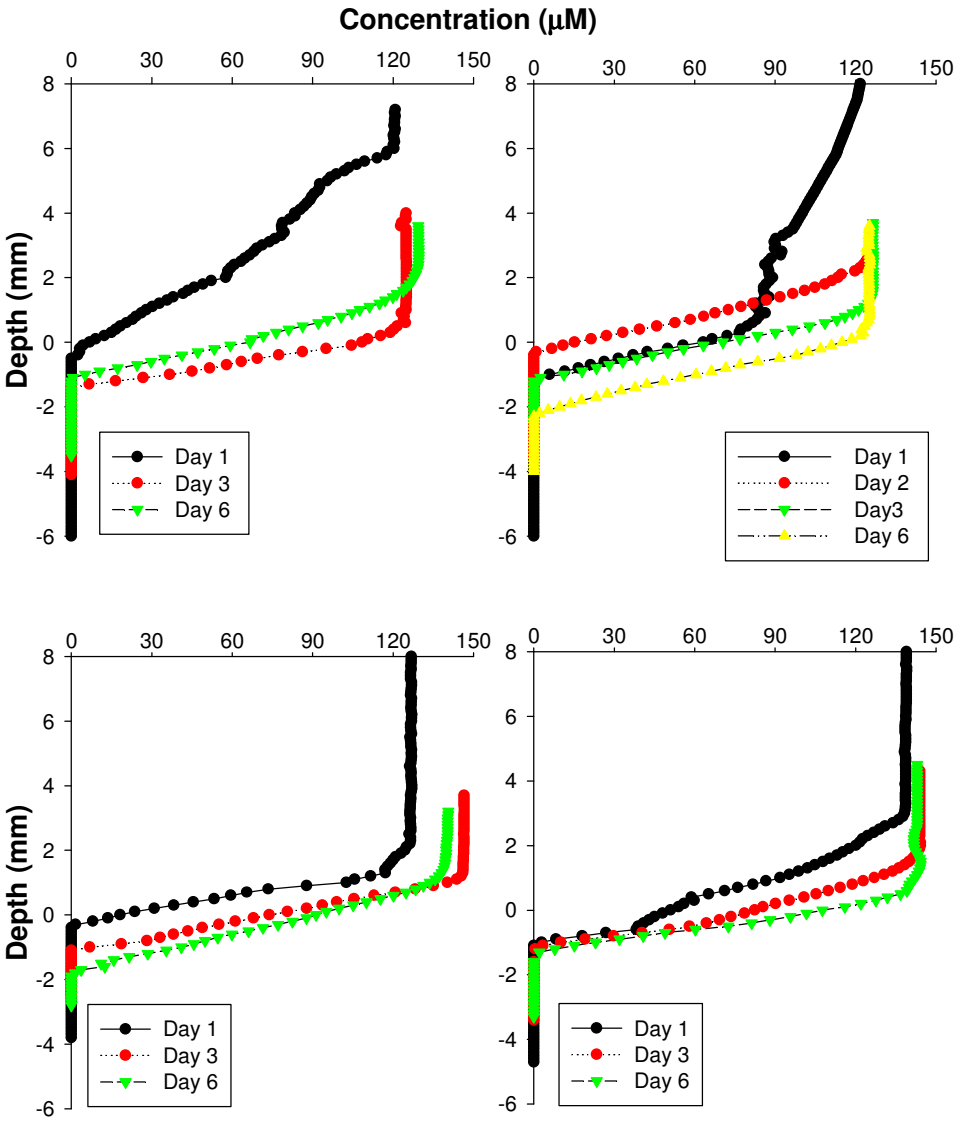


Figure 6.3. Evolution of the oxygen profile over time in the sediment cores studied in the first experiment. Depth zero is the water sediment interface.

6.4 Results: second sediment core experiment

During the first sediment core based experiment, the active aeration highly accelerated the oxygenation of the sediment, probably limiting the release of dissolved Fe and Mn in the phytodetritus-amended cores (Fig. 6.2). The short incubation time of the sediment cores might also have affected the biochemical transformations between particulate and dissolved metals (Fig. 6.2).

In the second experiment, cores were incubated without active aeration and for a longer (2 weeks) incubation period.

The pH measured 1 cm above the sediment surface was slightly lower than that measured 1 cm below the water surface, and the latter was closer to seawater values (8.0 to 8.2) (Appendix 8). The pH decreased in CO5, CO6 and CO7, whereas in CO8 it decreased until day 9 and then increased again over time.

6.4.1 Dissolved ferrous and ferric iron

The concentration of dissolved Fe(II) was initially higher in CO7 (0.52 ± 0.12 µM) compared to CO5 (0.25 ± 0.03 µM), CO6 (0.13 ± 0.02 µM) and CO8 (0.35 ± 0.05 µM). The concentration of dissolved Fe(II) ranged from 0.08 ± 0.03 nM in CO7 on day 4 to 3.9 ± 0.2 µM in CO5 on day 7 (Fig. 6.4). In CO5 the concentration of dissolved Fe(II) increased sharply over time from 0.31 ± 0.09 µM on day 4 to 3.9 ± 0.2 µM day 7 and then decreased again (Fig. 6.4). In CO6 and CO7 the concentration of dissolved Fe(II) initially decreased and then increased whereas in CO8 it was constant over time (Fig. 6.4).

The concentration of dissolved [Fe(II) + Fe(III)] was initially higher in CO7 (1.4 ± 0.2 µM) compared to CO5 (1.2 ± 0.15 µM), CO6 (0.8 ± 0.05 µM) and CO8 (1.1 ± 0.06 µM). The concentration of dissolved [Fe(II) + Fe(III)] ranged from 0.4 ± 0.03 µM in CO6 on day 7 to 4.4 ± 0.1 µM in CO5 on day 7 (Fig. 6.4). Dissolved [Fe(II) + Fe(III)] in CO5 was constant until day 4, then it increased very sharply until day 7 (4.4 ± 0.1 µM) and afterwards it decreased again towards the end of the experiments (Fig. 6.4). In CO6 the concentration of dissolved [Fe(II) + Fe(III)] decreased and then increased again over time (Fig. 6.4). Dissolved [Fe(II) + Fe(III)] decreased in CO7 and increased in CO8 over time throughout the experiments (Fig. 6.4).

6.4.2 Dissolved manganese

The initial concentration of dissolved manganese was similar in the water overlying the sediment cores and varied from 7.3 ± 0.54 to 8.80 ± 0.20 µM (Fig. 6.4). Dissolved manganese ranged from 3.9 ± 0.3 µM (CO6) to 12 ± 0.8 µM (CO5) (Fig. 6.4). The concentration of dissolved manganese in CO5 decreased initially, then increased from day 4 to day 7 (reaching 12.3 ± 0.8 µM) and decreased again towards the end of the experiments (Fig. 6.4). The concentration of dissolved manganese decreased in CO6 and was constant in CO7 and CO8, over time (Fig. 6.4).

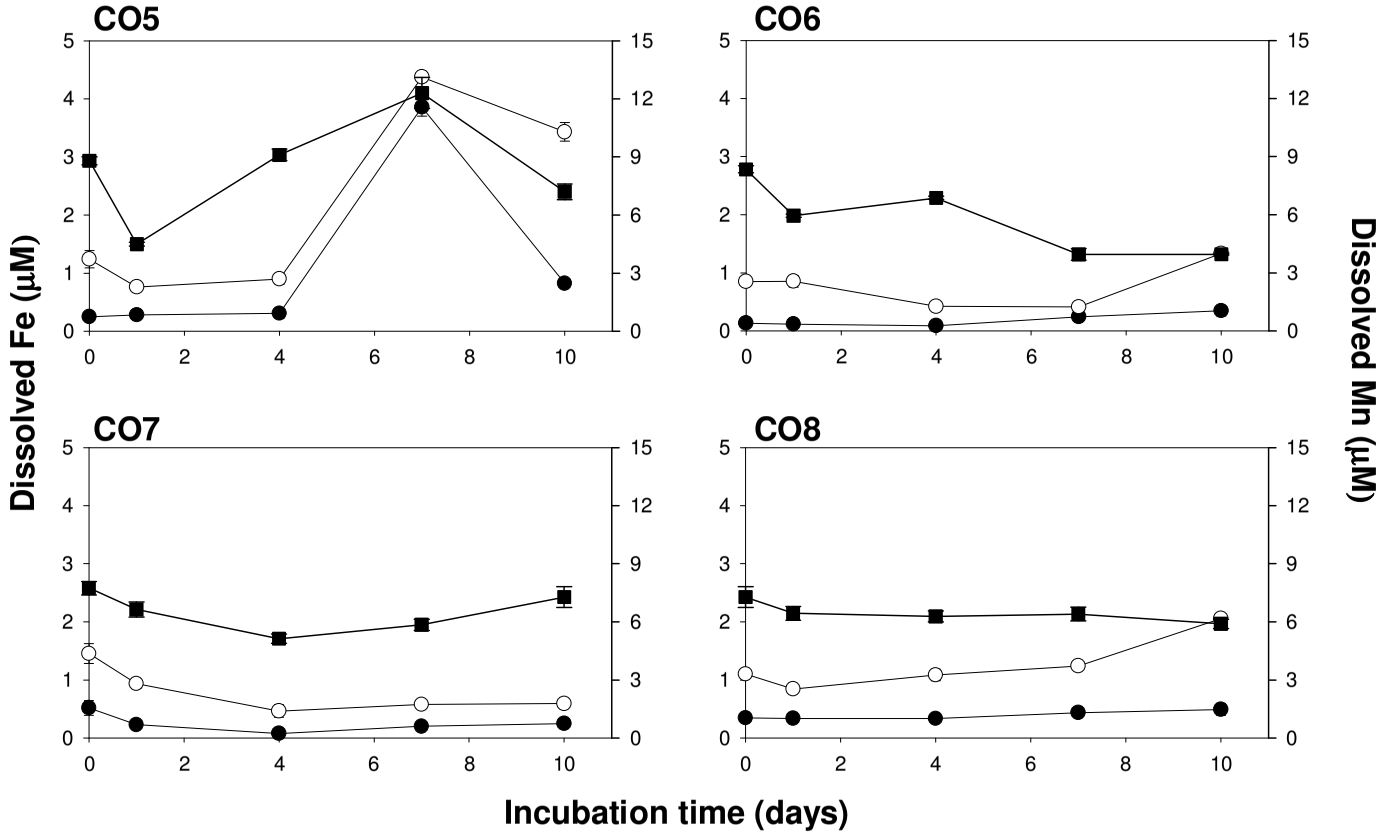


Figure 6.4. Concentration of (●) dissolved Fe(II), (○) dissolved [Fe(II)+ Fe(III)] and (■) total dissolved manganese in the seawater overlying the sediment cores during the second sediment core based experiment. Error bars show the standard deviations of triplicate measurements carried out on the same sediment core. If no bar is shown the standard deviation lay within the symbol size.

6.4.3 Oxygen gradient

The concentration of dissolved oxygen, measured in the 4 sediment cores, decreased from 180-220 µM in the seawater above the sediment to 0 µM below the sediment surface, with most of the sediment being anoxic and most of the overlying seawater being well oxygenated (≥160 µM). The vertical distribution of oxygen varied significantly over time and between one sediment core and another (Fig. 6.5). When experiments started oxygen was present to over 10 mm below the sediment surface in all the cores (Fig. 6.5).

The concentration of oxygen measured at the water-sediment interface was therefore highly variable, being fully oxygenated on day 0 in all the sediment cores and anoxic on day 2 in CO5 and CO6 and on day 6 in CO7 and CO8 (Fig. 6.5).

6.4.4 Diffusive boundary layer and Oxic/Anoxic interface

The DBL measured during the second experiment was found to be thicker than that measured for the first experiment (Fig. 6.6). In CO5 the DBL was 17 mm thick on day 0 and then decreased over time (Fig. 6.6). In CO6 and CO8 the DBL thickness also decreased over time whereas in CO7 it increased and then decreased again (Fig. 6.6).

The oxic/anoxic interface moved upward in CO5 until day 6, with more than 10 mm of seawater overlying the sediment being completely anoxic, and then it moved downward with oxygen being present below the sediment surface on day 9 and day 12 (Fig. 6.5). A similar trend was registered in CO6, CO7 and CO8: in CO6 the oxic/anoxic interface moved above sediment surface on day 2 and returned below the sediment surface afterwards (Fig. 6.5), and in CO7 and CO8 the oxic/anoxic interface was found above the sediment surface only on day 6 (Fig. 6.5).

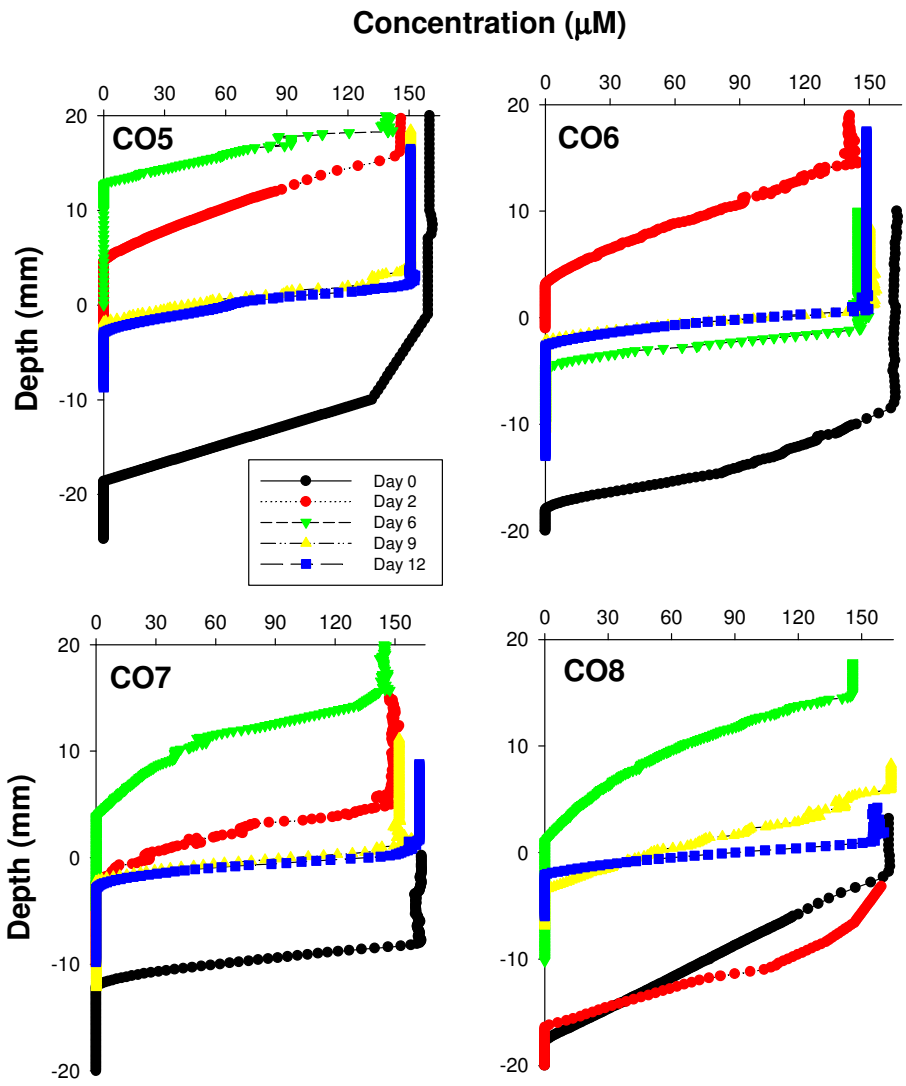


Figure 6.5. Evolution of the oxygen profile over time in the sediment cores studied in the second experiment. Depth zero is referred to the water sediment interface.

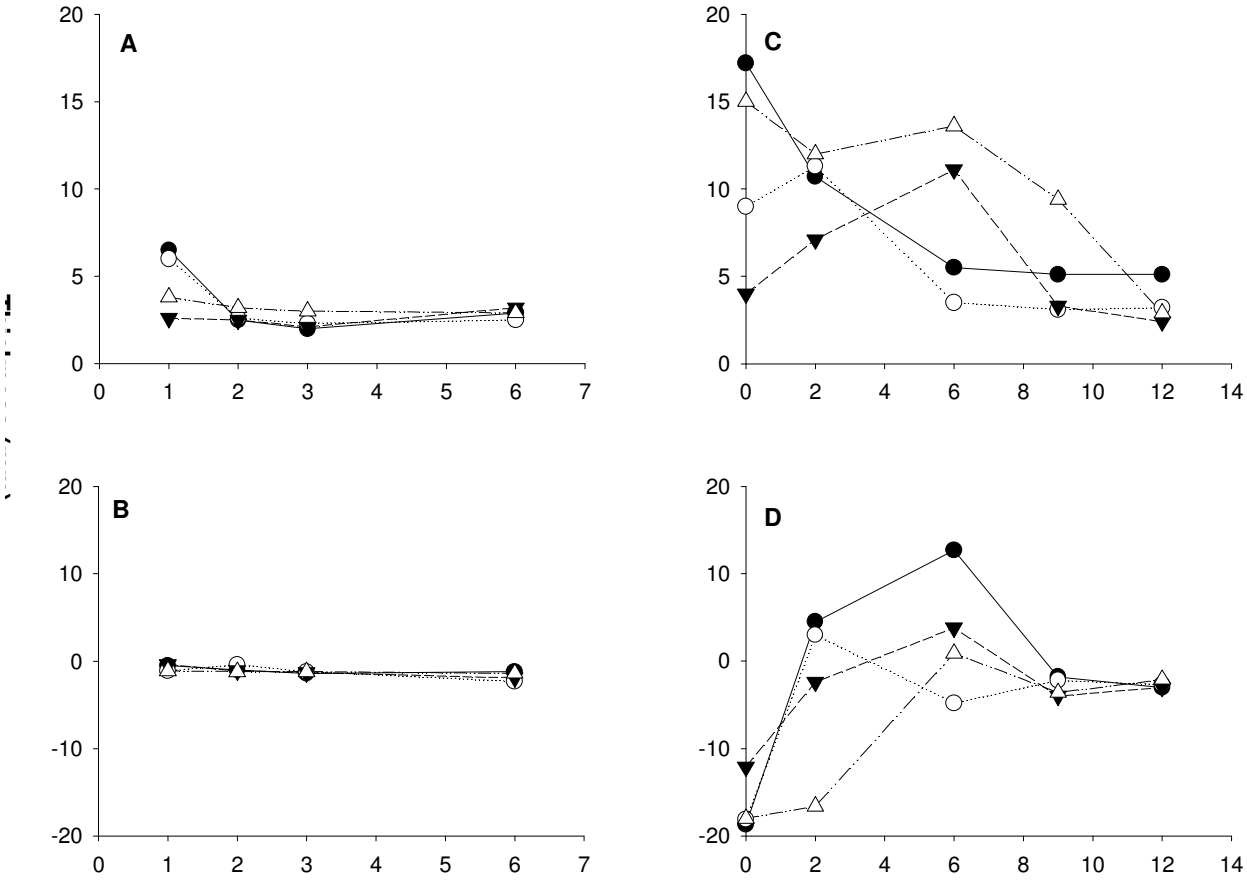


Figure 6.6. (A) Thickness of the diffusive boundary layer (DBL) for the oxygen and (**B**) position (relative to the sediment-water interface) of the oxic/anoxic interface in the sediment cores studied during the first experiment. Cores (●) Co1, (○) Co2, (▼) Co3 and (Δ) Co4. (**C**) Thickness of the DBL and (**D**) position of the oxic/anoxic interface in the sediment cores studied during the second experiment. Cores (●) Co5, (○) Co6, (▼) Co7 and (Δ) Co8. Note that the oxic/anoxic interface is defined as the point where the lowest detectable (≥ 1 μM) concentration of oxygen was measured.

6.3 Discussion

6.3.1 Diffusive Boundary Layer (DBL)

The behaviour of the DBL and the oxic/anoxic interface position suggest that the geochemical conditions were more stable when the cores were constantly aerated (first experiment), whereas in non aerated cores (second experiment) variable factors (see below) appear to control the consumption of OM.

6.3.2 Organic matter (OM) consumption

During the first experiments, the profile in dissolved oxygen was determined in the sediment cores after interruption of artificial bubbling to prevent disturbance in the measurements. After air bubbling was interrupted the Unisense microelectrode was rapidly deployed, however the sudden change in aeration might have altered the previously existing redox equilibrium. As a consequence the profile measured may not reflect a steady state related to previous aerating conditions.

The temporal variability in the oxygen profile measured in the cores (with and without phytodetritus added) indicates that the microbial processes occurring in the sediment dominated the oxygen consumption. The OM present in the sediment, rather than the phytodetritus, appears to be the main carbon source being oxidised. This is also confirmed by the similar pH values, registered for the phytodetritus-amended cores and the control cores (Appendix 8). Freshly formed aggregates obtained following the same method as the phytodetritus used here, were found to be depleted in labile fatty acid such as MUFA and PUFA (Chapter 5) suggesting that the OM present was already partially degraded and rather refractory.

The decrease of pH occurred in the second experiment on day 1 (Appendix 8) is likely associated with the respiration of OM which released CO_2. This hypothesis is supported by the lower pH values measured near the sediment surface compared to those found at the air-water interface (Appendix 8).

6.3.3 Redox state of the sediment cores

The similar trend in the concentration of dissolved Fe(II) (Figs. 6.2, 6.4) and the similar oxygen profile (Fig. 6.3, 6.5) measured in the cores with and without phytodetritus added for both experiments, indicate that the phytodetritus addition did not significantly affect the redox conditions of the cores. The presence of phytodetritus thus did not promote Fe(III) and Mn(IV) reduction in the cores. The sharp increase in dissolved Fe(II) and dissolved Mn registered between day 4 and day 7 (Fig. 6.4) in CO5 is associated with the fully anoxic conditions measured in the top 5-10 mm of seawater overlying the sediment on day 2 and day 6 (Fig. 6.5), and the subsequent decrease in dissolved Fe(II) (Fig. 6.4) is due to the aerobic conditions occurring on day 9 and day 12 in the seawater present in CO5 (Fig. 6.5).

Significant differences in the redox conditions occurred between the replicate cores, and thus led to the different concentrations in Fe(II) and Fe(III) measured.

Although equal volumes of homogenised phytodetritus were added to the replicate cores, different amounts of OM might have been present in the volumes added, leading to the supply of larger amounts of OM to CO5 compared to the other phytodetritus-amended cores.

6.3.4 Iron and manganese concentrations

The active aeration applied during the first experiment promoted the diffusion of oxygen by generating turbulence, and accelerated the oxidation of dissolved Fe(II), although its concentration was significant (100 to 300 nM) and was constant or even increased over time (Fig. 6.2). In the second experiment, higher concentrations of dissolved Fe(II), dissolved Fe(III) and dissolved Mn occurred (Fig. 6.4) compared to the first experiment (Fig. 6.2).

High levels of dissolved Fe(II) and dissolved Mn were present in all the waters overlying the sediment cores (Figs 6.2, 6.4), reflecting the high concentrations reported for Solent Water (Fang 1995) but there is also a role played by the sediment in maintaining these high concentrations. The release of reduced iron and manganese from aggregates demonstrated in the present research (Chapters 3-4, Balzano et al. 2009) appears to be contradictory to the lack of a significant release in dissolved Fe(II) and Mn to the seawater. The artificial aggregates studied previously (Chapter 3, Balzano et al. 2009) have been suggested to play a role in generating anoxic microenvironments and/or releasing Fe(II)-binding ligands. However in the present study, aggregates were mixed to obtain a homogeneous phytodetritus suspension, smaller particles were produced and anoxic microenvironments were thus unlikely to be generated, although the oxygen diffusion might have been limited within the phytodetritus layer formed. Moreover the addition of phytodetritus was not likely to significantly supply the sediment with bioavailable metals as higher concentrations of these are usually present in the sediment compared to seawater and OM.

In all the cores (from both experiments) except CO5, the seawater overlying the sediment was well oxygenated (Figs 6.3, 6.5), but high and stable concentrations of both dissolved Fe(II) and dissolved Fe(III) occurred. However both dissolved Fe(II) and dissolved Fe(III) are unstable under aerobic conditions and circumneutral pH (Sunda 2001). This behaviour likely suggests that (1) the amount of Fe(II) oxidised in the seawater equalised the amount of Fe(III) reduced in the sediment and/or (2) that Fe(II)-binding ligands were present and complexed the Fe(II) in solution thus retarding

its oxidation. As discussed in the previous chapters, a number of studies suggest the presence in seawater of Fe(II)-binding ligands which may retard the Fe(II) oxidation to Fe(III) (Croot et al. 2001, Moffett 2001, Statham et al. 2005, Croot et al. 2007, Croot et al. 2008, Roy et al. 2008, Balzano et al. 2009). Moreover the presence of Fe(III)-binding ligands has been demonstrated (Barbeau et al. 2001, Buck et al. 2007, Hunter & Boyd 2007), and are directly produced by bacteria or derive from OM degradation (Hunter & Boyd 2007). However siderophore production is controlled by iron levels (Reid et al. 1993) and might be inhibited under the high concentrations measured here.

6.3.5 Different trends in iron and manganese dissolution

In the present study, a different behaviour for dissolved Fe(II) and dissolved Mn has been found, with the former more stable and persistent than the latter under aerobic conditions. In particular the high levels of dissolved Fe(II) found on days 5 and 6 in CO1 and on day 6 in CO3 (Fig. 6.3) are not associated with high concentrations of dissolved Mn. This difference is quite surprising as dissolved Fe(II) is far more unstable than dissolved Mn in aerobic environment (Stumm & Morgan 1993, Morgan 2005).

The increases in both dissolved Fe(II) and dissolved Fe(III) found in CO1, CO2 and CO3 towards the end of the experiments (Fig. 6.2), may be associated with some mixing processes occurring between phytodetritus and sediment, driven by the active aeration occurring in these cores. Consistent with this, during the second experiments the same chemical species as above showed a range of behaviours without significant differences between the phytodetritus-amended cores and the control core (Fig. 6.4).

In contrast, as the concentration of dissolved Mn decreased over time in CO1, CO2, CO3 and CO6 but not in the control cores CO4 and CO8 (Figs. 6.2, 6.4), the presence of phytodetritus seems to have promoted the removal (precipitation and/or adsorption) of dissolved Mn from the system. The interactions of Mn with phytodetritus consist in a balance between production and removal processes. In seawater Mn can be removed by microbial oxidation, sorption to OM and sediment particles, and biological uptake.

Recent studies revealed that the enhanced bacterial activities occurring during phytoplankton blooms promote the microbial oxidation of dissolved Mn (Muller et al. 2005, Luengen et al. 2007). The oxidation of dissolved manganese can be catalysed by a range of microorganisms (Tebo et al. 2005), including *α-Proteobacteria* (Dick et al.

2008, Anderson et al. 2009) which are an important component of marine particles (DeLong et al. 1993, Grossart & Ploug 2001, Simon et al. 2002) and are present in artificial aggregates obtained following the same method as described here (Balzano et al. 2009). Among the *α-Proteobacteria*, Mn(II) oxidation has been well described in *Roseobacter* species (Hansel & Francis 2006) and artificial aggregates investigated in a previous study did contain a clone affiliated to *R. litoralis* (Chapter 3, Balzano e al. 2009). As the oxidation of reduced manganese is an autocatalytic process (Stumm & Morgan 1970), any particulate Mn produced is likely to enhance the oxidation rate of the remaining dissolved Mn. Moreover most of the dissolved Mn produced in the anoxic sediment might have been adsorbed in the phytodetritus layer rather than freely diffuse in the overlying seawater.

6.3.6 Sediment heterogeneity

Although the sediment had been sieved and mixed before the cores were prepared, it is possible that slight differences in grain size distribution and/or OM quantity/quality occurred between the different cores and led to different redox conditions and iron and manganese dissolution among the cores.

When experiments started the phytodetritus was gently poured above the undisturbed sediment and not mixed with it. The interactions between metals, mainly present in the sediment and the phytodetritus occurred thus only across the sediment-phytodetritus interface. The physical separation between sediment and phytodetritus might thus have limited the dissolution of metals, making most of the bacteria associated with sediment unlikely to be in contact with the phytodetritus. In previous studies based on phytodetritus amended sediment core, the phytodetritus was mixed with sediment and homogenised prior to addition to sediment cores (Sun et al. 1997, Sun & Wakeham 1998, Sun et al. 1998).

In natural waters, after phytodetritus deposition events, a range of factors including deep currents, convective and advective movements of the interstitial seawater (Huettel et al. 1998, Huettel et al. 2003) and faunal bioturbation (Beaulieu 2002) promote the mixing of phytodetritus with the underlying sediment. Faunal bioturbation enhances also the bacterial abundances and activities and therefore the oxygen consumption rates (Hansen & Kristensen 1998, Goni-Urriza et al. 1999).

6.4 Conclusion and future work

The current results indicate that the mere addition of phytodetritus material to sediment cores does not necessarily affect the oxygen consumption and the dissolution of iron and manganese. A crucial role in metal cycling in the sediment is indeed played by the quality of the OM (labile vs refractory) present, which controls the redox conditions, and the mixing processes occurring between the phytodetritus and the sediment. Other factors, along with the presence of phytodetritus, appear to control the degradation of sedimentary OM and consequent distribution of oxygen, iron and manganese.

Future experiments should be carried out to investigate the impact of phytodetritus deposition on the dissolution of Fe and Mn. The OM content in the sediment should be determined and it should be reasonably low compared to the OM present in the phytodetritus. In addition, labile phytodetritus, prepared from exponentially-growing rather than senescent algal cultures should be used. It is also useful to pre-mix the phytodetritus with sediment or to promote the partial mixing using mechanical devices or benthic animals (bivalves, polychaetes, echinoderms).

A wider range of physical (sediment granulometric composition) chemical (nutrients, DOC, particulate metals) and biological (microbial community composition, total bacterial number) parameters should be determined and complemented with the measurements of dissolved Fe and Mn to give a more definitive interpretation of the data.

Current results also suggest that dissolved Fe and Mn might have been differently complexed; the different forms of Fe and Mn could be determined using sequential column techniques (Öztürk et al. 2003). The concentrations of Fe- and Mn-binding ligands can also to be determined using published techniques including competitive ligand exchange-adsorptive cathodic stripping voltammetry (Rue & Bruland 1995, Buck et al. 2007).

Chapter 7. Conclusions and future work

7.1 Conclusions

The combination of inorganic (nutrients, dissolved iron and manganese) and organic (lipids) chemistry with microbiology (16S rRNA and anaerobic culturing) techniques has permitted an interdisciplinary investigation on artificial aggregates. The role of aggregates in the release of reduced species under dark conditions has been assessed and the bacteria likely involved in these processes characterised. Moreover the compositional changes in fatty acids occurring in degrading aggregates have been investigated.

7.1.1 Production of reduced species

Artificial aggregates released nitrite, most likely derived from nitrate reduction or oxidation or organic nitrogen, and contained microorganisms capable of growing through the reduction of NO_3^- and NO_2^-. However it is not clear to what extent these microorganisms are active under seawater conditions and if they further reduce NO_2^- to gaseous nitrogen species (denitrification *sensu stricto*) or NH_4^+ (ammonification) (Zumft 1997).

Marine aggregates can release dissolved Fe(II) and dissolved Mn into their surrounding seawater. The releases found are variable and do not occur in all cases. For example aggregates incubated under settled conditions (Chapter 3) released both dissolved Fe(II) and dissolved Mn. In contrast aggregates kept under conditions of constant velocity shear (Chapter 4) did not affect the iron cycling (Figs 4.3-4.4) but released significant amounts of dissolved Mn (Fig. 4.5). The dynamic conditions likely promoted the diffusion of solutes, including oxygen, and limited the production of Fe(II) but not that of dissolved Mn. This reflects the higher stability of reduced manganese in aerobic environment compared to reduced iron.

In contrast significant amounts of microaggregates (phytodetritus) settled on the sediment might play a role in the removal of dissolved Mn from seawater (Chapter 6), and microbial Mn(II) oxidation rates are indeed governed by biochemical parameters that vary spatially and temporally (Clement et al. 2009). The deposition of phytodetritus on the seafloor does not necessarily promote the release of dissolved

Fe(II) and dissolved Mn from the sediment, other factors including bioturbation and interstitial seawater diffusion appear to be more important for these metals.

Aggregates are likely to play a role in the release of organic compounds produced by bacteria (siderophores) or derived from the degradation of refractory OM (tetrapyrroles) which complex and stabilise both dissolved Fe(III) and dissolved Mn (Hutchins et al. 1999, Barbeau 2006, Duckworth & Sposito 2007, Hunter & Boyd 2007). Similar ligands are also suggested to bind dissolved Fe(II) and retard its oxidation to Fe(III) (Statham et al. 2005, Roy et al. 2008, Balzano et al. 2009).

The reducing processes demonstrated for artificial aggregates appear to occur at a very small scale, associated with anoxic microzones presumably in close proximity to the cell surface of bacteria (Balzano et al. 2009). Alternatively, some bacteria seem able to reduce Fe(III) even in the presence of low oxygen concentrations. In either scenario, diffusion of oxygen to the reduction site must be limited or inhibited, or the reduced species complexed by ligands.

The concentration of oxygen inside aggregates is governed by a balance between oxygen diffusion and consumption, whilst the overall diffusivity of gases within aggregates has been recently reported to be similar to that occurring in seawater (Ploug & Passow 2007), small scale solute barriers may be present and locally limit the diffusion of oxygen. In addition the lability of the OM in aggregates is a crucial factor that controls the microbial respiration and therefore the O_2 consumption. Microenvironments with limited diffusivity and rich in labile OM, when present within aggregates are most likely to mediate the reducing processes described in the present thesis.

7.1.2 Compositional changes in lipids

Consistent with previous studies on natural sinking particles (Wakeham 1995, Wakeham et al. 1997, Kiriakoulakis et al. 2001) artificial aggregates incubated in the dark are rapidly depleted in labile fatty acids (*n*SCFA, MUFA and PUFA, Fig. 5.4), and internal anoxic microenvironments were thus likely generated during the early stages of degradation, when a rapid consumption of labile compounds occurred. The lower abundance of bacterial fatty acids (brFA, Kaneda 1991), similar to that occurring in natural aggregates sinking in aerobic rather than anaerobic waters (Wakeham 1995), confirms that the degradation processes taking place were mainly aerobic. Moreover *n*LCFA and LbrFA persist in degrading aggregates, the former might have been

partially produced *in situ* by phytoplankton (Dunstan et al. 1992, Volkman et al. 1998), bacteria (Volkman et al. 1988) and heterotrophic eukaryotes (Rezanka et al. 1987) rather than derive only from higher plants (Meyers 1997), whereas the latter are rarely reported in geochemical literature and appear to derive from bacteria (Brinis et al. 2004, Tolosa et al. 2004, Fukushima et al. 2005, Pancost et al. 2006). A branched monounsaturated fatty acid (11-methyloctadecenoic acid, Fig. 5.3) not previously reported in marine particles, and likely derived from *α*- and *γ-Proteobacteria* (Rontani et al. 2005), was also found in artificial aggregates in the present study.

7.1.3 Microbial populations

Although artificial (and similarly natural) aggregates were formed under oxic conditions, anaerobic bacteria, mainly affiliated to *γ- and δ-Proteobacteria* were found among the attached microorganisms. These aggregate-attached bacteria have the potential for a range of processes including glucose fermentation and respiration of Fe(III) (Balzano et al. 2009), Mn(IV), sulfate (Grossart & Ploug 2001) and nitrate (Wolgast et al. 1998, Balzano et al. 2009).

As aggregates are likely to be subjected to highly variable redox conditions, facultative rather than obligate anaerobes are adapted to actively consume organic matter and respire either oxygen or other electron acceptors. Facultative anaerobes affiliated to *γ-Proteobacteria* were isolated from aggregates and were potentially involved in the reducing processes found. In particular *Marinobacter* species, which were detected in artificial aggregates, are able to respire a surprisingly wide range of electron acceptors (Lloyd et al. 2009), including NO_3^-, Fe(III) and Mn(IV), and can be also active under aerobic conditions oxidising OM or even Fe(II) (Edwards et al. 2003) with important biogeochemical implications.

7.2 *Future work*

7.2.1 Nitrogen cycling

Aggregates play an important role in nitrogen cycling and a mass balance study on nitrogen is necessary. During aggregate incubation experiments the concentrations of the different nitrogen species (NO_3^-, NO_2^-, NH_4^+ and organic nitrogen) should be determined over time to further investigate the loss in nitrate from aggregates highlighted in the present thesis. The addition of stable isotopes ($^{15}NO_3^-$) to aggregates

could be applied to evaluate the relative importance of denitrification and ammonification to the nitrate loss.

In addition ^{15}N-labelled phytodetritus could be used to assess the oxidation of DON and the importance of anaerobic ammonium oxidation (Strous et al. 1999, Woebken et al. 2007) in aggregates. Planctomycetes which are responsible for anaerobic ammonium oxidation (Strous et al. 1999), are present in marine aggregates (DeLong et al. 1993); the presence of anammox in aggregates could be investigated using specific primers (Neef et al. 1998, Woebken et al. 2007) and lipid biomarkers such as ladderanes (Sinninghe Damste et al. 2002).

7.2.2 Iron and manganese dissolution

To better understand the impact of marine aggregates to the global cycling of Fe and Mn, microcosm experiments aimed at calculating a mass balance (i.e. conversion between different forms in the microcosm) are necessary to estimate the reduction rates of Fe(III) and Mn(IV). Aggregates could be incubated under dark conditions and both particulate and dissolved Fe and Mn measured along with particulate and dissolved organic carbon to associate the reducing processes with the biomass involved. Radio-isotopes (^{55}Fe- and ^{54}Mn-oxides) could be added to calculate specific rates of reduction, and lifetime in the water of reduced species. The isotopic composition of the iron and the manganese present could be subsequently determined using a liquid scintillation counter for ^{55}Fe (Zubkov et al. 2007) and by gamma counting for ^{54}Mn (Simpson & Morris 2004). Amorphous, rather than crystalline iron and manganese oxides would be used as the chemical properties of particulate metals in seawater are highly dependent on the nanoscale mineral structure where they are present (Hochella et al. 2008). Moreover the intracellular particulate iron and manganese could be discriminated from that adsorbed to the cell membranes using Oxalate Reagent (Tovar-Sanchez et al. 2003).

7.2.3 Microscale measurements

The biogeochemistry of iron and manganese in marine aggregates should be investigated at a smaller scale. Microscale Lab-On-A-Chip (~100 µL) analysers (available at NOCS) could be used to measure both Fe and Mn in the interstitial seawater of aggregates. These analyses should be complemented with measurements of pH, pE, dissolved oxygen and sulfide through aggregates using Unisense

microelectrodes. Confocal microscopy of aggregates stained with redox specific indicators could be used to estimate the redox conditions across/through an aggregate also in relation to presence of bacteria. This study would show whether or not anoxic microenvironments are present and associated with (bacterial) cell surfaces.

The elemental composition and therefore the Fe/C ratio, of specific microbial cells in aggregates would be determined, for both phytoplankton and bacteria, using single cell microprobes (Twining et al. 2004, Twining et al. 2007).

7.2.4 Fe- and Mn-binding ligands

The ability of organic compounds to bind Fe(II) retarding its oxidation has been suggested in the present thesis. The presence of these ligands associated with aggregates should be investigated. Moreover the role of aggregates in the production of Fe(III)-binding ligands would be assessed. Freshly formed aggregates host significant bacterial activities and are thus likely to release siderophores. In contrast aged aggregates, are rich in refractory organic matter (Lee et al. 2004) and are likely to produce a second class of ligands (including tetrapyrroles) typical of Deep Ocean and derived from OM degradation (Hutchins et al. 1999, Hunter & Boyd 2007). The concentration of ligands (eventually released from aggregates) could be measured using cathodic stripping voltammetry (Gledhill & van den Berg 1994, van den Berg 2006).

7.2.4 Microbial populations

The bacteria present in marine aggregates should be better characterised by isolation and cultivation using selected substrates (supplied at seawater concentrations) and variable oxygen concentrations. Previous studies suggest that *Planctomycetes* and *γ-Proteobacteria* appear to be adapted to the redox changing conditions and high nutrient concentrations present in marine snow (Nold & Zwart 1998, Kuypers et al. 2005, Woebken et al. 2007).

Co-cultures of two or more bacterial strains isolated from aggregates could be set to investigate the ecological relationships among microbial populations attached to aggregates. For example, as fermentation products (such as acetate or lactate) can serve as substrates for Fe(III) and Mn(IV) reductions, a mixed culture of fermenters and Fe(III)-reducing bacteria, isolated from aggregates, would elucidate the importance of this microbial association.

Molecular analyses of the 18S rRNA gene are also necessary to assess the microbial eukaryotic populations present in aggregates. In particular marine yeasts are also important in the degradation of detrital material (Fell & Newellm 1998) and their presence and activity in marine aggregates has never been investigated. Single cell studies using NanoSIMS techniques (Lechene et al. 2007, Popa et al. 2007, Behrens et al. 2008) could also be used to determine the activities of single bacterial cells.

7.2.5 Stable Isotopes to investigate aggregates degradation

A good approach to study the degradation of organic matter in the laboratory microcosms is based on stable isotopes. The degradation of phytodetritus has already been investigated in the laboratory (Harvey & Macko 1997, Rontani et al. 1997, Rontani et al. 1999, Rontani et al. 2003) using stable (^{13}C and ^{15}N) isotopes (Sun 2000, Sun et al. 2002, Sun & Dai 2005) but these studies mainly focused on phytol and sterols degradation rather than the compositional changes of the overall lipid composition.

Specific diatom strains known to be involved in aggregation events (e.g. *Thalassiosira, Chaetoceros, Skeletonema, Navicula,* Grossart and Ploug 2001, Grossart et al. 2006) could be cultivated axenically with labelled $NaH^{13}CO_3$ as the sole carbon sources. Cultures should then be mixed with a natural seawater sample and aggregates formed. Aggregates should then be incubated in the dark and active microorganisms would incorporate labelled carbon. The DNA extracted from active microorganisms (both eukaryotes and prokaryotes) could be discriminated from that extracted from senescent microorganisms using stable isotope probing (Neufeld et al. 2007).

The concentration of DOC, POC, nutrients, pigments and their successors could be measured along with that of lipids. As sugars and aminoacid biomarkers for plankton freshness and bacterial activities can occur in seawater (Goutx et al. 2007) the changes in concentrations of carbohydrates and aminoacids should also be assessed. The isotopic enrichment of specific sugar, aminoacids and lipid biomarkers would provide evidence for the incorporation of phytoplankton biomass and can be determined using stable isotope GC-MS.

The fluctuating concentrations of cholesterol (Fig. 5.5) in dark incubated aggregates suggest that eukaryotic microorganisms were likely to be active in degrading aggregates. These experiments would investigate the role of any microorganism (prokaryote or eukaryote) likely involved in the degradation of

aggregates and the production of LbrFA and *n*LCFA from decaying phytodetritus. A range of marine yeasts, bacteria and mixotrophic microalgae were shown to produce *n*LCFA (Rezanka et al. 1987, Yokoyama et al. 2001). LbrFA likely produced *in situ* were found associated with superficial suspended marine particles (Brinis et al. 2004, Tolosa et al. 2004). However it is not clear which microorganisms synthesise both *n*LCFA and LbrFA and under which conditions.

Microorganisms potentially involved in *n*LCFA and/or LbrFA production would then be isolated and cultivated in order to demonstrate their effective role in the synthesis of such fatty acids and to elucidate the conditions promoting or limiting the production of *n*LCFA and/or LbrFA in marine environment.

The combination of stable isotope probing of DNA and lipid analyses has been successfully applied to investigate sediment microbial communities (Webster et al. 2006) but not for marine particles.

7.2.6 Phytodetritus degradation during a natural flocculation event

Phytoplankton blooms are not completely predictable; however they are likely to occur in spring in coastal and estuarine systems in temperate waters. A sediment trap study could be conducted to collect sinking particles throughout a natural flocculation event. The changes in species compositions would be assessed by light microscopy (microplankton and nanoplankton) and flow cytometry (picoeukaryotes and bacteria) (Olson et al. 1993, Marie et al. 1997). The community succession during the development of the bloom would be investigated by molecular (16S and 18S rRNA gene) analyses. Temperature, salinity, nutrients and oxygen are also to be measured.

It is expected that diatoms initially dominate the phytoplankton populations, one or few species are then likely to contribute for most of the community during the bloom. If flocculation occurs free-living microalgae would be likely to differ from those involved in the flocculation (Najdek et al. 2002). The fatty acid composition of collected aggregates would help understanding the physiological state of the phytoplankton community (senescent vs. exponentially growing). At the end of the bloom higher proportions of nanoplankton and picoplankton over microplankton as well as dinoflagellates over diatoms would be expected. The fatty acid composition of particles collected at the end of the bloom would be likely to include more degraded fatty acids and higher concentrations of zooplankton and bacterial biomarkers. Lipids

could be analysed using "Isotope ratio GC-MS" to infer the origin (marine vs. terrestrial) of the compounds analysed.

Phospholipid fatty acids (PLFA) are associated with active microorganisms as they are rapidly removed from seawater after the death of cells (White et al. 1979). In addition to total fatty acids, PLFA should thus be analysed from natural aggregates.

Archea have been shown to be abundant in marine systems (DeLong et al. 1994, Massana et al. 1997, DeLong 2007) and suspended particles were shown to contain archeal biomarkers (Hoefs et al. 1997). A range of biogeochemical processes, including anaerobic methane oxidation (Pancost et al. 2000, Wakeham et al. 2003), and aerobic ammonium oxidation (Konneke et al. 2005, Wuchter et al. 2006, DeLong 2007) appear to be driven by *Archaea* and aggregates can contain significant concentrations of NH_4^+ (Karl et al. 1984) and CH_4 (Karl & Tilbrook 1994). The 16S rRNA gene could be amplified from aggregates using archaeal specific primers. The analysis of archeal biomarkers such as ether lipids and isoprenoids is also important to ascertain the contribution of archeal over bacterial and eukaryotic lipids.

These experiments suggested would provide further information on the microbial populations involved in the degradation of phytodetritus along with the compositional changes in lipids.

Appendix 1. Media composition

Composition of the f/2 medium used to cultivate phytoplankton strains.

Component	Concentration
$NaNO_3$	880 µM
NaH_2PO_4 H_2O	36 µM
Na_2SiO_3 $9H_2O$	100 µM
$FeCl_3$ $6H_2O$	12 nM
Na_2EDTA* $2H_2O$	12 nM
$CuSO_4$ $5H_2O$	39 pM
Na_2MoO_4 $2H_2O$	26 pM
$ZnSO_4$ $7H_2O$	76 pM
$CoCl_2$ $6H_2O$	42 pM
$MnCl_2$ $4H_2O$	0.9 nM
thiamine HCl (vitamin B_1)	150 pM
biotin (vitamin H)	1 pM
cyanocobalamin (vitamin B_{12})	0.2 pM

* Ethylenediaminetetraacetic acid

Marine minimal medium used to cultivate the anaerobic bacteria present in aggregates. The medium is a modification of the recipe of Caccavo et al. 1984).

Ingredient	Quantity (L^{-1})
$NaHCO_3$	30 mmol
NH_4Cl	4.7 mmol
$NaH_2PO_4.H_2O$	4.3 mmol
KCl	1.3 mmol
Vitamin mix[a]	10 ml
Mineral mix[b]	10 ml
NaCl	0.34 mol
$MgCl_2.6H_2O$	15 mmol
N_2-CO_2	80:20
Glucose[c]	20 mmol
Electron donors[d]	
$NaCH_3COO$	15 mmol
Sodium lactate	15 mmol
Electron acceptors[d]	
Fe(III)-NTA[e]	10 mmol
FeOOH[f]	10 mmol
$NaNO_3$	15 mmol
Na_2SO_4	20 mmol
δ-MnO_2[g]	1 mmol
Redox and pH conditions	
N_2-CO_2	80:20
pH	~6.8-7.0

[a] Prepared as described in Balch et al. 1984
[b] Prepared as described in Lovley et al. 1984
[c] Supplied only to the cultures for glucose-fermenting bacteria
[d] Only one electron donor and one electron acceptor were added for each culture, no electron acceptors or donors were added to the cultures for glucose-fermenting bacteria.
[e] Ferric nitriloacetic acid (Frederickson et al. 2000)
[f] Amorphous Fe(III) oxyhydroxide (Lovley and Phillips 1986)
[g] Synthetic vernardite (Villalobos et al. 2003)

Composition of the Difco medium, used to isolate bacteria in the present work[a].

Ingredient	Quantity (L^{-1})
Peptone	5 g
Yeast extracts	1 g
Ferric citrate	0.1 g
NaCl	19 g
$MgCl_2 \cdot 6H_2O$	5.9 g
$MgSO_4$	3.2 g
$CaCl_2$	1.8 g
KCl	0.55 g
$NaHCO_3$	0.16 g
KBr	80 mg
SrCl	34 mg
H_3BO_3	22 mg
Na_2HPO_4	8 mg
$NaSiO_4$	4 mg
NaF	2.4 mg
NH_4NO_3	1.6 mg

Appendix 2. List of samples

List of samples studied in the present thesis.

Sample	Formation date [a]	Incubation volume [b]	Sample type	Provenance
A1	19/01/2006	0.25 L	Aggregates	*A. tamarense*
A3	15/06/2006	0.25 L	Aggregates	*A. tamarense*
A4	15/06/2006	0.25 L	Control [c]	*A. tamarense*
T3	27/01/2006	0.25 L	Aggregates	*T. weissflogii*
C4	19/01/2006	0.25 L	SW Control [d]	Southampton Water
P1	02/05/2006	0.5 L	Aggregates	*P. poucheti*
P2	02/05/2006	0.5 L	SW Control	*P. poucheti*
P3	15/06/2006	0.5 L	Aggregates	*P. poucheti*
P4	15/06/2006	0.5 L	SW Control	*P. poucheti*
TA1	30/05/2006	0.5 L	Aggregates	*T. weissflogii* and *A. tamarense*
TA2	30/05/2006	0.5 L	Control	*T. weissflogii* and *A. tamarense*
TA7	30/05/2006	2L	Aggregates	*T. weissflogii* and *A. tamarense*
N1	28/04/2006	0.5 L	Aggregates	Cultivated Southampton Water [e]
N2	28/04/2006	0.5 L	Control	Cultivated Southampton Water
N3	08/02/2006	0.25 L	Aggregates	Cultivated Southampton Water
N4	08/02/2006	0.25L	Control	Cultivated Southampton Water
N5	28/04/2006	0.5 L	Aggregates	Cultivated Southampton Water + Fe(III) [f]
N6	28/04/2006	0.5 L	Control	Cultivated Southampton Water + Fe(III)
N7	28/04/2006	2L	Aggregates	Southampton Water
N10	10/05/2006	2L	Aggregates	Southampton Water
N14	28/06/2006	0.25 L	Aggregates	Southampton Water + colloidal Fe(III)
N16	02/10/2006	1L	Aggregates	Southampton Water
N17	02/10/2006	1L	Aggregates	Southampton Water + colloidal Fe(III)
N18	02/10/2006	1L	Aggregates	Southampton Water + *Shewanella* mix. [g]
N19	02/10/2006	1L	Aggregates	Southampton Water + colloidal Fe(III) +*Shewanella* mix.
N20	02/10/2006	1L	Sterile Aggregates [h]	Southampton Water
SOT1	15/05/2007	0.25 L	Aggregates	Southampton Water [i]
SOL1	09/05/2007	0.25 L	Aggregates	Solent [i]
SOT2	15/05/2007	0.25 L	Control	Southampton Water [i]
SOL2	09/05/2007	0.25 L	Control	Solent [i]

A	18/11/2008	2L	Aggregates	Solent
B	18/11/2008	2L	Aggregates	Solent
C	18/11/2008	2L	Aggregates	Solent
D	18/11/2008	2L	Aggregates	Solent
E	18/11/2008	2L	Aggregates	Solent
F	18/11/2008	2L	Control	Solent
G	18/11/2008	2L	Control	Solent

[a] Aggregates were incubated after their formation on the roller table.
[b] Aggregates were always formed in 1L or 2L cylindrical polycarbonate bottles, where the volume indicated is lower than 1L, aggregates were transferred in such smaller container after their formation.
[c] Seawater control. Obtained from 10-µm filtered medium where senescent algae had been cultivated
[d] Seawater control, obtained from 10-µm filtered Southampton Water.
[e] Mixed phytoplankton assemblages (<20µm) naturally present in Southampton Water.
[f] 100mM $FeCl_3$ in autoclaved Southampton Water were added to the aggregates mesocosm to reach a final concentration of 100µM Fe(III).
[g] A mixture of cultures from three different *Shewanella* species was added to the aggregates.
[h] Once formed aggregates were sterilised by autoclaving.
[i] In May 2007 a phytoplankton bloom occurred in both Southampton and Solent Estuary. Aggregates were formed from phytodetritus naturally collected and rotated on the roller table.

Species used in the present study: *Alexandrium (A.) tamarense, Thalassiosira (T. weissflogii), Phaeocystis (P.) poucheti*

Appendix 3. Nutrients

Concentration of nutrients (µM) in artificial aggregates studied on Chapter 3.

NO_3^-

Sample Day	N1	N2	TA1	TA2
0	83 ± 10	83 ± 8	103 ± 8	107 ± 10
1	86 ± 7	97 ± 16	98 ± 9	120 ± 14
2	75 ± 10	135 ± 8	61 ± 3	120 ± 7
3	71 ± 13	197 ± 13	32 ± 6	100 ± 9
4			24 ± 2	110 ± 12
5	70 ± 7	181 ± 21	22 ± 3	120 ± 1
6	81 ± 13	203 ± 15	20 ± 2	100 ± 8
7	82 ± 9	196 ± 21	28 ± 3	110 ± 14

PO_4^{3-}

Sample Day	N1	N2	TA1	TA2
0	1.2 ± 0.2	1.2 ± 0.2	0.5 ± 0.2	0.5 ± 0.2
1	1.1 ± 0.2	1.0 ± 0.2	0.6 ± 0.2	0.2 ± 0.1
2	1.5 ± 0.2	1.0 ± 0.1	1.3 ± 0.1	0.3 ± 0.1
3	2.2 ± 0.5	1.2 ± 0.1	0.9 ± 0.1	0.1 ± 0.1
4			1.5 ± 0.1	0.2 ± 0.1
5	3.1 ± 0.3	1.1 ± 0.2	1.6 ± 0.1	0.1 ± 0.1
6	3.7 ± 0.7	1.3 ± 0.1	1.3 ± 0.1	0.1 ± 0.1
7	4.0 ± 0.5	1.3 ± 0.2	1.9 ± 0.1	0.1 ±0.1

NO_2^-

Sample Day	N1	N2	TA1	TA2
0	2.7 ± 0.4	2.6 ± 0.2	2.0 ± 0.2	2.0 ± 0.4
1	3.0 ± 0.3	2.7 ± 0.3	2.0 ± 0.1	1.9 ± 0.1
2	3.3 ± 0.1	2.4 ± 0.3	2.0 ± 0.1	1.7 ± 0.1
3	3.9 ± 0.2	2.1 ± 0.1	2.1 ± 0.1	1.5 ± 0.1
			2.2 ± 0.1	1.4 ± 0.1
5	3.9 ± 0.6	2.2 ± 0.1	2.1 ± 0.1	1.3 ± 0.1
6	4.1 ± 0.3	2.3 ± 0.2	2.5 ± 0.1	1.6 ± 0.1
7	4.1 ± 0.2	2.4 ± 0.2	2.6 ± 0.1	1.3 ± 0.1

Concentration of NO_3^- and NO_2^- (mM) in the anaerobic cultures of aggregates N1, incubated with NO_3^- as the sole electron acceptor (Chapter 3).

NO_3^-

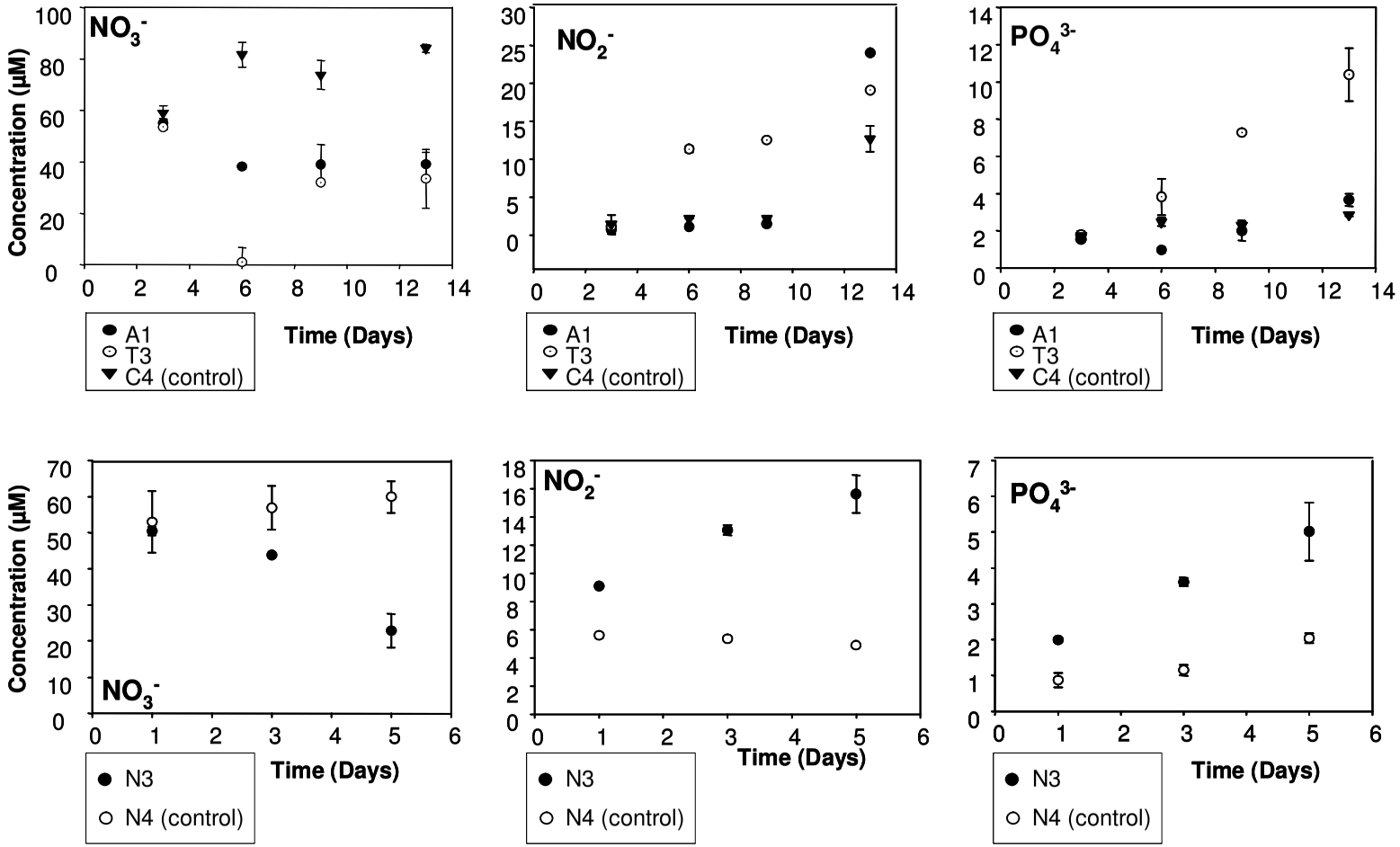
Sample Day	Lac[a]	Ac[b]	Blank[c]
0	15	15	15
10	7.1 ± 5.7	14 ± 1.8	14 ± 2.1
20	1.7 ± 2.9	13 ± 1.5	14 ± 1.5
30	0.0	13 ± 0.8	14 ± 1.5

NO_2^-

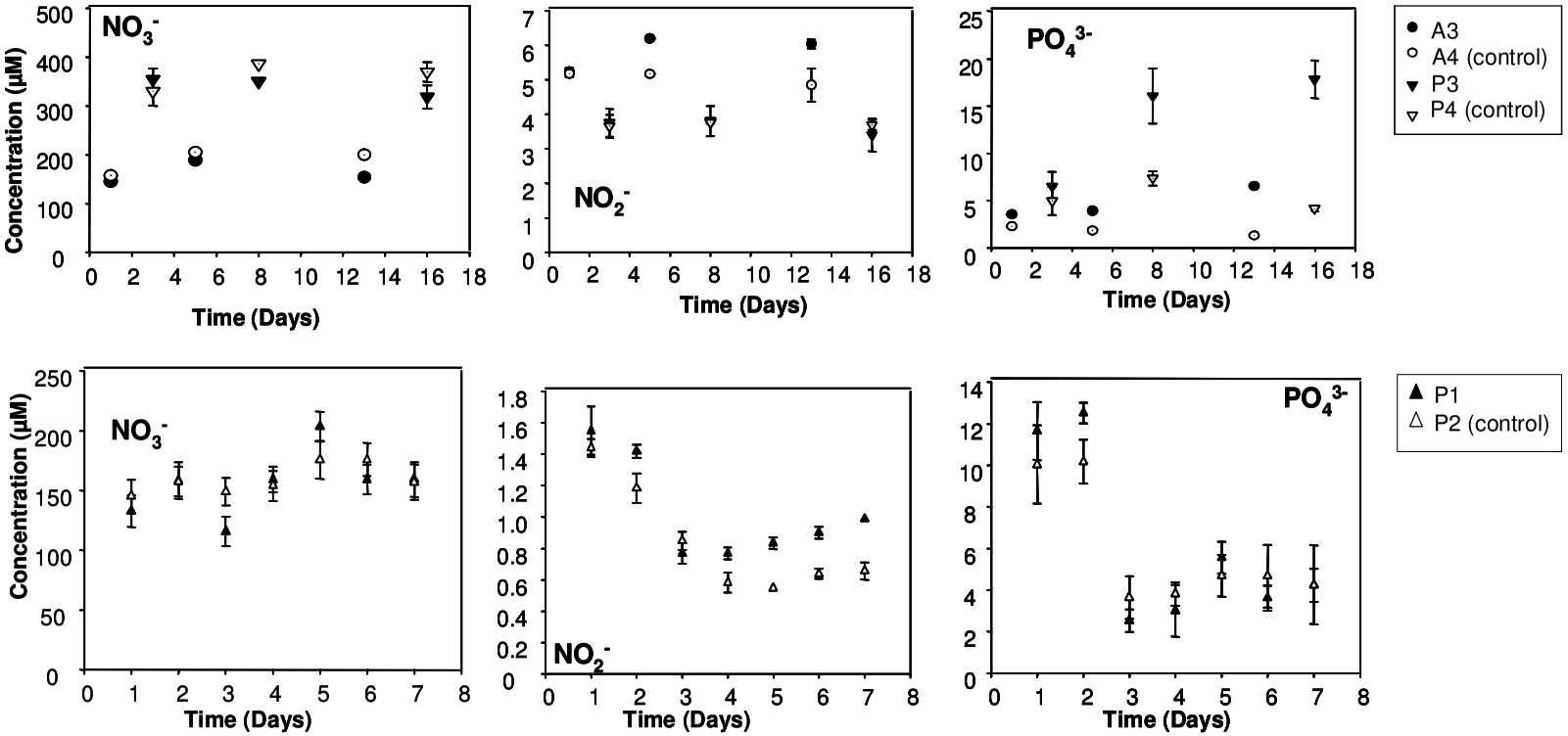
Sample Day	Lac .	Ac	Blank*
0	0.0	0.0	0.0
10	0.6 ± 0.6	0.1	0.0
20	0.7 ± 1.1	0.0	0.0
30	0.1	0.0	0.0

[a] Acetate supplied as electron donor
[b] Acetate supplied as electron donor
[c] Abiotic control (both acetate and lactate were supplied as electron donors)

Concentration of nitrate, nitrite and phosphate in the flasks containing aggregates (A1, T3 and N3) and their related seawater controls (C4 and N4).



Concentration of nitrate, nitrite and phosphate in the flasks containing aggregates (A3, P3 and P4) and their related seawater controls (A4, P4 and P2).



Concentration of nutrients (µM) in artificial aggregates studied on Chapter 5.

NO_3^-

Sample Day	N7	N10	TA7
0	86 ± 7	28 ± 2	21 ± 3
2	75 ± 10	25 ± 1	22 ± 6
4	71 ± 13	28 ± 1	24 ± 2
6	70 ± 7	30 ± 2	22 ± 3
8	81 ± 13	25 ± 1	20 ± 2
10	82 ± 9	30 ± 2	28 ± 3

PO_4^{3-}

Sample Day	N7	N10	TA7
0	1.1 ± 0.2	0.4 ± 0.2	2.0 ± 0.1
2	1.5 ± 0.2	0.3 ± 0.1	1.6 ± 0.3
4	2.2 ± 0.3	0.2 ± 0.1	1.7 ± 0.2
6	3.1 ± 0.3	0.2 ± 0.1	1.8 ± 0.4
8	3.7 ± 0.7	1.1 ± 0.1	1.9 ± 0.1
10	4.0 ± 0.5	0.6 ± 0.4	2.1 ± 0.2

NO_2^-

Sample Day	N7	N10	TA7
0	3.0 ± 0.3	1.5 ± 0.3	1.3 ± 0.3
2	3.3 ± 0.1	1.8 ± 0.4	0.9 ± 0.1
4	3.9 ± 0.2	1.6 ± 0.3	1.5 ± 0.2
6	3.9 ± 0.6	1.6 ± 0.2	1.6 ± 0.1
8	4.1 ± 0.3	2.0 ± 0.1	1.3 ± 0.1
10	4.1 ± 0.2	1.6 ± 0.6	1.9 ± 0.1

Appendix 4. Dissolved ferrous and ferric iron

Concentration of dissolved Fe(II) (µM) in artificial aggregates N1 and TA1 (Chapter 3).

Sample Day	N1	N2	TA1	TA2
0	0.10 ± 0.01	0.09 ± 0.01	0.22 ± 0.03	0.20 0.02
1	0.15 ± 0.02	0.15 ± 0.02	0.20 ± 0.01	0.20 ± 0.03
2	0.22 ± 0.01	0.14 ± 0.04	0.28 ± 0.06	0.18 ± 0.02
4			0.30 ± 0.03	0.25 ± 0.01
5	0.33 ± 0.04	0.16 ± 0.03		
6			0.45 ± 0.05	0.14 ± 0.02
8	0.47 ± 0.06	0.19 ± 0.03	0.64 ± 0.05	0.14 ± 0.07
11	0.54 ± 0.02	0.15 ± 0.02		
14	0.42 ± 0.01	0.17 ± 0.03		
17	0.21 ± 0.04	0.18 ± 0.03		

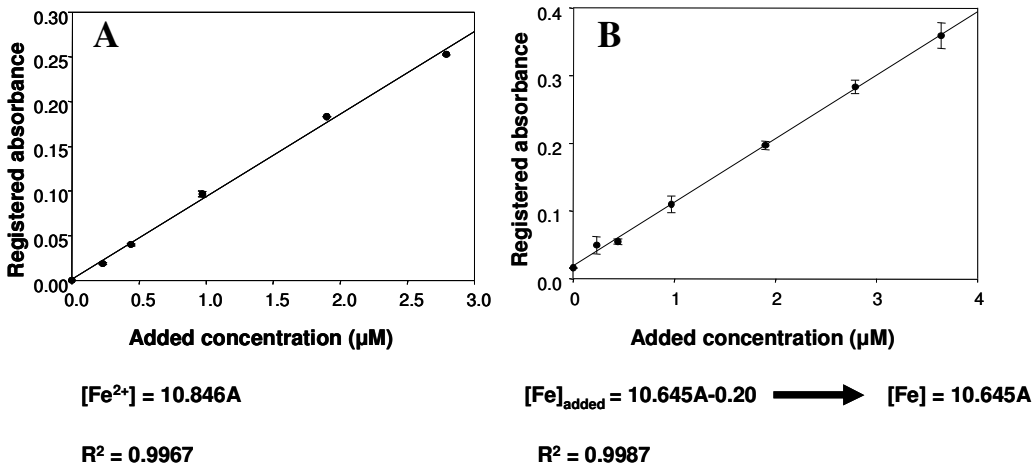
Concentration (nM) in N16 to N20 (Chapter 3).

Sample Day	N16	N17	N18	N19	N20
0	108 ± 34	108 ± 34	108 ± 34	108 ± 34	108 ± 34
2	81 ± 13	152 ± 15	1048 ± 47	6275 ± 203	42 ± 8
3	120 ± 18	135 ± 54	1556 ± 241	5147 ± 663	
4	93 ± 5	117 ± 14	1466 ± 76	3428 ± 29	47 ± 9
7	93 ± 12	98 ± 7	220 ± 11	2342 ± 53	54 ± 5
8	81 ± 13	119 ± 32	311 ± 25	2711 ± 77	66 ± 5
9	79 ± 5	114 ± 2	1245 ± 31	2807 ± 31	
15	66 ± 9	56 ± 19	1076 ± 5	1663 ± 23	39 ± 4

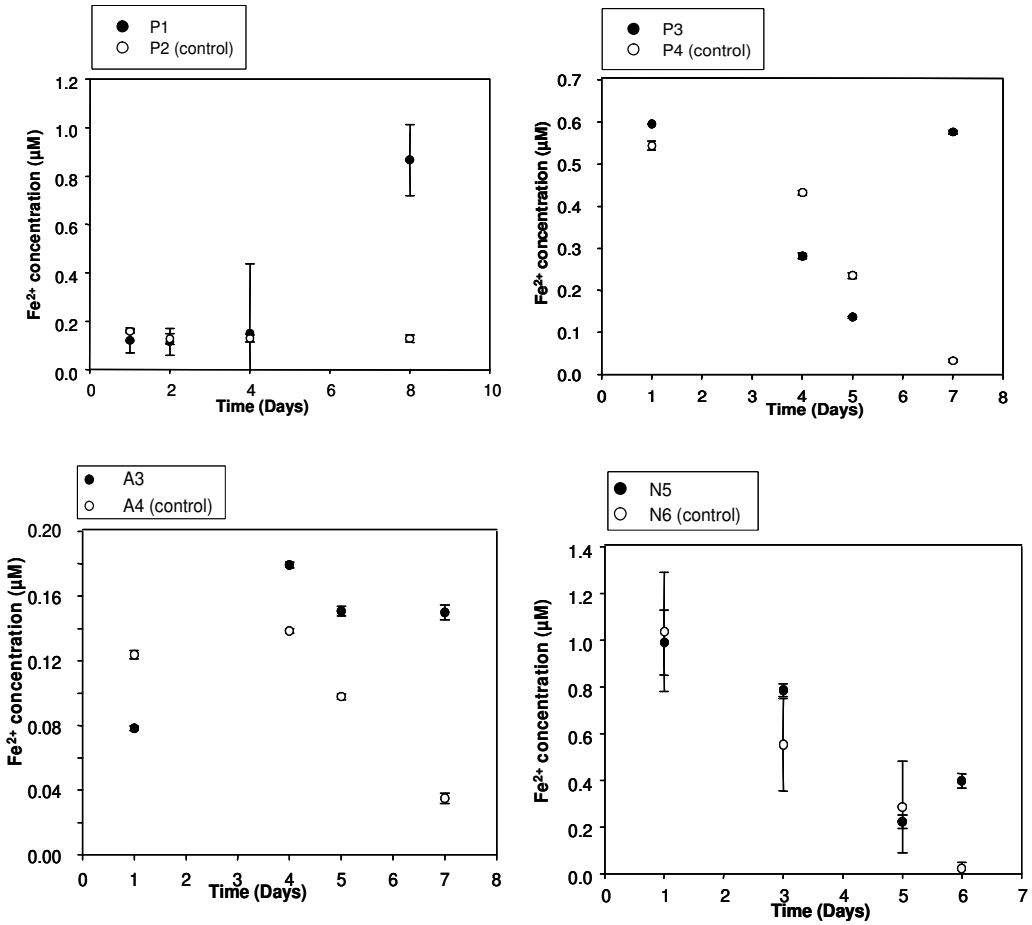
Concentration (nM) of Fe(II) in samples A to F (Chapter 4)

Sample Day	A	B	C	D
-4	45 ± 6	51 ± 11	40 ± 8	51 ± 8
0	70 ± 6	127 ± 6	134 ± 8	148 ± 8
2	96 ± 3	85 ± 8	84 ± 5	135 ± 19
4	52 ± 5	78 ± 5	73 ± 5	115 ± 5
6	39 ± 9	59 ± 12	40 ± 3	107 ± 14
8	25 ± 5	39 ± 10	23 ± 6	69 ± 10

Sample Day	E	F	G
-4	44 ± 3	44 ± 3	44 ± 3
0	136 ± 5	82 ± 3	30 ± 3
2	102 ± 16	73 ± 10	43 ± 9
4	61 ± 3	73 ± 5	63 ± 5
6	60 ± 12	21 ± 7	30 ± 7
8	52 ± 5	0	0



Calibration curves of the ferrozine method for MQ Water (A) and seawater (B)



Effect of marine aggregates on Fe(III) reduction. Samples N5 and N6 were originally spiked with 100 µM $FeCl_3$.

Concentration of dissolved [Fe(II) + Fe(III)] in samples A to F (Chapter 4)

Sample Day	A	B	C	D
-4	170 ± 30	170 ± 30	170 ± 30	170 ± 30
-2	253 ± 8	255 ± 16	235 ± 14	216 ± 37
0	221 ± 8	293 ± 14	312 ± 3	286 ± 16
2	166 ± 3	228 ± 24	157 ± 9	244 ± 3
4	204 ± 7	222 ± 4	209 ± 7	225 ± 15
6	78 ± 10	71 ± 15	108 ± 25	129 ± 14
8	157 ± 12	155 ± 3	183 ± 14	180 ± 4
11	176 ± 8	143 ± 11	129 ± 6	230 ± 9

Sample Day	E	F	G
-4	170 ± 30	170 ± 30	170 ± 30
-2	274 ± 16	270 ± 16	239 ± 17
0	249 ± 3	288 ± 23	288 ± 27
2	130 ± 3	162 ± 5	65 ± 3
4	75 ± 8	84 ± 5	122 ± 3
6	82 ± 14	95 ± 8	95 ± 6
8	217 ± 4	157	180 ± 4
11	204 ± 19	141 ± 12	157 ± 14

Concentration of HCl extractable Fe(II) (mM) in the anaerobic cultures of aggregates N1 and TA1, incubated with Fe(III) as the sole electron acceptor (Chapter 3).

	Ac[a]/NTA[b]		**Lact[c]/NTA**		**Ac/FeOOH[d]**		**Lact/FeOOH**	
	Av.[f]	St. dev.[g]	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
Blank	1.25	0.06	1.21	0.05	1.15	0.02	1.33	0.26
TA1	3.28	0.08	2.97	1.07	3.10	0.02	1.91	0.15
N1	1.52	0.05	1.81	0.49	1.64	0.06	1.27	0.27

[a] Acetate supplied as electron donor.
[b] Fe(III)-Nitrilotriacetic acid supplied as electron acceptor.
[c] Lactate supplied as electron donor.
[d] Amorphous FeOOH supplied as electron acceptor.
[f] Average
[g] Standard deviation

Concentration of HCl extractable Fe(II) (mM) in the anaerobic cultures of aggregates A (Agg3) and B (Agg1), incubated with Fe(III)-NTA as electron acceptor and acetate as electron donor (Chapter 4).

Sample Day	AGG1(I)		AGG1(II)		AGG1(III)		AGG3(I)		AGG3(II)		AGG3(III)		BL1	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.08	0.09	0.18	0.01	0.08	0.01	0.08	0.01	0.12	0.01	0.09	0.02	0.11	0.01
8	0.16	0.03	0.20	0.03	0.40	0.12	0.20	0.09	0.20	0.12	0.14	0.05	0.21	0.10
16	0.24	0.06	0.24	0.08	5.54	0.74	0.34	0.20	0.23	0.09	0.25	0.13	0.20	0.05
28	0.38	0.12	0.48	0.14	9.05	0.15	0.13	0.04	0.10	0.13	0.08	0.09	0.15	0.02

Concentration of HCl extractable Fe(II) (mM) in the anaerobic cultures of aggregates A (Agg4) and B (Agg2), incubated with Fe(III)-NTA as electron acceptor and lactate as electron donor (Chapter 4).

Sample Day	AGG2(I)		AGG2(II)		AGG2(III)		AGG4(I)		AGG4(II)		AGG4(III)		BL2	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.15	0.01	0.20	0.01	0.09	0.02	0.10	0.01	0.11	3.74e-3	0.12	3.70e-3	0.11	0.01
8	0.16	0.07	1.24	0.06	0.26	0.09	0.19	0.04	0.19	0.08	0.46	0.08	0.24	0.05
16	1.19	0.28	7.82	0.45	0.98	0.22	0.34	0.17	0.20	0.12	0.81	0.08	0.33	0.06
28	2.35	0.14	9.24	0.29	1.93	0.11	0.13	0.09	0.14	0.08	0.43	0.13	0.28	0.06

Concentration of HCl extractable Fe(II) (mM) in the anaerobic cultures of aggregates A (Agg5) and B (Agg7), incubated with FeOOH as electron acceptor and acetate as electron donor (Chapter 4).

Sample Day	AGG5(I)		AGG5(II)		AGG5(III)		AGG7(I)		AGG7(II)		AGG7(III)		BL3	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.62	0.09	0.52	0.07	0.65	0.05	0.55	0.17	0.90	0.15	0.69	0.55	0.77	0.07
20	1.49	0.22	1.24	0.04	1.85	0.07	2.01	0.12	2.85	0.18	1.44	2.01	0.96	0.02

Concentration of HCl extractable Fe(II) (mM) in the anaerobic cultures of aggregates A (Agg6) and B (Agg8), incubated with FeOOH as electron acceptor and lactate as electron donor (Chapter 4)

Sample Day	AGG6(I)		AGG6(II)		AGG6(III)		AGG8(I)		AGG8(II)		AGG8(III)		BL4	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.57	0.05	0.92	0.04	0.46	0.08	0.77	0.05	0.91	0.05	0.70	0.08	0.62	0.11
20	1.77	0.25	1.79	0.16	1.22	0.18	3.64	0.44	4.63	0.43	3.38	0.09	0.72	0.14

Concentration of extractable Fe(II) (mM) in the strains isolated from artificial aggregates(Chapter 4).

Strain	**Is-2**		**Is-3**		**Is-4**		**Is-6**		**Is-7**		**Is-8**	
Day												
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.20	0.10	0.22	0.11	0.25	0.08	0.21	0.08	0.19	0.08	0.23	0.10
11	0.30	0.17	0.36	0.17	1.00	0.22	0.70	0.11	0.18	0.11	0.40	0.20
40	0.21	0.10	0.88	0.05	2.50	0.15	0.90	0.54	0.21	0.04	0.13	0.07
60	1.60	0.14	1.50	0.23	3.70	0.38	1.00	0.12	0.14	0.03	0.07	0.06

Appendix 5. Dissolved manganese

Concentration of dissolved manganese in aggregates obtained from natural phytodetritus (Chapter 3).

Sample Day	SOL1	SOL2	SOT1	SOT2
1	143 ± 9	153 ± 2	253 ± 20	224 ± 35
4	171 ± 26	126 ± 1	433 ±18	202 ± 41
6	190 ± 5	109 ± 8	446 ± 40	307 ± 26
7	213 ± 5	113 ± 2	524 ± 46	303 ± 38
8	235 ± 23	105 ± 5	501 ± 24	301 ± 23
0	126 ± 14	126 ± 3	208 ±35	208 ± 35

Concentration of dissolved manganese in aggregates A to F (Chapter 4).

Sample Days	A	B	C	D
-2	63 ±10	63 ± 10	63 ± 10	63 ± 10
0	72 ± 4	65 ± 2	78 ± 5	89 ± 7
2	333 ± 11	847 ± 11	643 ± 46	1240 ± 139
4	71 ± 1	1280 ± 49	343 ± 56	635 ± 58
6	66 ± 4	779 ± 8	440 ± 67	891 ± 29
8	137 ± 55	324 ± 30	423 ± 43	769 ± 34

Concentration of dissolved Mn (mM) in the anaerobic cultures of aggregates A (Agg11) and B (Agg9), incubated with δ-MnO_2 as electron acceptor and acetate as electron donor (Chapter 4).

Sample Day	AGG9(I)		AGG9(II)		AGG9(III)		AGG11(I)		AGG11(II)		AGG11(III)		BL5	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.16	0.04	0.28	0.04	0.19	0.06	0.16	0.05	0.28	0.04	0.20	0.06	0.12	0.05
4	0.20	0.02	0.28	0.05	0.18	0.04	0.20	0.03	0.29	0.03	0.25	0.04	0.12	0.04
8	0.43	0.05	0.24	0.04	0.26	0.04	0.23	0.04	0.27	0.03	0.27	0.04	0.14	0.06
20	0.36	0.04	0.37	0.05	0.42	0.05	0.27	0.05	0.34	0.03	0.31	0.05	0.17	0.04
26	0.61	0.05	0.69	0.07	0.70	0.06	0.31	0.04	0.35	0.05	0.34	0.05	0.17	0.04

Concentration of dissolved Mn (mM) in the anaerobic cultures of aggregates A (Agg12) and B (Agg10), incubated with δ-MnO_2 as electron acceptor and lactate as electron donor (Chapter 4).

Sample Day	AGG10(I)		AGG10(II)		AGG10(III)		AGG12(I)		AGG12(II)		AGG12(III)		BL6	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.33	0.03	0.21	0.03	0.32	0.04	0.29	0.04	0.21	0.04	0.35	0.05	0.26	0.05
4	0.21	0.06	0.23	0.04	0.31	0.04	0.24	0.05	0.23	0.03	0.31	0.05	0.27	0.04
8	0.47	0.04	0.32	0.04	0.34	0.07	0.37	0.04	0.24	0.02	0.34	0.06	0.24	0.05
20	0.75	0.05	0.62	0.05	0.42	0.05	0.35	0.05	0.21	0.04	0.32	0.06	0.30	0.04
26	0.72	0.04	0.80	0.05	0.63	0.04	0.37	0.04	0.23	0.06	0.40	0.05	0.32	0.03

Concentration of dissolved Mn (mM) in the strains isolated from artificial aggregates(Chapter 4).

Strain / **Day**	**Is-2**		**Is-3**		**Is-4**		**Is-6**		**Is-7**		**Is-8**	
	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.	Av.	St. dev.
0	0.10	0.01	0.13	0.01	0.11	0.02	0.13	0.02	0.12	0.02	0.15	0.04
50	0.19	0.05	0.29	0.01	0.38	0.01	0.47	0.01	0.30	0.02	0.26	0.03
80	0.22	0.05	0.39	0.05	0.65	0.04	0.74	0.07	0.30	0.03	0.24	0.05

Appendix 6. Molecular analyses

Taxonomic affiliation of 16S rRNA ribotypes identified in aggregates collected from Flask N1 after analysis of the partial 16S rRNA gene fragment*

Closest matching microorganism	NCBI** accession number	Class	Identities (% matches)	Percentage present
Anaeromyxobacter dehalogenans strain FRC-D1.	CP001359	*δ-Proteobacteria*	142/193 (73%)	81.6
Uncultured clone TDNP_LSbc97_121_1_46	FJ516825	*δ-Proteobacteria*	112/163 (68%)	15.8
Uncultured clone PI_6G45	AY374682	*δ-Proteobacteria*	295/310 (95%)	2.6

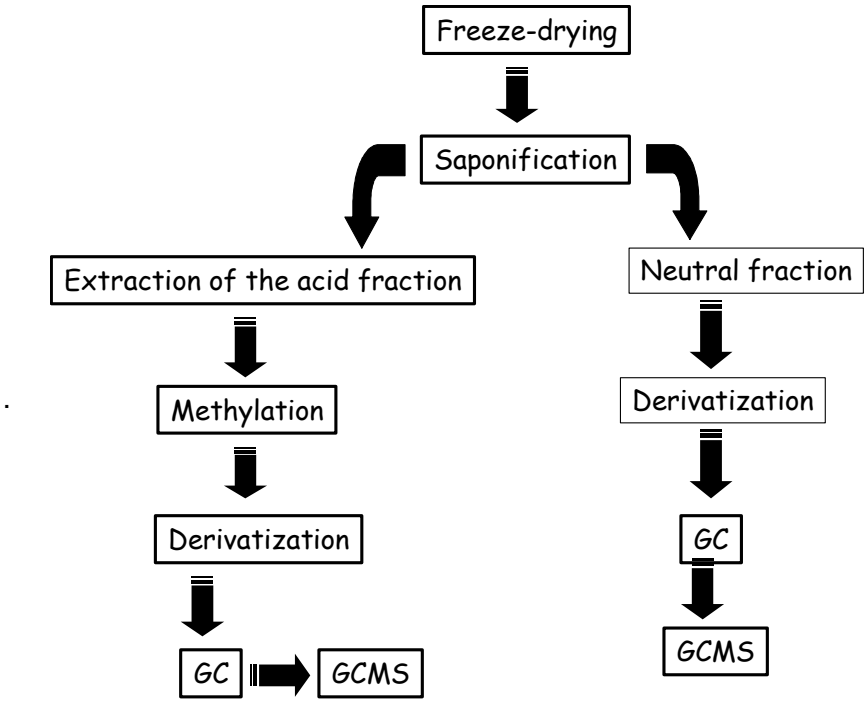
* Amplified using *Geobacter* specific primers Geo564F and Geo840R.
**National Center for Biotechnology Information

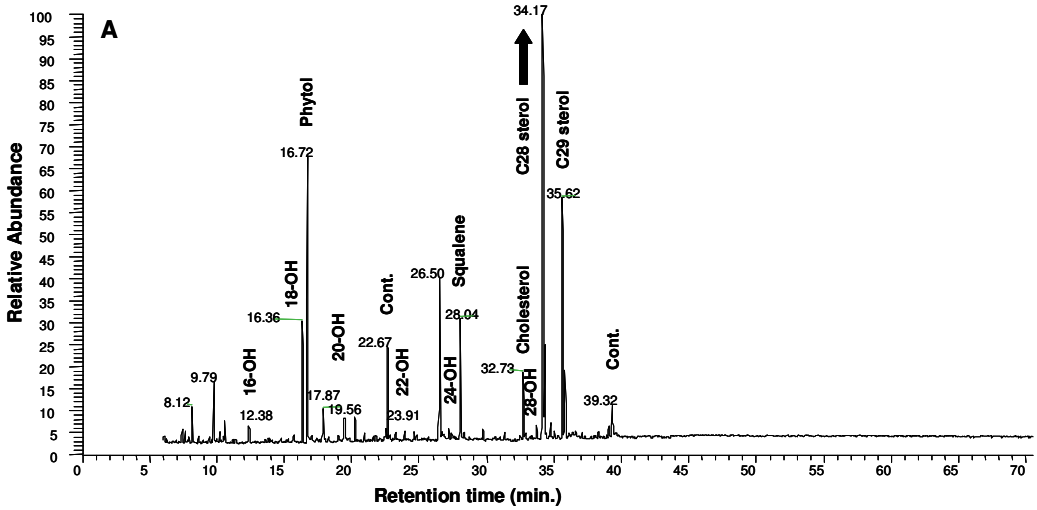
Taxonomic affiliation of 16S rRNA ribotypes identified in aggregates collected from Flask N14 after analysis of the partial 16S rRNA gene fragment*

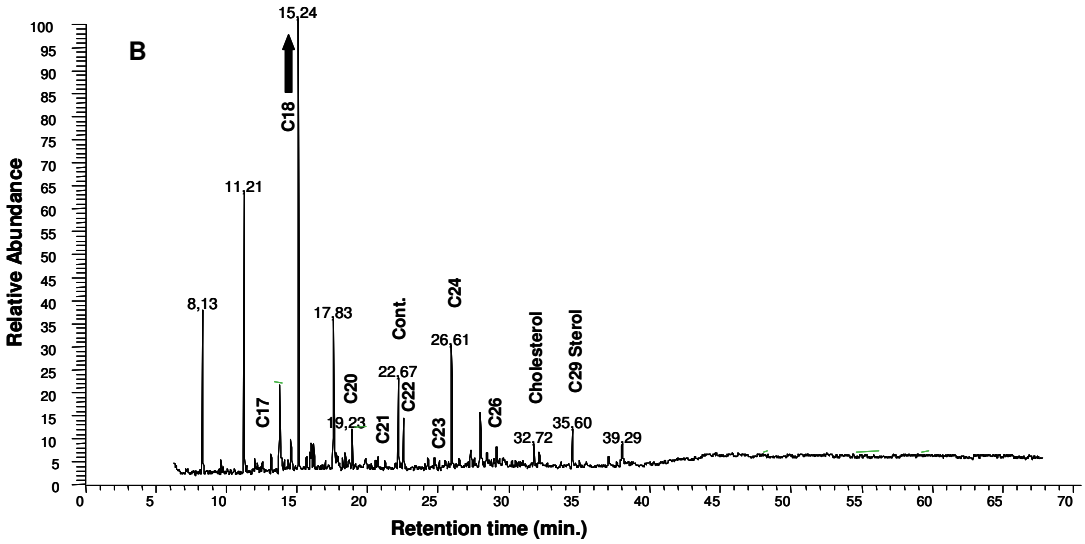
Closest matching microorganism	NCBI** accession number	Class	Identities (% matches)	Percentage present
Planctomycete GMD16E07.	AY162118	*Planctomycetes*	394/415 (94%)	2
Planctomycete GMD14H10.	AY162122	*Planctomycetes*	107/119 (89%)	66
Uncultured bacterium clone B97.	FJ206546	Unknown	24/24 (100%)	32

Appendix 7. Lipids

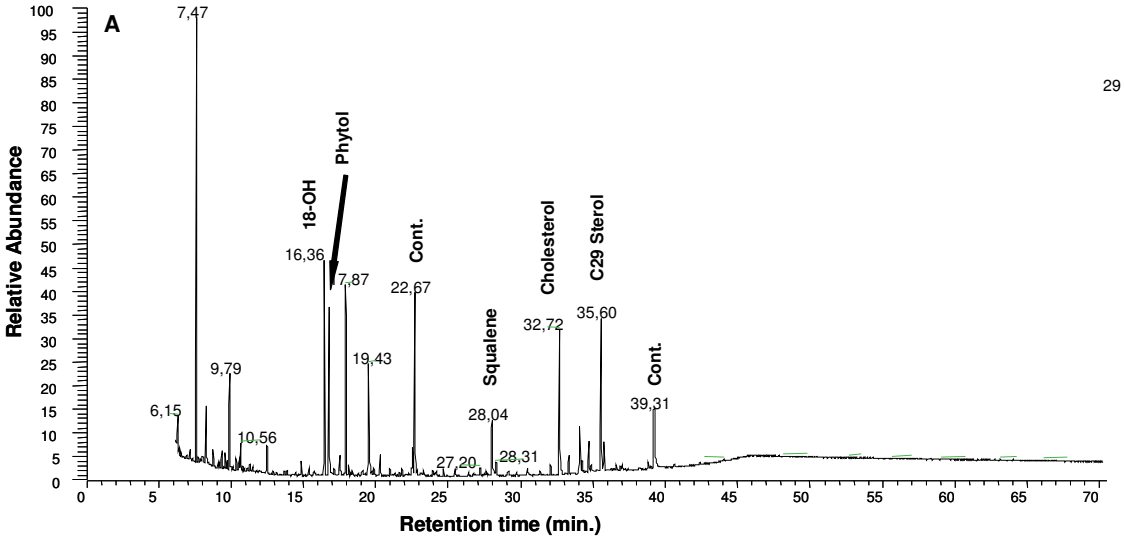
Flow chart of the analytical scheme, yielding fatty acids and neutral lipids (alcohols, sterols and hydrocarbons) used in the present thesis.

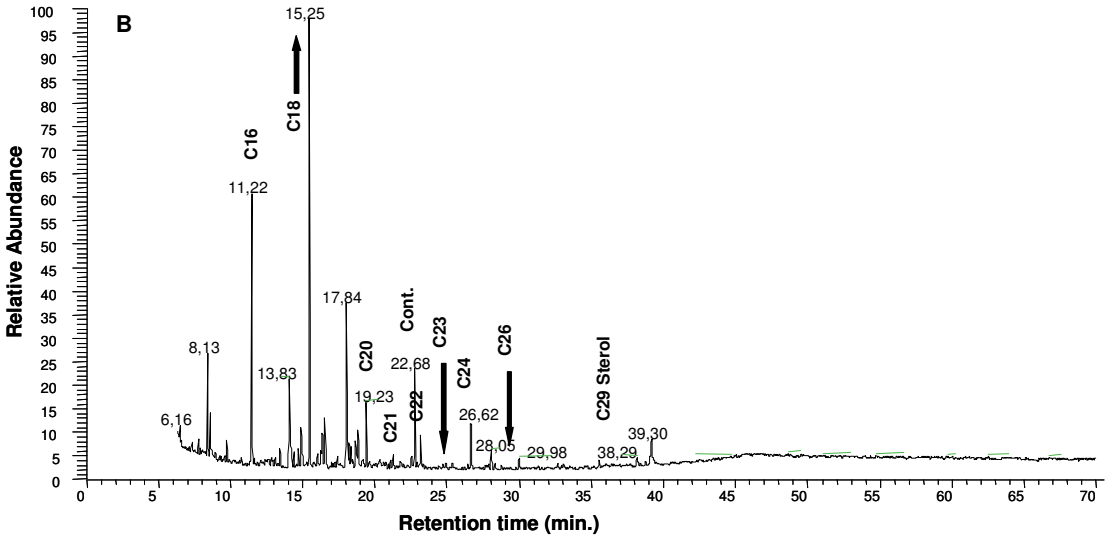






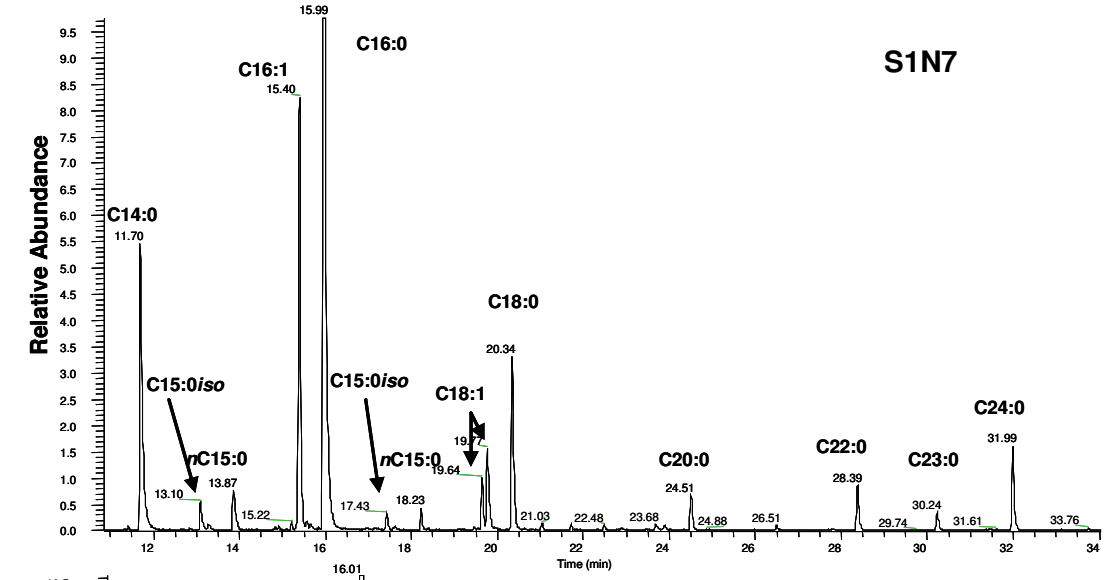
Lipid composition of sample T1. A, neutral fraction. B, acid fraction

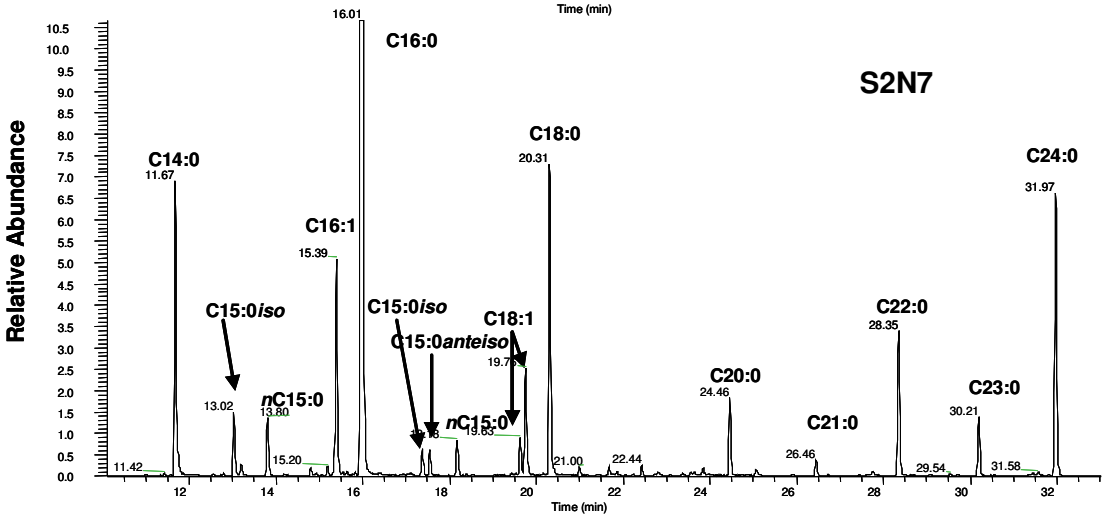


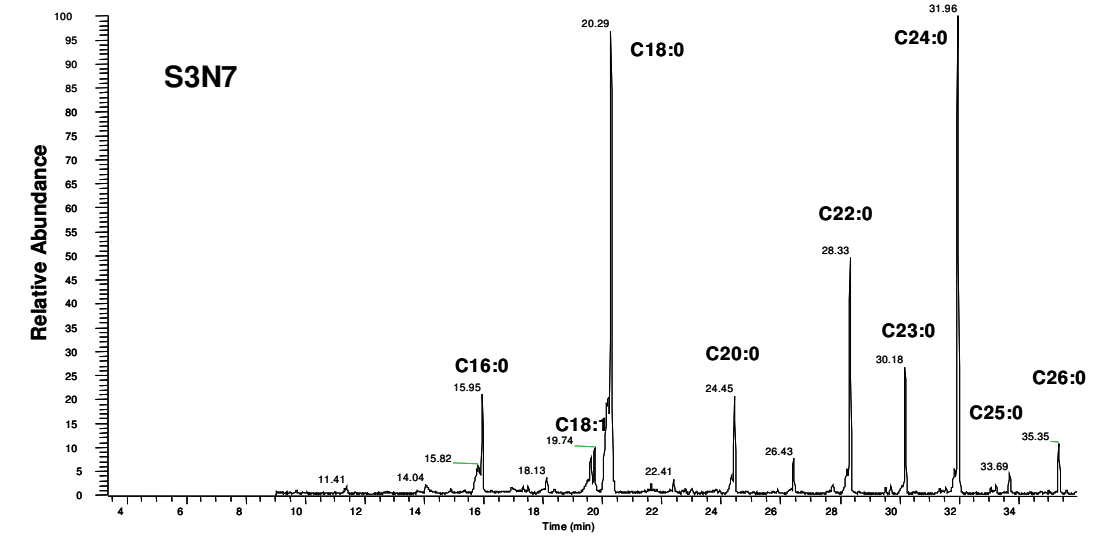


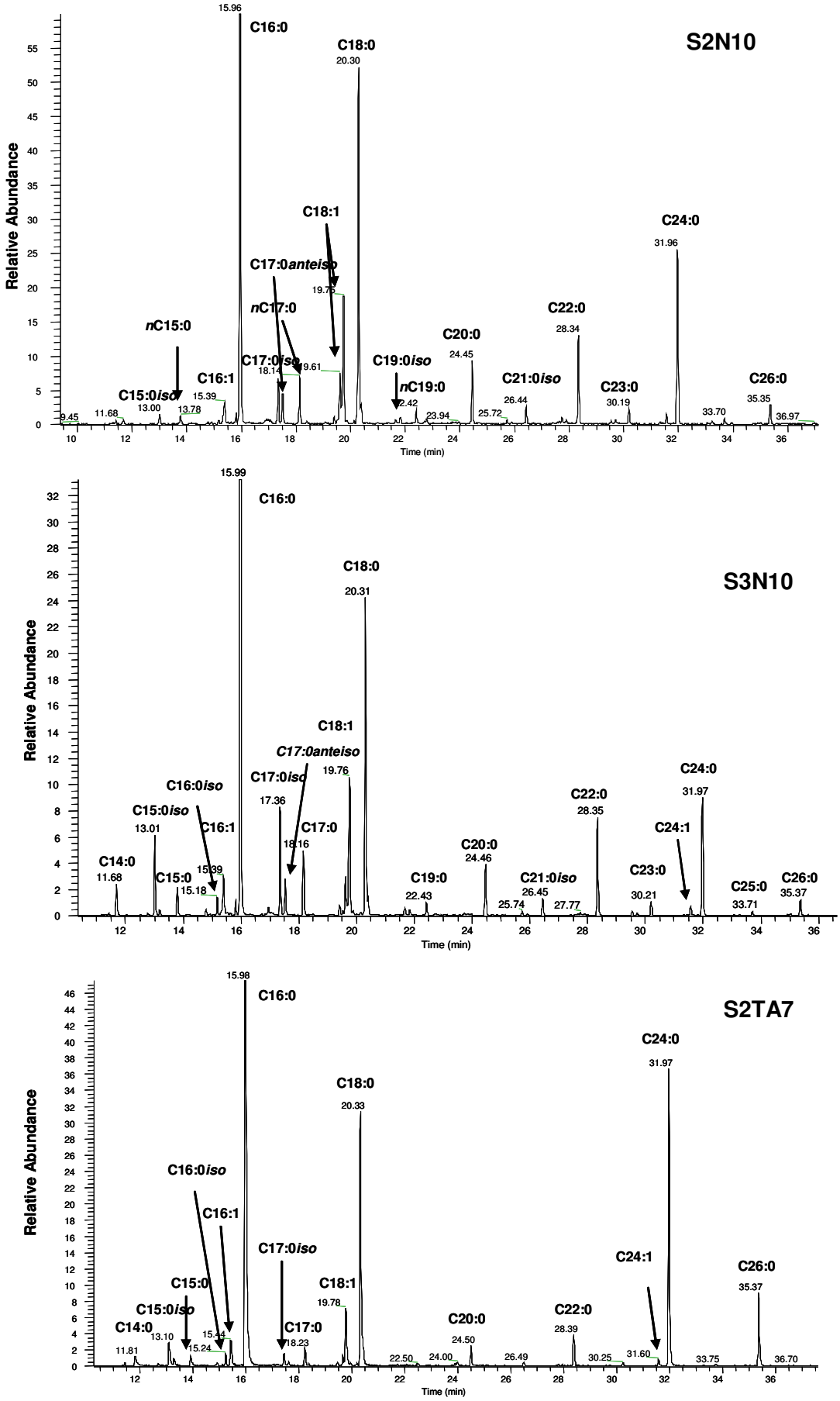
Lipid composition of sample N3.A, neutral fraction. B, acid fraction.

Partial *m/z* 74 mass chromatograms of the acid fraction of samples. The peaks of monounsaturated and polyunsaturated fatty acids are not well represented in *m/z* 74 mass chromatograms compared to saturated ones. Note that the peaks are normalized to C16:0 (=100%) which is off scale in all the figures.








S2N10
Relative Abundance
Time (min)
15.96
C16:0
C18:0
20.30
C18:1
C24:0
31.96
C17:0anteiso
19.75
nC17:0
C22:0
28.34
nC15:0
C20:0
24.45
C17:0iso
19.61
C19:0iso
C16:1
nC19:0
C21:0iso
C23:0
30.19
C26:0
35.35
C15:0iso
13.00
15.39
13.78
11.68
9.45
26.44
23.94
25.72
33.70
36.97
S3N10
Relative Abundance
Time (min)
15.99
C16:0
C18:0
20.31
C18:1
C17:0anteiso
C24:0
31.97
C16:0iso
C17:0iso
19.76
17.36
C22:0
28.35
C15:0iso
13.01
C16:1
C17:0
18.16
C24:1
C20:0
24.46
C14:0
11.68
C15:0
15.39
15.18
C19:0
22.43
C21:0iso
26.45
C23:0
30.21
C25:0
33.71
C26:0
35.37
25.74
27.77
S2TA7
Relative Abundance
Time (min)
15.98
C16:0
C24:0
31.97
C18:0
20.33
C16:0iso
C16:1
C17:0iso
C24:1
C26:0
35.37
C15:0
C18:1
19.78
C15:0iso
C22:0
28.39
C14:0
13.10
C17:0
C20:0
24.50
15.44
15.24
18.23
11.81
22.50
24.00
26.49
30.25
31.60
33.75
36.70

Appendix 8. Sediment cores

pH measured in the cores during the first sediment core based experiments.

Day	Co1		Co2		Co3		Co4	
	A/W*	Sed.**	A/W	Sed.	A/W	Sed.	A/W	Sed.
0	7.7	7.8	7.6	7.6	7.7	7.6	7.6	7.7
1	7.8	7.9	7.6	7.8	7.9	7.9	7.8	7.8
2	7.7	7.8	7.7	7.8	7.9	7.9	7.8	7.9
3	7.6	7.4	7.7	7.4	7.5	7.1	7.6	7.3
4	7.7	7.9	8.0	7.9	7.9	8.0	7.9	8.0
5	7.8	7.8	8.0	7.9	8.1	8.0	8.3	8.0
6	7.8	8.1	7.9	8.1	8.0	8.2	8.2	8.3

* Air/water interface
** 1 cm above the sediment

pH measured in the cores during the second sediment core based experiments.

Day	Co5		Co6		Co7		Co8	
	A/W	Sed.	A/W	Sed.	A/W	Sed.	A/W	Sed.
0	8.2	8.0	8.0	7.9	8.0	8.0	8.1	7.9
1	8.0	7.7	7.8	7.7	7.8	7.3	7.9	7.7
2	7.8	7.7	8.1	7.7	7.7	7.1	7.8	7.1
6	7.5	7.3	7.7	7.4	7.7	7.5	7.8	7.7
9	7.7	7.5	7.7	7.5	7.5	7.6	7.5	7.0
12	7.7	7.7	7.7	7.7	7.6	7.7	7.8	7.9

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