

**Microscale spatial
distributions of microbes
in marine intertidal
sediments and photosynthetic
microbial mats**

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I mean yeah but no but yeah but no.

Vicky Pollard

You should make something.

You should bring something into the world that wasn't in the world before.

It doesn't matter what it is.

It doesn't matter if it's a table or a film or gardening - everyone should create.

You should do something, then sit back and say, 'I did that.'

Ricky Gervais

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General Introduction

General introduction

1.1. Intertidal sediments and photosynthetic microbial mats

The microbial activity and abundances found in the marine sediments exceeds those of the pelagic oceans (Jørgensen & Boetius 2007). At the sediment surface, it has been observed that prokaryotic abundances decrease with water depth, generally related to a decrease in particulate organic matter (POM) input with depth, and distance from the coast (Jørgensen & Boetius 2007). Below the photic zone sediment microbiology is mainly driven by heterotrophic processes (Canfield et al. 2005). However, sediments exposed to light (coastal and estuarine locations) are dominated by photoautotrophs (prokaryotic and eukaryotic microbes performing oxygenic and anoxygenic photosynthesis) mostly concentrated in the top 10 mm (Mir et al. 1991, Underwood & Kromkamp 1999). In these systems, exudation and cell death, particularly of the oxygenic photoautotrophic community, contribute to the input of organic matter which is decomposed by a series of different metabolic pathways occurring in the sediment (Canfield et al. 2005, Jørgensen 2006).

Intertidal sediments tend to be dominated by eukaryotic benthic diatoms both at the sediment surface and deeper (Underwood & Kromkamp 1999). However, under more extreme conditions (e.g. hot springs, sea ice, supratidal sandy beaches) (Stal 1994, Des Marais 2003, Dobretsov et al. 2010, Andersen et al. 2011), prokaryotic cyanobacteria are likely to thrive (Stal 1995, Dijkman et al. 2010, Bühring et al. 2014, Bolhuis et al. 2015). When under such conditions, grazers are low (or absent), and more permanent photosynthetic microbial mats can develop (Fig. 1.1) (Fenchel et al. 2012). In the photosynthetic microbial mats found on intertidal flats, cyanobacteria dominate, diatoms are present, and green eukaryotic microalgae are typically only a minor component (Fig. 1.1) (Jørgensen et al. 1983, Mir et al. 1991, Bolhuis et al. 2013, Bolhuis et al. 2015).

Oxygen produced by the oxygenic photoautotrophs is used as an electron acceptor by heterotrophic prokaryotes (Canfield et al. 2005). Therefore, oxygen typically depletes rapidly in the first few millimetres (mm) (due to the aerobic respiration and diffusional limitation) and the heterotrophic prokaryotes are forced to

use a gradient of electron acceptors (NO_3^- , Mn^{4+} , Fe^{3+} , SO_4^{2-} , CO_2) for anaerobic metabolism (Canfield et al. 2005). In the interface between oxygen and sulfide, some prokaryotes (e.g. green sulfur bacteria, purple sulfur bacteria) will photosynthesise without the production of oxygen. Their anoxygenic photosynthesis results in the production of sulfate or elemental sulfur (Canfield et al. 2005). The cascade of metabolic pathways typically results in colourful layering of microbial diversity, ending with the anoxic black and grey layers, as a result of the activity of sulfate-reducing bacteria (Fig. 1.1). The black layer is caused by the reaction of hydrogen sulfide, resultant from sulfate reduction, with metal ions, such as iron, forming iron sulfide (FeS). The reaction between hydrogen sulfide and FeS results in pyrite (FeS_2), and the characteristic grey layer (Canfield et al. 2005). The presence of different functional groups of prokaryotes has been confirmed using measurements of chemical variables and fluxes (Glud et al. 1999, Revsbech 2005). Besides the photoautotrophs and heterotrophic bacteria, Archaea are also present in photosynthetic microbial mats (Bolhuis & Stal 2011). Furthermore, it was recently discovered that shallow sediments contain “cable bacteria” (Nielsen et al. 2010, Pfeffer et al. 2012). These are filamentous bacteria that link distant biogeochemical processes in sediments, by electric currents. The result is a connection between oxygen consuming bacteria at the sediment surface, with bacteria performing oxidation of hydrogen sulfide and organic carbon several centimetres deeper in the sediment (Nielsen et al. 2010). In the same way, it has been shown that “cable bacteria” in photosynthetic biofilms adjust their position in response to daily oxygen changes (Malkin & Meysman 2015).

Photosynthetic microbial mats and shallow sediments are often characterized by the presence of exopolymeric substances (EPS), which glue cells together, and thereby create a complex and durable biofilm environment (Wolfaardt et al. 1999). EPS production has several advantages for the organisms inhabiting the mat: 1) it stabilises the sediment particles, 2) it helps protect cells against pathogens (e.g. viruses, prokaryotes, fungi) and external toxic compounds (the low diffusion rates slow the metabolites dispersal), 3) it protects cells from desiccation, and 4) it keeps excreted enzymes and substrates close to the cells (Wolfaardt et al. 1999, Decho 2000). In these multispecies environments, synergistic effects have been observed.

For example, with overall biomass increase due to metabolic cooperation, or by extending protection to neighbour cells from an antimicrobial agent (Burmølle et al. 2006, Elias & Banin 2012). However living in this biofilm-like structure also has disadvantages for cells, such as the lack of space and strong competition for resources (Costerton & Lewandowski 1995). These interactions lead to the production of biosurfactants, metabolites, and enzymes that inhibit cell adhesion, disrupt populations dispersion and cell-cell communications, and degrade the extracellular matrix (Rendueles & Ghigo 2012). All of these interactions contribute to the spatial distribution of microbes in shallow benthic habitats.

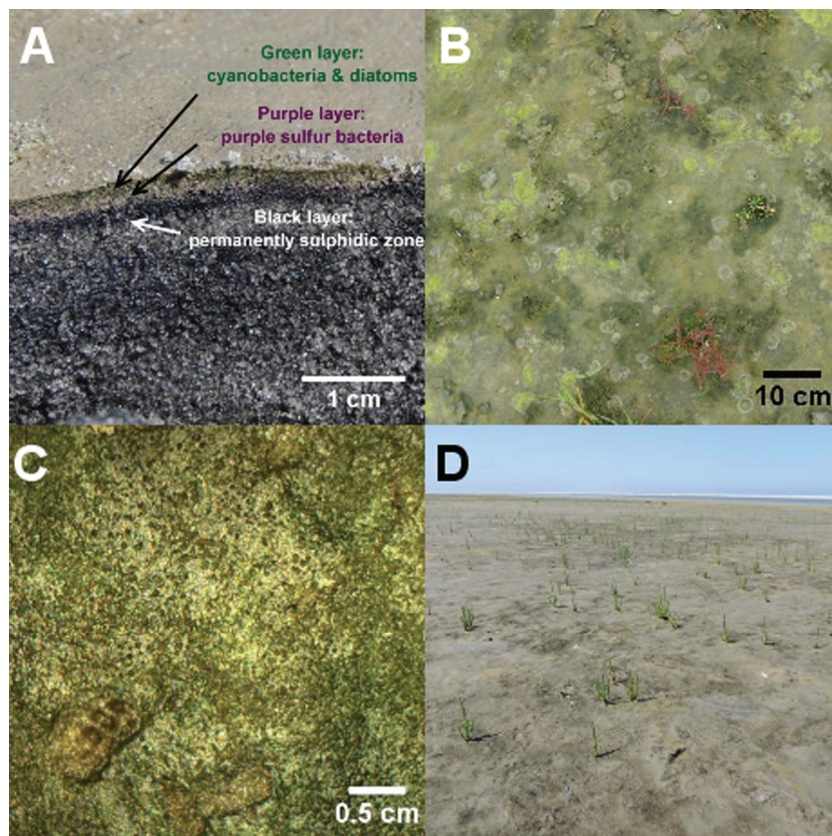


Figure 1.1. Side view of the photosynthetic microbial mat found in Schiermonnikoog (Wadden Sea Island, The Netherlands) with the different characteristic microbial layers (A); Colour image of top view of photosynthetic microbial mats (B), a magnified area (C); and view of the microbial mats in Schiermonnikoog (D).

1.2. Microbial spatial ecology

Landscape ecology is the study of the relation between spatial patterns and ecological processes (Turner 2005). Depending on the process to be studied, the scale of spatial resolution has to be adjusted accordingly (Wiens 1989). In marine microbial ecology, prokaryotes control biogeochemical cycles in the oceans, manipulating the Earth's environment. However these effects are the product of interactions at the cell level (Stocker 2012). It is therefore essential to describe microbial ecology at microscales (μm - to mm) in order to capture the actual microbial diversity and production in the marine ecosystems (Paerl & Pinckney 1996, Azam & Malfatti 2007). Studies in the water column have demonstrated that the distribution of microbes and organic matter at microscales is heterogeneous (Long & Azam 2001, Seymour et al. 2004, Azam & Malfatti 2007). This is, in great part, due to the chemotactic behaviour of prokaryotes (movement towards a chemical cue), which creates a heterogeneous distribution with hot spots of elevated activity (Fenchel 2002). Microscale dissolved organic matter (DOM) degradation has been suggested to increase twofold as a result of prokaryotic chemotactic behaviour (Fenchel 2002). Another study predicted that phytoplankton production is higher in heterogeneous conditions, as compared to a homogeneous situation (Brentnall et al. 2003), probably as a result of chemotactic prokaryotes that supply phytoplankton with nutrients (Stocker 2012). Such findings have implications for the way we frame marine biogeochemical cycling by microbes. Nevertheless, in most microbial ecology studies, this heterogeneity is averaged out when using traditional sampling methods (e.g. Gasol et al. 2009). In shallow sediments, the use of microelectrodes and optical sensors, revealed that the microbial activity varies at scales smaller than generally sampled (cm to metre - m) (Glud et al. 1999, Revsbech 2005). Yet, few studies actually describe the microscale distribution of microbes in sediments. For example, in intertidal sediments, oxygenic photoautotrophs (as inferred from chlorophyll *a* concentration and imaging) have been found to be distributed in patches, ranging from mm to cm (Seuront & Leterme 2006, Chennu et al. 2013). To my knowledge, no data on microscale spatial distributions of other microbes in intertidal sediments and of the key players in photosynthetic microbial mats are yet available. Therefore, in

this thesis I focus on microscale spatial distributions of major oxygenic photoautotrophic groups, prokaryotes and viruses in intertidal sediments and photosynthetic microbial mats.

Equally important to describing microbial ecosystems at microscale is to understand what drives the patterns of distribution. Physico-chemical factors (e.g. light, oxygen, sediment type, input of organic matter) are the primary factors that allow or restrict microbial life in any shallow benthic ecosystem. However, as communities are established, biological interactions and activities (e.g. grazing, pathogenicity, bioturbation) become important for the distribution of microbes. In the next section, I will describe some physico-chemical and biological drivers determining the distribution of microbes in intertidal sediments and photosynthetic microbial mats.

1.2.1. Physico-chemical drivers of microbial distribution

To survive all organisms need a source of energy, electrons, and carbon (Canfield et al. 2005), therefore these are the crucial primary factors for the development of a biological community. These resources are distributed in the sediment, and prokaryotes move to reach or retract from patches of chemical gradients (Barbara & Mitchell 1996, Fenchel 2008). Organic matter is central to the development of any heterotrophic microbial community. Sources of organic matter in intertidal sediment and microbial mats come mostly from exudation and cell lysis (although in intertidal sediments it might also come from invertebrates fecal pellets) (Volkman et al. 2000, Fenchel et al. 2012). Therefore its distribution is heterogeneous horizontally, but it decreases with depth as it is being consumed (Danovaro et al. 2001, Jonkers et al. 2003).

Higher concentrations of organic matter in the sediment increases the light attenuation as the organic material absorbs light (Haardt & Nielsen 1980). Light intensity, quality, and penetration depth is of major importance for the spatial distribution of the photoautotrophs (Kühl et al. 1994a). As diatoms seem to resist higher light intensities than cyanobacteria, they will typically be found at the surface, with cyanobacteria just beneath (Jørgensen et al. 1983, Kühl et al. 1994b). The type of sediment also influences the occurrence of oxygenic photoautotrophs, e.g.

cohesive silty-clay sediment usually harbours diatoms, whereas in non-cohesive sandy sediments, cyanobacteria predominate (Guerrero et al. 1994, Stal 1994, Watermann et al. 1999, Jesus et al. 2009). Additionally, smaller-sized sediment particles attenuate more light compared to larger particles, and red and infrared light penetrates deeper in the sediment than low wavelength light (Fenchel & Straarup 1971). Other dominant microbial populations are vertically stratified in the typical microbial mat relative to their requirements for electron acceptors (like oxygen and sulfate). Oxygen availability changes throughout a daily cycle, forcing microbes to adjust their position (Fenchel 2002). Oxygen is produced by the oxygenic photoautotrophs at the top of the mat and is the most favourable electron acceptor thermodynamically (Canfield et al. 2005). In such sediments, strong diurnal variations in oxygen concentration in the top sediment are often observed. During the day oxygen concentration is supersaturated in the top sediments due to photosynthesis, whereas, at night it is often depleted due to respiration (Van Gemerden 1993, Jørgensen 2006). Oxygen has been shown to be heterogeneously distributed on small vertical scale in sediments (Wenzhöfer & Glud 2004, Hancke et al. 2014) and microbial mats (Glud et al. 1999), due to microscale differences in microbial oxygen consumption rates and irrigation of the sediment. Oxygen can stimulate or inhibit prokaryote growth, depending on whether prokaryotes are aerobic or anaerobic respectively, and thus spatially separating the different prokaryotic populations (Paerl & Pinckney 1996).

Once oxygen is depleted in the sediment, the next most common electron acceptor is sulfate. Anaerobic sulfate respiration results in sulfide production, which can react with iron producing various iron sulfide phases. Several reactions and stages occur between the reduction of sulfate to sulfide, and the oxidation of sulfide back to sulfate (Jørgensen & Kasten 2006). These reactions are performed by different groups of prokaryotes that will distribute themselves in the sediment depending on the available conditions, such as light, oxygen or carbon sources (Jørgensen 2006).

1.2.2. Biological drivers of microbial distribution

Grazing, competition, and pathogenicity are biological factors that cause cell removal, thereby structuring microbial mat communities. Microorganisms, such as oxygenic photoautotrophs may be grazed upon by invertebrates, or killed by viruses, algicidal bacteria or fungi. The impact of grazers on the spatial distribution of benthic oxygenic photoautotrophs and prokaryotes have been well studied (e.g. Sommer 2000, Liess & Hillebrand 2004, Weerman et al. 2011). However, the spatial distribution of microbial pathogens and their impact on oxygenic photoautotrophs and prokaryotes has been understudied thus far. In this thesis, I focused on the role of viruses and fungi as drivers of spatial distributions of the numerically dominant prokaryotes and oxygenic photoautotrophs in intertidal sediments and photosynthetic microbial mats, respectively.

1.2.2.1. Viruses

About 25 years ago it was discovered that high viral abundances occurred in marine aquatic systems (Torrella & Morita 1979, Bergh et al. 1989). This triggered a new research field within aquatic microbial ecology, to study viral diversity, population dynamics, community composition, food web efficiency and biogeochemical cycling (Middelboe et al. 2003, Suttle 2007, Brussaard et al. 2008). Viruses are small (20 - 1000 nanometre) obligate parasites that form the largest pool of genetic diversity globally (Breitbart 2012). With an average 10^{23} viral infections per day in the oceans they are responsible for the release of 10^8 - 10^9 tonnes of carbon per day (Suttle 2007). Viruses are composed of DNA or RNA enveloped in a protein capsid with varying shapes. Viruses cannot move, respire, or grow, thus to survive viruses must infect a host with its genome which then directs viral replication within the host cell. Replication essentially occurs via a lytic or lysogenic cycle (Weinbauer 2004, Paul & Weinbauer 2010). In a lysogenic infection, the viral genome is incorporated into the host genome and the genetic material is transmitted with every cell division, until external stimuli prompts it to activate and go into the lytic cycle (Paul & Weinbauer 2010). In a lytic infection, the virus overtakes the host and uses the host's machinery

for replication, producing progeny, which takes place directly after infection. Upon lysis of the host cell, the newly produced virus particles are released into the surrounding environment. Viral infection, life cycle and production are influenced by physico-chemical (e.g. temperature, UV-light, nutrient availability, organic and inorganic particles) and biological (e.g. grazing and host morphology) factors (Mojica & Brussaard 2014).

Marine aquatic viruses have been shown to affect biogeochemical cycles by recycling organic matter and nutrients in the microbial loop (“viral shunt”; Suttle 2005, Brussaard et al. 2008), but also impact the diversity of microbial communities (“kill the winner” theory; Thingstad 2000). In this way no single species dominates the system but an array of species can thrive, thereby contributing to the coexistence of competing species (“Hutchinson paradox”; Hutchinson 1961). This leads to a constant battle between viruses and microbes labelled the “arms race” theory, which predicts that both players will be constantly evolving in order to overcome each other’s mechanisms of defence/attack (Dawkins & Krebs 1979, Stern & Sorek 2011).

Most work in marine viral ecology has been concentrated in water column studies, but observations of large densities ($10^7 - 10^{10} \text{ g}^{-1}$) and activities of viruses in marine sediments have suggested that viruses also play key roles in these environments (Suttle 2005, Danovaro et al. 2008). The high viral load in sediments is most likely the result of the higher volumetric microbial activity observed in sediments and microbial mats (Jørgensen et al. 1983, Haglund et al. 2002). Viruses in coastal sediments were shown to correlate with prokaryotic abundance and activity (Middelboe et al. 2003, Mei & Danovaro 2004, Middelboe & Glud 2006). Natural benthic microalgae were found to be negatively affected by viruses, as photosynthesis decreased after enrichment with viral concentrates (Hewson et al. 2001).

In general the average virus to prokaryotes ratio (VPR) of 7 in sediments is somewhat higher than in the water column (Weinbauer 2004, Danovaro et al. 2008). This difference may possibly be linked to the need to counteract the higher losses from dispersal in liquid environment, in contrast to benthic systems, where viruses are more “stuck” into the biofilm. In sediments, virus dispersal could be achieved by

transport with interstitial water, diffusion, cell to cell transmission, or “virus biofilms” (Mothes et al. 2010, Thoulouze & Alcover 2011).

Although several studies have examined the impact of viruses in sediment systems, there is virtually no information available on viruses and their ecological role in photosynthetic microbial mats. Moreover, most sediment sampling, thus far, has been performed on cm scale at best, while it is clear that microbes in sediments and microbial mats are active at microscale (e.g. Revsbech 2005).

1.2.2.2. Fungi

In terrestrial environments fungi are the main remineralisers of organic matter, however in aquatic systems their role has been largely ignored until recently (Sigee 2005). Fungal trophic states can be symbiotic (e.g. lichens), saprophytic, parasitic or predatory (Sigee 2005). Saprophytic fungi can degrade refractory organic matter to labile organic matter or to inorganic matter. However, parasitic fungi (nutrients extraction before killing host) or predatory fungi (nutrient extraction after host is killed) tend to attack prokaryotes, oxygenic photoautotrophs, other fungi, and protozoa. Parasitic microscopic fungi (chytrids) have been found to control freshwater diatoms and cyanobacterial bloom decline (Canter & Lund 1948, Gerphagnon et al. 2013), and to relate to phytoplankton succession in lakes (Kagami et al. 2007). The fungus genera *Acremonium* and *Emericellopsis* inhibited growth or caused lysis of several cyanobacteria and green algae species by producing a heat stable extracellular compound thought to be the antibiotic cephalosporin C (Safferman & Morris 1962, Redhead & Wright 1978, Redhead & Wright 1980). It was suggested that the fungal lysing agent was only effective when close to the cells, due to the difficulty of the antibiotic penetrating the mucus surrounding the cells.

Fungi have been found in more extreme environments such as deep-sea hydrothermal systems and hypersaline microbial mats (Cantrell et al. 2006, Le Calvez et al. 2009), and it has been suggested that fungi in these mats are an active and important component of organic carbon degradation (Cantrell & Duval-Pérez 2013). Figure 1.2 shows a suggested microbial loop for sediments and microbial mats where fungi are also included. However, more studies are needed to elucidate

how and to what extent fungal activity affects the spatial distribution of microbial mat members.

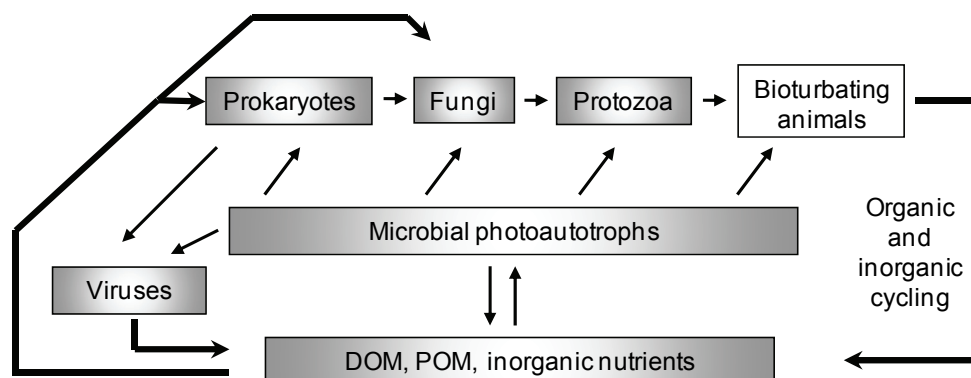


Figure 1.2. Suggested microbial loop scheme for microbial mats and sediments showing energy flow (adapted from Azam et al. 1983). Grey squares represent the loop in microbial mats, whereas grey and white squares represent the loop in sediments.

1.3. Outline of thesis

Benthic ecosystems are structured habitats with the microbial activity being compacted into the top centimetres. To accurately study such environments we must examine them at the relevant microscales at which microbes interact. Furthermore, it is crucial to understand the factors influencing this microscale distribution of microbes. Much work has been done regarding physico-chemical parameters structuring microbial activity and distribution in benthic habitats. However, detailed studies on biological interactions and loss factors, other than grazing, that underlie the horizontal and vertical distribution of oxygenic photoautotrophs and prokaryotes, is still largely missing (partly due to the lack of appropriate methods thus far). The work presented in this thesis focuses on the microscale distribution of intertidal benthic microbial communities, and the biological factors affecting their distribution. To address this, new methodologies were established, microscale sampling performed, and the role of viruses and fungi as structuring agents examined.

In **Chapter 2** I studied microscale (mm) distribution of viruses, prokaryotes and oxygen in a tidal sediment from Dunstaffnage Bay (Scotland). It was observed that the horizontal and vertical distribution of prokaryotes and viruses is highly

variable and parallel studies of viral and prokaryote temporal dynamics suggested that viral activity contributed to the microscale spatial heterogeneity.

Extraction of viruses from photosynthetic microbial mats using the existing sediment methods proved difficult to obtain good quality and reproducible results. Therefore, I developed an extraction method for viruses from photosynthetic microbial mats (**Chapter 3**). The method also provided better results than obtained with existing methods when applied to sediment samples. Furthermore, the developed method allowed easy application of flow cytometry (FCM), thereby providing a fast and accurate method that can open the research field of viral ecology in microbial mats.

In **Chapter 4** I describe a new method to distinguish between the two main oxygenic photoautotrophs (filamentous cyanobacteria and benthic diatoms) in microbial mats, by using autofluorescence imaging. This method allowed study of the spatial distribution of cyanobacteria and diatoms in a simple, fast and non-destructive way, both in culture and in natural mats.

In **Chapter 5** the microscale distribution of viruses, prokaryotes and oxygenic photoautotrophs were studied spatially and temporally (November 2012, April, and July 2013) in an intertidal microbial mat on the Wadden Sea Island Schiermonnikoog, The Netherlands, using the newly developed methods presented in Chapters 3 and 4. The viral abundances in the photosynthetic microbial mat system belonged to one of the highest reported in a natural system. Additionally, the spatial and temporal distribution of viruses in photosynthetic mats suggested that viruses are important structuring components in these productive environments.

During field work, ring-like structures were observed on intertidal photosynthetic microbial mats from Schiermonnikoog, The Netherlands. In **Chapter 6** I studied in more detail these ring-like structures. A fungus (*Emericellopsis* sp.) was identified as the cause of the ring structures by degrading the oxygenic photoautotrophic layer, leaving a cleared area which was initially re-colonised by diatoms. The implications of the fungal degradation are discussed with regard to the microbial mat succession and productivity.

Finally in **Chapter 7** I present and discuss the main findings of this thesis, but also future prospects in viral microbial mats research.

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Chapter 2

Heterogeneous distribution of prokaryotes and viruses at the microscale in a tidal sediment

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Abstract

In this study we show for the first time the microscale (mm) 2- and 3-dimensional spatial distribution and abundance of prokaryotes, viruses and oxygen in a tidal sediment. Prokaryotes and viruses were highly heterogeneously distributed with patches of elevated abundances surrounded by areas of ~3-fold lower abundance within distances of < 2 mm. Abundances of prokaryotes and viruses ranged from $1.3 - 4.2 \times 10^9$ cells cm^{-3} and $4.1 - 13.1 \times 10^9$ viruses cm^{-3} , respectively. The results showed oxygen concentration and uptake rates to be heterogeneously distributed at the same spatial scale, with the oxygen penetration depth varying from 1.5 to 5.8 mm and with an average diffusive oxygen uptake of $18.9 (\pm 6.4)$ $\text{mmol m}^{-2} \text{d}^{-1}$. Locally, prokaryotes, viruses and oxygen were found to be positively, negatively or not correlated, but overall no significant relationship was detected. The lack of consistent spatial correlation between viruses and prokaryotes was explained by a temporal experiment using organic carbon-enriched homogenised sediment samples. Here it was documented that enhancement in metabolic activity and the proliferation of prokaryotes and viruses were not completely phased. These results suggest that local nourishment is likely to be an important driver of a high microscale heterogeneity in abundance and dynamics of benthic viruses and prokaryotes. This is expected to influence the rates and regulation of benthic virus-host interactions and thus microbial biogeochemical cycling.

Key words: Spatial heterogeneity, temporal dynamic, hot spots, virus-host interactions

2.1. Introduction

Virus abundance in marine sediments are generally 10 - 100 times higher than in the pelagic zone (Weinbauer 2004), yet still very little is known about their role in benthic environments. The conditions in sediments differ in many ways from those of free water masses, but three factors are of particular importance for prokaryote-virus interactions: sediments hold an enormous surface area, have low transport coefficients and the volume-specific microbial activity of surface sediments are orders of magnitude higher than those in water columns (Glud 2008).

Generally the abundance of viruses correlates positively with prokaryote abundance (e.g. Middelboe et al. 2006, Danovaro et al. 2008) and activity (Middelboe et al. 2003) in sediments, and viruses have been shown to be a dynamic component of microbial communities in marine sediments with potentially large impact on prokaryote mortality and biogeochemical cycling (Hewson et al. 2001, Fischer et al. 2003, Glud & Middelboe 2004).

Prokaryote and viral abundance normally decrease with sediment depth, as resolved by traditional vertical sediment slicing at cm to m resolution (e.g. Glud & Middelboe 2004, Hewson et al. 2001, Middelboe et al. 2011). Horizontally, viral distribution has been monitored along transects at spatial scales ranging from a few cm to km distance (e.g. Hewson et al. 2001, Middelboe & Glud 2006, Middelboe et al. 2006). These studies concluded that despite overall positive relation between trophic status and the abundance of prokaryotes and viruses, most of the variability is expressed on a much smaller spatial scale, with large differences in abundances of prokaryotes and viruses in sediment patches separated by a few cm (Hewson et al. 2001, Middelboe & Glud 2006, Middelboe et al. 2006). Since the microbial metabolic activity is driven by a highly patchy microscale distribution of both electron donors and acceptors (Fenchel & Glud 2000, Glud et al. 2005, Glud et al. 2009) none of these studies have focused on the most relevant spatial scale for interactions between microbial communities and viruses.

In the present study we examined the microscale (mm) 2- and 3-dimensional spatial distribution and abundance of prokaryotes and viruses in combination with oxygen measurements in shallow coastal sediments to map microscale distribution in

microbial abundance and activity. Additionally, to resolve possible lack of correlation between virus and prokaryote abundance, we also examined successive changes in the abundance of prokaryotes and viruses in homogenized sediment samples as a function of the community metabolic activity.

2.2 Material and methods

Study site and sediment characteristics

Sampling was conducted in November 2009 at low tide in Dunstaffnage Bay (West Coast of Scotland) (56°27.0"N, 5°26.4"W) at a water temperature of 9°C. The sediment was sampled using a custom made sampler, allowing the transfer of intact sediment cores into the flow chambers. Three cores were sampled and used for general characterization of sediment porosity and the content of organic matter. Briefly, 3 sediment cores were sampled and sliced every 0.5 cm to a maximum depth of 5 cm. Porosity (ϕ) was determined from the density and the weight loss after drying at 105°C for 24 h, and decreased from 0.6 v/v at the sediment surface to 0.40 v/v at 5 cm depth. The content of organic matter was measured from the weight loss of dried samples after combustion at 450°C for 24 h, and was highest in the top 0.5 cm (2.3 % dry weight) gradually declining to ~ 1.7 % at 5 cm depth. At the same time, undisturbed, intact sediment was sampled and immediately transferred into pre-constructed flow chambers (20 × 15 × 8 cm; H × L × W). Once in the laboratory, the sediment was kept submerged under re-circulating seawater at *in situ* temperature (9 ± 1°C) and O₂ concentration for 2 days before experiments were carried out.

Two experiments (2-dimensions, 2D; and 3-dimensions, 3D) were performed with the sediment. In each experiment, abundance of prokaryotes and virus were measured in parallel to oxygen (O₂) concentrations and dynamics. The measurements of O₂ concentrations and dynamics were performed using planar optodes (2D) and microelectrodes (3D).

Planar optode imaging

The principles for planar optode imaging have been previously described in detail (Glud et al. 1996, Holst et al. 1998, Holst & Grunwald 2001) and is only briefly described below. The imaging system was based on a 1.3 megapixel fast gatable, 12-bit, Peltier cooled, CCD camera (Charged Couple Device) (SensiCam, www.PCO.de) equipped with a 25 mm/1.4 Nikon wide-angle lens equipped with 690 nm long-pass dichroic colour filter (UQG optics, www.UQGoptics.com) (Frederiksen & Glud 2006). Excitation light was delivered from 4 high power LED's (light emitting diode) (λ -peak = 445 nm, LXHL-LR3C, Luxeon, F = 340 mW at IF = 700 mA), equipped with a 475 nm short-pass dichroic colour filter (UQG optics, www.UQGoptics.com). To allow measurements of the relative short-lived phosphorescent lifetime, the camera and a LED power supply was synchronized with custom made trigger box. The trigger box was connected to a PC and controlled by the software Look@Molli (Holst & Grunwald 2001). The phosphorescent lifetime was inferred from two well defined time frames configured by the Look@Molli software. Recorded images were calibrated using the phosphorescent lifetime recorded in two areas where the foil was exposed to known O₂ concentrations (100 % air-saturation in the overlying water and 0 % air saturation in deep anoxic sediment layers), and using a rearranged modified Stern-Volmer equation.

$$C = \frac{T_0 - \tau}{K_{SV} \cdot (\tau - \tau \cdot \alpha)} \quad (1)$$

Where τ_0 is the phosphorescent lifetime in the absence of O₂ and τ is the lifetime in the presence of any given O₂ concentration (C), K_{SV} the bi-molecular quenching constant and α , the non-quenchable fraction of the phosphorescent signal, which was experimentally determined to be 0.14. All images were recorded in darkness to avoid potential interference from ambient light. Images were recorded using a 16 image average to increase the signal to noise ratio. The maximum theoretical spatial resolution that could be achieved with the given optical setup was $\sim 100 \times 100 \mu\text{m}$.

Microelectrode profiling

O₂ profiles were measured by Clark type microelectrodes equipped with an internal reference and a guard cathode (Revsbech 1989). The sensors had external tip diameters of 10 - 20 µm, a stirring sensitivity < 2 % and a 90 % response time of < 1 s (Gundersen et al. 1998). The sensors were mounted on a motor driven micromanipulator and the sensor currents were measured using a picoammeter (PA 2000, Unisense) and transferred via an A/D-converter to a PC. The O₂ microprofiles were measured at a vertical resolution of 100 µm. The sensor values were calibrated against the known O₂ concentration of the overlying water and zero values recorded in deep anoxic sediment layers. The diffusive oxygen uptake (DOU) was calculated using Fick's first law of diffusion, $J = D_0(dC/dz)$, where dC/dz is the O₂ concentration at a given depth within the Diffusive Boundary Layer (DBL) and D_0 is the molecular diffusion coefficient corrected for salinity and temperature (Glud 2008). Two sensors were mounted on the micromanipulator, thus 2 independent profiles were acquired simultaneously. Profiling of the 30 samples took approximately 6 h. Contour plots were made in Surfer 9.0.

Microscale spatial sampling of prokaryotes and virus

Microscale samples for prokaryotes and viral abundance were taken using a purpose-designed minicore. The plastic minicore had an outer diameter of 3.6 mm and an inner diameter of 3.0 mm and a maximum length of 80 mm. As the objective of this study was to sample both in 2D and 3D, two different devices were used. For the 2D sampling 9 minicores were fixed and aligned side by side separated by a centre distance of 5.6 mm (Fig. 2.1A). The 9 cores penetrated the sediment to a maximum depth of 27 mm. A hand pump was used to keep vacuum and avoid displacement of sediment in the minicores, during recovery (each minicore was connected to the pump by a plastic tube). Each of the nine minicores was sliced vertically at a depth resolution of 3 mm, with a total of 9 slices. Planar optode images were taken on the exact same location before sampling.

In the 3D experiment, the minicores were inserted one by one through a plexiglas plate with a grid of sampling holes (Fig. 2.1B). The plate was fixed to the wall of the flow channel and a total of 30 minicores were recovered, and separated from each other's by an internal centre distance of 11.2 and 5.6 mm in length and width, respectively. Each minicore was inserted to a depth of 6 mm and subsequently sliced in two depth intervals: 0 - 3 mm and 3 - 6 mm. Microelectrode profiling in the same grid holes was done prior to sampling.

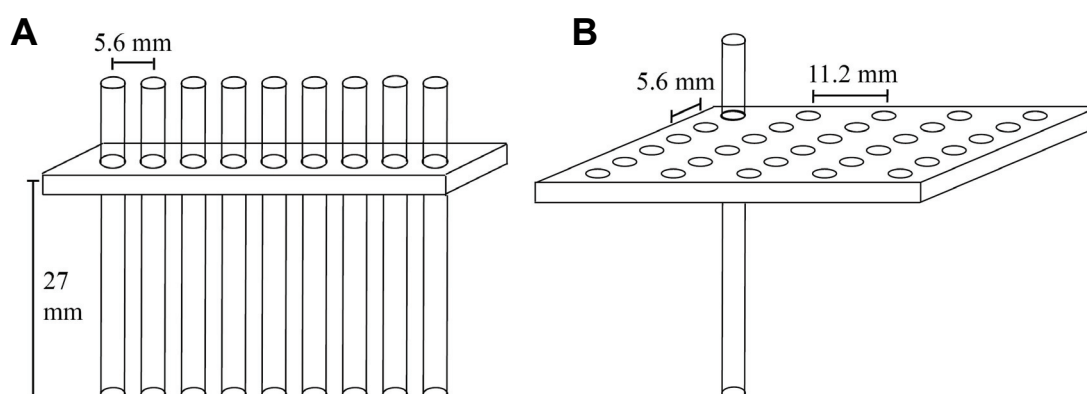


Figure 2.1. Schematic diagram of sampling devices. (A) Two-dimensional structure where the 9 minicores, separated by a centre distance of 5.6 mm, were inserted down to 27 mm depth and along a 48 mm transect. (B) Three-dimensional structure where each minicore was inserted down to 6 mm depth, separated by an internal distance of 11.2 and 5.6 mm in length and width, respectively, in a total of 30 holes within 1706 mm².

Sample preparation and analysis of prokaryotes and viral counts

Each sediment slice (~23 μ L) was placed into a sterile 2 mL tube and fixed with 2 % glutaraldehyde (final concentration) for 15 min at 4°C. The samples were then snap frozen in liquid nitrogen and kept at -80°C until further analysis. Prokaryotes and viruses were extracted and treated from the sediment, according to Danovaro & Middelboe (2010) by the addition of Tetrasodiumpyrophosphate (10 mM final concentration) in the dark for 15 min and sonicated using a probe sonicator (Sonicprep 150; 4 μ m amplitude) in three cycles of 1 min sonication and 30 sec of manual shaking in an ice bath. Then, 5 μ L of the extract were diluted in 1 mL of 0.02 μ m filtered seawater (virus free) and filtered onto a 0.02 μ m Anodisc-filter. Filters were stained in a drop of 10 % SYBR Gold (Noble & Fuhrman 1998) for 15 min and

subsequently rinsed three times in a series of MilliQ water drops. Finally, filters were mounted on a glass slide with an anti-fade solution (50 % glycerol; 50 % PBS pH = 7.5; 1 % *p*-phenyldiamine) and preserved at -20°C. Prokaryotes and viruses were counted on an epifluorescence microscope (Olympus) at 600 - 750 x magnification. 400 prokaryotes and viruses or at least 10 fields were counted per sample. Contour plots were made in Surfer 9.0.

Regression analyses were performed on prokaryote and virus abundance, and O₂ concentration, using the best-fit between the two variables X and Y obtained by regression model I as described in Sokal & Rohlf (1995). Normality was checked and the confidence level was set at 95 % with all statistical analyses conducted in SigmaPlot 12.0.

Temporal dynamics in homogenized sediment

To understand the potential lack of correlation between prokaryotes and virus, another experiment was established where prokaryote and viral abundance and production, and community respiration (Dissolved inorganic carbon - DIC) were followed over time under enriched conditions at two different temperatures (8 and 13°C). The two temperatures were applied to see if the same temporal pattern in virus and prokaryote abundance developed at two different metabolic activity levels. Approximately 500 g sediment from 3 - 6 cm depth was homogenized, enriched with yeast extract (~12.5 mmol C kg⁻¹) and transferred to duplicate gas tight Würgler bags (Hansen et al. 2000) under a nitrogen atmosphere in a glove bag. Samples for prokaryote and viral abundance, and DIC concentration were collected every 4 h for the first 24 h where after the sampling frequency was reduced until the end of the experiment after 136 h. For practical purposes involving multiple sampling of the same homogenised sediment samples under anoxic condition, and to overcome microscale heterogeneity that may develop in the bags, a larger sediment volume was sampled (10 g) and processed according to Danovaro & Middelboe (2010). For DIC measurements, pore water from sediment was retrieved after centrifugation (5 min, 2000 x g), and preserved in 2 mL gas-tight vials containing 20 µL saturated HgCl₂ at 5°C until further analysis on an infrared gas analyser (ADC-225-MK3)

(Glud & Middelboe 2004). Rates of respiration were calculated by linear regression of the increase in DIC concentration over time, whereas exponential rates of prokaryote and viral net production and decay were calculated by linear regression of changes in ln-transformed plots.

2.3. Results

Microscale 2-dimensional spatial distribution

Oxygen images resolved an O₂ penetration depth (OPD) that varied from 1.5 to 2.3 mm along the primary interface (Fig. 2.2). As a result of irrigating fauna a few patches of elevated O₂ concentration were observed in the deep otherwise anoxic sediment. The contour plots showed microscale variability in the distribution of prokaryotes and viruses (Fig. 2.3A, B) across distances as small as 2 mm over the 2D area sampled (1307 mm²), i.e. $1.3 - 4.2 \times 10^9$ prokaryotes cm⁻³ and $4.1 - 11.3 \times 10^9$ viruses cm⁻³, respectively. The spatial dispersal of both prokaryotes and viruses was characterized by patches with higher abundance surrounded by areas with 3-fold lower abundances. The distribution varied from similar patterns of prokaryotes and viruses (i.e. high prokaryote abundances associated with high viral abundances and vice versa) to opposite patterns (i.e. high prokaryotes - low viruses or low prokaryotes - high viruses) (Fig. 2.3A, B).

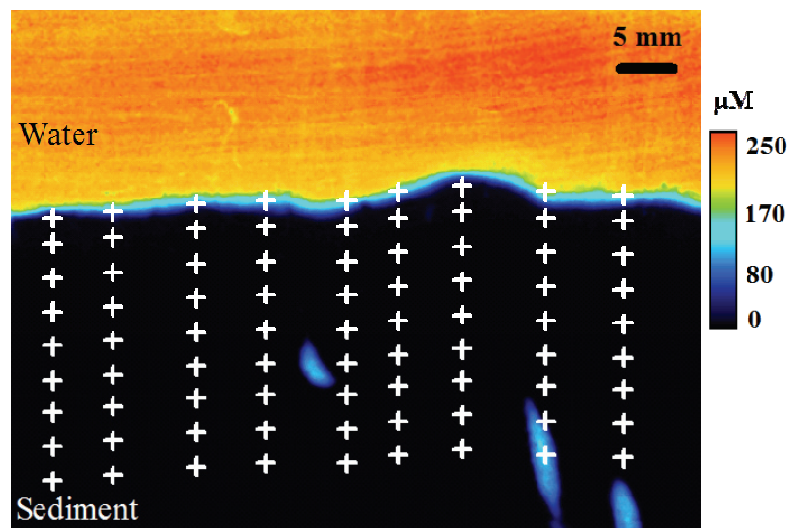
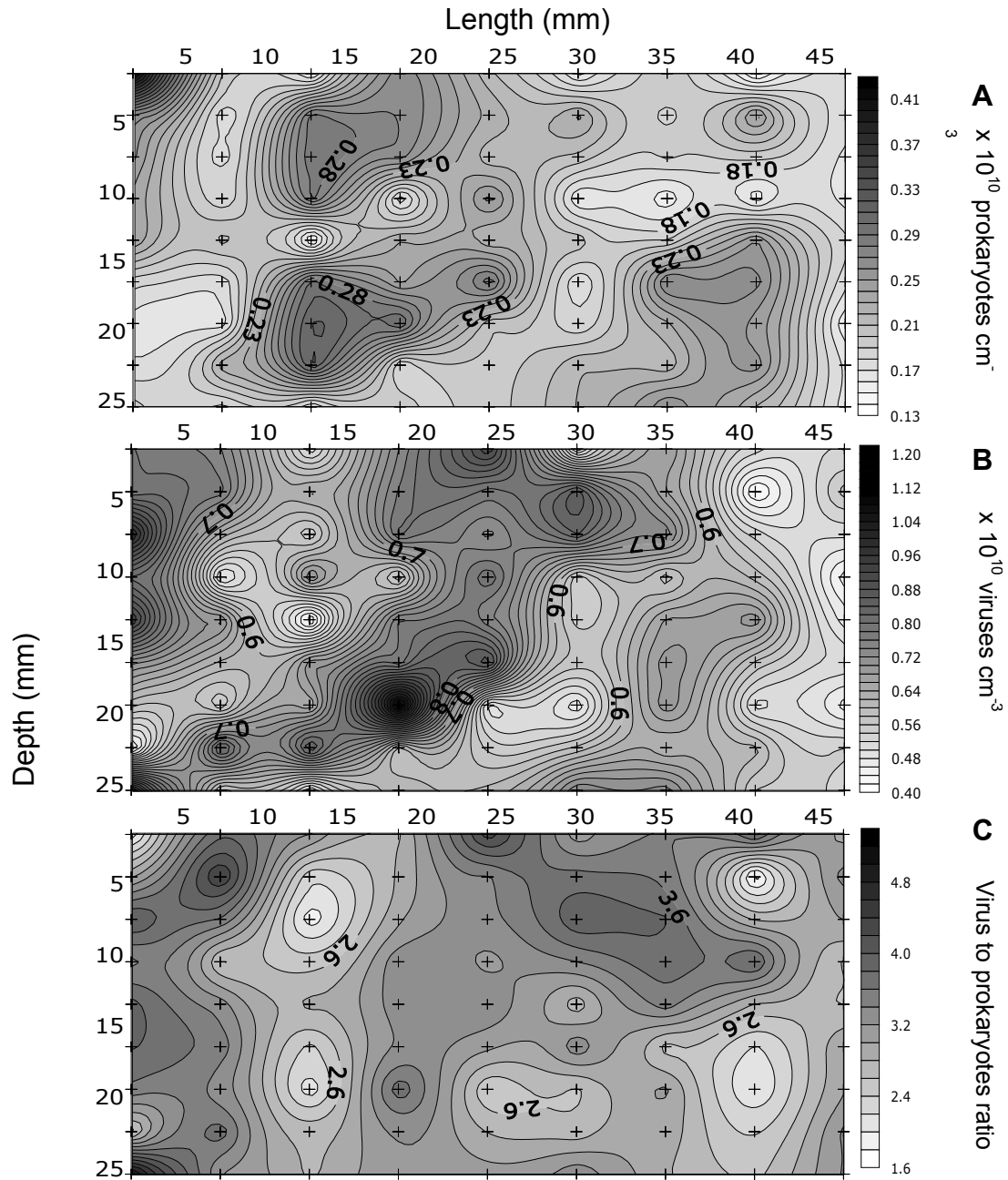


Figure 2.2. Planar optode image representing the average two-dimensional O_2 concentration distribution in the surface sediments as calculated from the 166 optode images, recorded previously to the microscale sampling. Crosses represent the position of the microscale sampling for prokaryotes and virus in the sediment with water above. Calibration bar represent O_2 concentration in μM .

The subsequent range in virus to prokaryotes ratio (VPR) was between 1.7 and 5.1, without a clear spatial pattern (Fig. 2.3C). Prokaryotes abundance, viral abundance and O_2 concentrations did not exhibit statistically significant correlations ($p > 0.05$, $n = 83$).



the top layer (0 - 3 mm; Fig. 2.5A). O₂ concentration in the bottom layer (3 - 6 mm; Fig. 2.5B) was mostly below 1 µM of O₂, although areas with elevated O₂ concentration were observed in a few profiles. The mean diffusive O₂ uptake derived from the O₂ concentration gradient of the DBL was $18.9 \pm 6.4 \text{ mmol m}^{-2} \text{ d}^{-1}$, which is a typical value for coastal marine sediments.

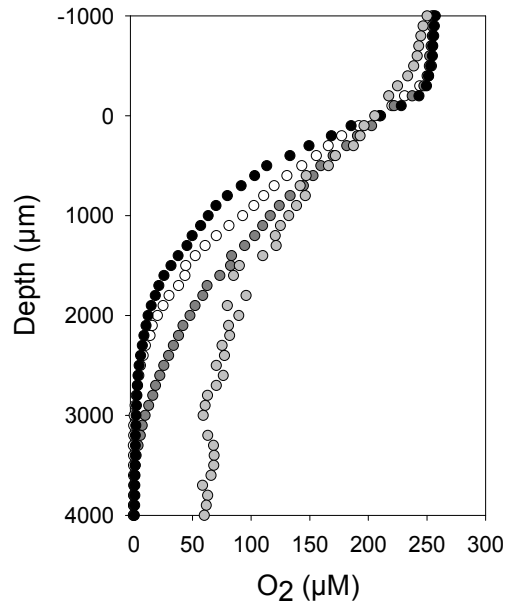


Figure 2.4. Representative O₂ profiles recorded with the microelectrode from the 3-dimensional experiment. Profiles were recorded in all sampling spots prior to sampling. The position Y = 0, represents the sediment surface.

Prokaryotes and viral abundances in the top layer (0 - 3 mm layer) varied from 1.4 to $2.8 \times 10^9 \text{ cells cm}^{-3}$ and 4.4 to $13.1 \times 10^9 \text{ viruses cm}^{-3}$, respectively (Fig. 2.6A and 2.7A). In the bottom layer (3 - 6 mm) they ranged from 1.5 - $3.3 \times 10^9 \text{ prokaryotes cm}^{-3}$ and 4.4 - $12.8 \times 10^9 \text{ viruses cm}^{-3}$ (Fig. 2.6B and 2.7B). The spatial distribution was characterised by areas of elevated prokaryotes or viral abundance occurring next to low-density areas within a few mm distance.

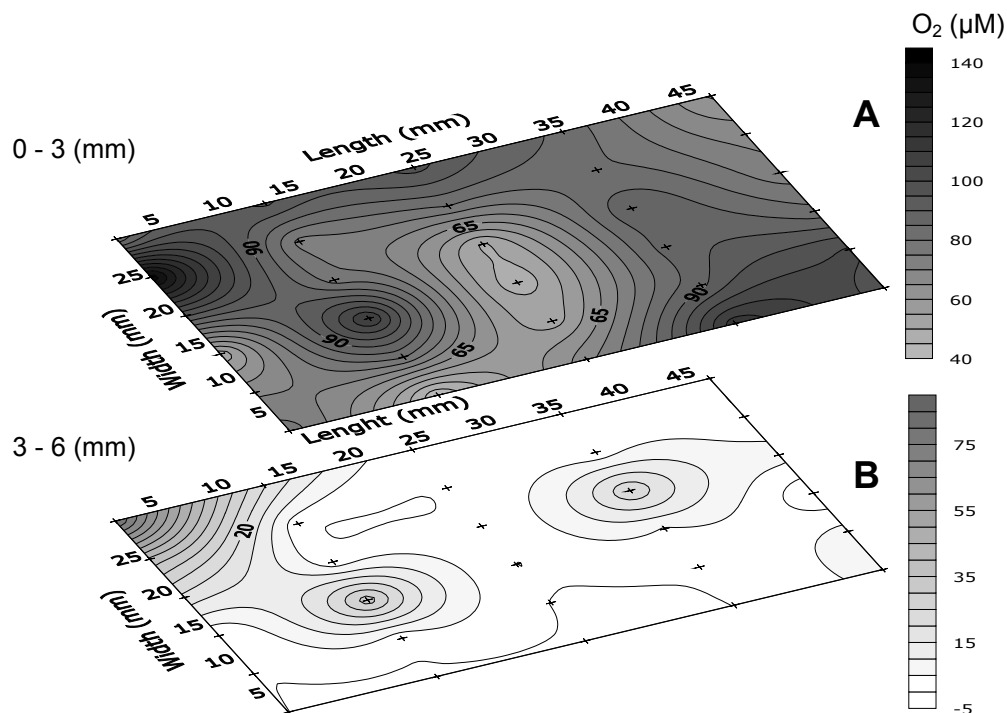


Figure 2.5. Three-dimensional (48.4 x 31.6 x 6 mm) average O_2 concentration in the (A) 0 - 3 mm and (B) 3 - 6 mm sediment layer, composed of 30 individual microelectrode profiles. Crosses represent position of the individual oxygen profiles. Calibration bar represents oxygen concentration in μM .

Comparing the spatial distribution of prokaryotes and viruses in the top layer (0 - 3 mm, Fig. 2.6A and 2.7A), showed a few locations with similar patterns (high prokaryotes - high viruses or low prokaryotes - low viruses), but mostly there was a spatial uncoupling of prokaryotes and viral abundance (high prokaryotes - low viruses; low prokaryotes - high viruses), and overall, no significant correlation between prokaryotes and viruses or oxygen was observed ($p > 0.05$, $n = 26$). In the bottom layer (3 - 6 mm), viral abundances tended to follow the prokaryotes distribution (Fig. 2.6B and 2.7B) although the relationship between prokaryotes and viruses in this anoxic layer overall was not significant ($p > 0.05$, $n = 27$).

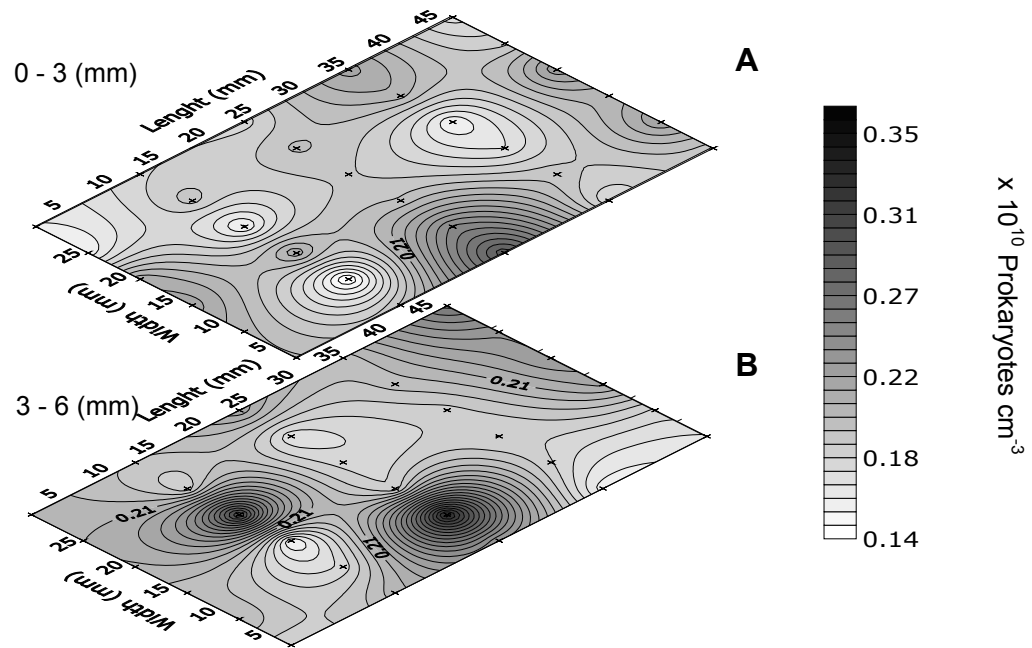


Figure 2.6. Three-dimensional (48.4 x 31.6 x 6 mm) distribution of prokaryotes abundance in the (A) 0 - 3 mm and (B) 3 - 6 mm sediment layer. Crosses represent the position of the microscale sampling. Scale bar represents prokaryotes $\times 10^{10} \text{ cm}^{-3}$.

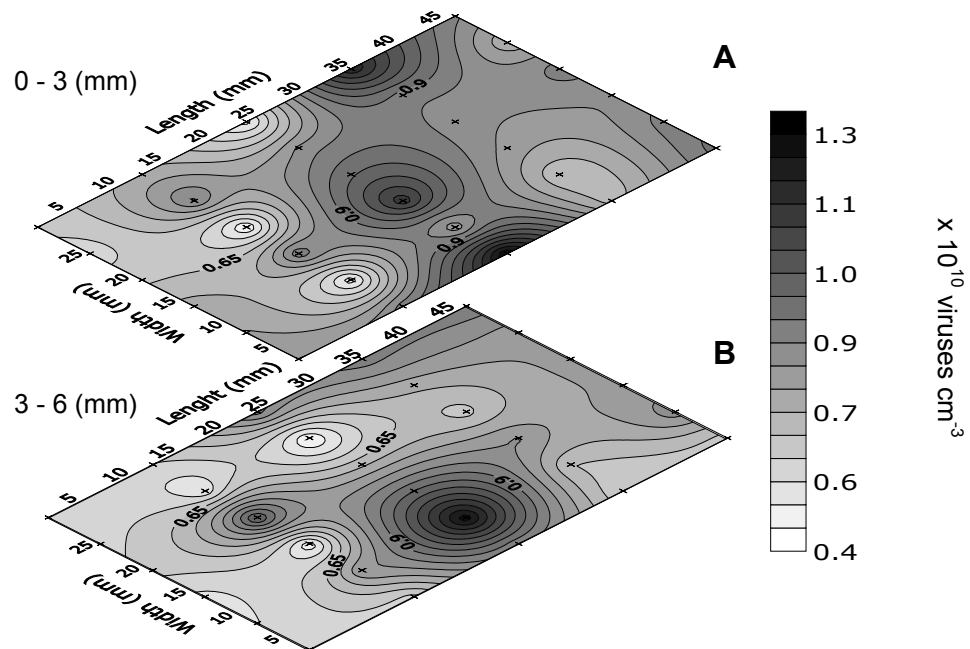


Figure 2.7. Three-dimensional (48.4 x 31.6 x 6 mm) distribution of viral abundance in the (A) 0 - 3 mm and (B) 3 - 6 mm sediment layer. Crosses represent the position of the microscale sampling. Scale bar represents viruses $\times 10^{10} \text{ cm}^{-3}$.

The VPR was rather similar in the oxic and the anoxic layer, varying from 2.7 to 5.8 in the surface layer (Fig. 2.8A) and from 2.6 to 5.2 in the bottom layer (Fig. 2.8B). The experiment was repeated using a parallel sediment sample, which showed a similar spatial heterogeneity in viral and prokaryote abundances (data not shown).

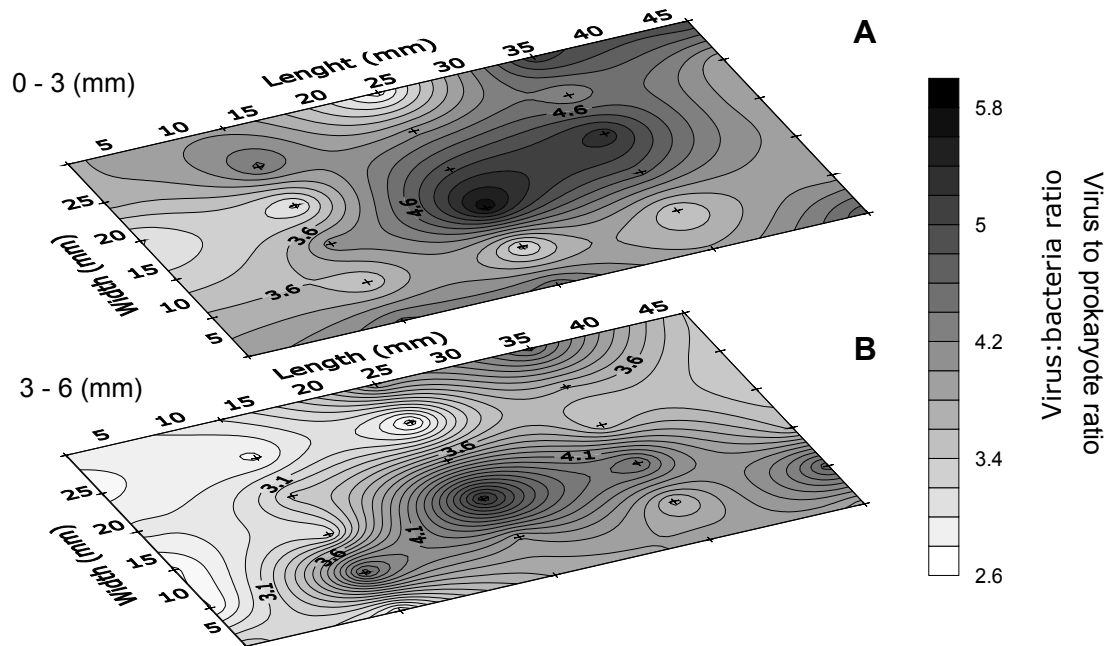


Figure 2.8. Three-dimensional (48.4 x 31.6 x 6 mm) distribution of virus to prokaryote ratio (VPR) in the (A) 0 - 3 mm and (B) 3 - 6 mm sediment layer. Crosses represent the position of the microscale sampling.

Temporal dynamics in homogenized sediment

Changes in prokaryotes and viral abundances in response to community respiration were examined in enriched, homogenized sediment to explore possible mechanisms for the observed spatial uncoupling between their abundances in natural sediments. The rates of change in prokaryotes and viral abundance, and community respiration varied considerably and systematically over the 4 main time periods in both experiments, exhibiting the same temporal pattern at both temperatures (Fig. 2.9A, B). In the initial period (0 - 8 h) prokaryotes, viruses and DIC increased, followed by a decrease in prokaryotes net production, whereas viral

abundance continued to increase (8 - 20 h). Thereafter (20 - 38 h), prokaryotes net production ceased concomitant with net decay in viral abundance despite relatively high community respiration. By the end of the experiment (38 - 135 h), the incubations reached stationary phase without significant rates of changes in abundances or activities.

As a consequence of these differences in the successions of prokaryotes and viruses, the VPR increased from ~4 to 5.5 during the first 20 h of the incubation and then rapidly declined to around 3 (Fig. 2.9C), thus covering the range of VPR obtained in the spatial study (Fig. 2.3C and 2.8).

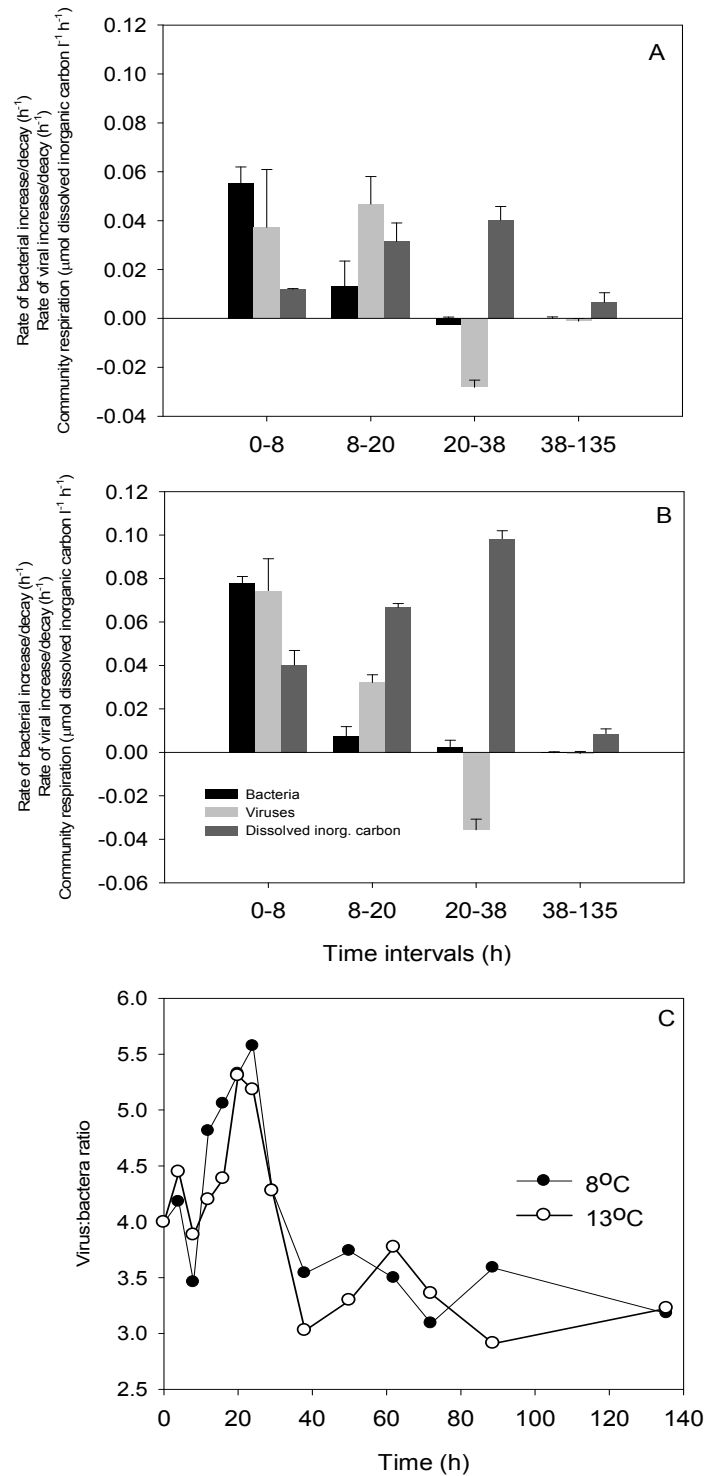


Figure 2.9. Temporal development in community respiration (DIC production), and rates of prokaryotes and viral production and decay during incubations of homogenized sediment samples enriched with labile organic carbon in anaerobic bags at 8°C (A) and 13°C (B). Changes in virus to prokaryotes ratios over time in the same incubations (C).

2.4. Discussion

Here we showed that prokaryotes and viruses in tidal sediments are highly heterogeneously distributed even at the mm scale. Until now spatial variations in prokaryotes and viral abundances have always been monitored in homogenized sediment samples at cm to m scale resolution, and reported values have typically represented average abundances in 30 - 100 cm³ sediment slices (Hewson et al. 2001, Glud & Middelboe 2004). On these spatial scales significant decreases in viral abundance and production have been observed with sediment depth or along trophic gradients (e.g. Hewson et al. 2001, Middelboe & Glud 2006), reflecting reduced microbial activity as availability of organic matter declines, and suggesting prokaryote abundance and metabolic activity as predictors of viral dynamics.

However, benthic microbial and viral dynamics occur on a much smaller scale and are considered to be related to microniches induced by e.g. sedimentation of aggregates, fauna activity, etc (Fenchel & Glud 2000, Glud et al. 2009). At this spatial scale, our data revealed a highly heterogeneous distribution of microbial abundances and oxygen concentration in the upper 27 mm of a temperate, coastal tidal sediment with areas of elevated abundances (hot spots) extending a few mm in vertical and horizontal directions. Prokaryotes and viral abundance did not correlate across the vertical and horizontal gradients analysed, and no clear vertical or horizontal patterns in their distribution were observed at this spatial resolution within the investigated sampling grids. Consequently, microscale viral distribution and the relation to the activity and abundance of their prokaryotes hosts followed a highly different pattern than the distribution patterns obtained from homogenized sediment samples on large spatial scales. The microscale spatial variation found in this study thus supports the accumulating evidence of highly heterogeneous microscale distribution of organic matter (Fenchel 2008) and microbial diagenetic activity in surface sediments as resolved by O₂ and DIC measurements obtained using microsensors and targeted incubation approaches (Fenchel & Glud 2000).

Our observations are in line with the 2D-distribution of prokaryotes and viruses obtained at a similar spatial scale in pelagic environments (Seymour et al. 2006, Seymour et al. 2008) demonstrating an equally heterogeneous and non-correlated

distribution of prokaryotes and viruses, which is consistent with observations of organic matter heterogeneously distributed in aggregates providing microzones of elevated prokaryotes activity (Blackburn et al. 1998). A similar heterogeneous horizontal distribution of prokaryotes abundance was observed at the cm scale in a coastal surface sediment without any correlation to the distribution of their potential food sources, suggesting a time lag between organic matter input and the microbial response (Danovaro et al. 2001). The current study thus emphasizes that microscale microbial dynamics and local diagenetic hotspots are an inherent feature of marine ecosystems, which should be better recognized when evaluating dynamics in diagenetic processing and microbial community distribution.

Viral replication may depend strongly on its host metabolic activity and thus elevated viral abundances are theoretically expected in association with observed niches of intensified prokaryotes activity (Glud et al. 2005). However, due to the time lag from infection to viral proliferation, and to a variable life time of prokaryotes and viral particles in the environment, abundances of prokaryotes and viruses may be uncoupled in time and space. Their distribution thus reflects the recent history of the specific environment rather than the actual activity of the micro habitat. The examination of temporal dynamics in net prokaryote and viral production and community respiration in homogenised sediment showed indeed an uncoupling over time in microbial abundances related to prokaryotes activity in response to organic carbon enrichment. A sequence of events in response to local sediment enrichment resulted in a temporal uncoupling between viral and prokaryotes abundances in the homogenized sediment, leading to 4 distinct scenarios of virus-prokaryote interactions within 48 h: (1) High prokaryotes abundance and low viral abundance (0 - 8 h) as a result of the initial stimulation of prokaryotes activity and a delay in the subsequent viral production, (2) high VPR due to a bloom of viruses in response to the elevated prokaryotes activity (8 - 20 h), 3) high prokaryotes abundance but low viral abundance reflecting a fast decay or irreversible particle-attachment of viruses following the bloom conditions (20 - 38 h), and (4) low prokaryotes and viral abundance during the period of low microbial activity and reduced contact rates (> 38 h).

The study validated that pulses of viral production and decay may indeed occur in response to local organic enrichment in marine sediments creating transient hotspots of elevated viral abundances associated with stimulation of prokaryotes activity (Glud & Middelboe 2004, Siem-Jørgensen et al. 2008). We suggest therefore that the uncoupled microbial abundances and heterogeneous distribution of prokaryotes and viruses at mm scale in surface sediments are driven by the dynamics of formation and degradation of patches of labile organic matter in the sediment, which stimulates prokaryotes respiration and abundance and subsequently viral abundance. The traditional use of larger-scale samples averages out this microscale variability, which may have important implications for the ecology at the microscale relevant for the microbial communities in the sediment.

On a large spatial scale, the positive correlation between viral and prokaryotes abundance and activity suggests that the general level of viral abundance in a given benthic environment is constrained by the levels of average microbial activity in that same environment. On a microscale, on the other hand, the lack of correlation suggests that virus-prokaryote interactions occur as periodic elevations and declines of viral and prokaryotes densities associated with a continuous redistribution of microscale resource patches. Microscale variability in microbial distribution and dynamics has implications for microbial biogeochemical cycling that extends beyond the specific microhabitat, ultimately determining the benthic carbon cycling on the global scale. Caution is therefore required when assessing the role of viruses from studies of viral and prokaryotes activity based on homogenized samples, and there is a need for development of methods to measure *in situ* viral production at the scale and conditions at which they occur.

Acknowledgements

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Chapter 3

Counting viruses and prokaryotes in photosynthetic microbial mats

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Abstract

Viral abundances in benthic environments are the highest found in aquatic systems. Photosynthetic microbial mats represent benthic environments with high microbial activity and possibly high viral densities, yet viral abundances have not been examined in such systems. Existing extraction procedures typically used in benthic viral ecology were applied to the complex matrix of microbial mats but were found to inefficiently extract viruses. Here, we present a method for extraction and quantification of viruses from photosynthetic microbial mats using epifluorescence microscopy (EFM) and flow cytometry (FCM). A combination of EDTA addition, probe sonication and enzyme treatment to a glutaraldehyde fixed sample resulted in substantially higher viral (5 to 33-fold) extraction efficiency and reduced background noise as compared to previously published methods. Using this method it was found that in general, intertidal photosynthetic microbial mats harbour very high viral abundances ($2.8 \pm 0.3 \times 10^{10} \text{ g}^{-1}$) compared with benthic habitats ($10^7 - 10^9 \text{ g}^{-1}$). This procedure also showed a 4.5 and 4-fold increased extraction efficacy of viruses and prokaryotes, respectively, from intertidal sediments, allowing a single method to be used for the microbial mat and underlying sediment.

Keywords: viral abundance, epifluorescence microscopy, prokaryotes, photosynthetic microbial mats, sediment, flow cytometry

3.1. Introduction

Photosynthetic microbial mats are vertically stratified benthic microbial communities that are found worldwide from hot springs to sea ice (e.g. Van Gemerden 1993). The top layer of these mats is mostly composed of oxygenic photoautotrophs (filamentous cyanobacteria and eukaryotic microalgae) that produce organic carbon, which is decomposed in a succession of layers of different heterotrophic prokaryotes reflecting concentration gradients in oxygen and other electron acceptors (e.g. Van Gemerden 1993, Stal 1994, Teske & Stahl 2002, Canfield et al. 2005). The intertwined filamentous cyanobacteria in the top layer and the excretion of exopolymeric substances (EPS), make the microbial mats very stable and resistant to wind and wave erosion (De Brouwer et al. 2002). Viruses are diverse, abundant and ecologically important components of microbial communities, acting as major drivers of biodiversity and organic matter flux (e.g. Larsen et al. 2001, Rohwer & Thurber 2009, Lønborg et al. 2013). In sediments, viruses have been shown to affect prokaryotes host mortality (Danovaro et al. 2008), spatial distribution (Carreira et al. 2013) and biogeochemical cycling (Middelboe & Glud 2006). However, while microbial mats have been intensively studied in regard to their biogeochemistry and biodiversity (e.g. Des Marais 2003, Ward et al. 2006), studies on the ecological role of viruses in these mats are to our knowledge lacking.

One of the challenges of assessing the role of viruses in sediments and other surface associated environments, as photosynthetic mats, is the need for reliable quantitative measures to determine their abundance. Depending on the type of sediment (intertidal, coastal, or deep sediments; Lunau et al. 2005, Kallmeyer et al. 2008, Danovaro & Middelboe 2010), different methods have been used to extract viruses and prokaryotes. In microbial mats, EPS bind microorganisms, viruses and particles together in a complex matrix (Decho 2000) making it more challenging to extract viruses and prokaryotes than from bulk sediments. To allow detailed studies of viruses in microbial mats, modifications to protocols currently used for quantitative assessment of benthic viruses are necessary (Hewson & Fuhrman 2003, Helton et al. 2006, Danovaro & Middelboe 2010). Here, we report an improved assay allowing efficient extraction and enumeration by epifluorescence microscopy (EFM) or flow

cytometry (FCM) of viruses from photosynthetic microbial mats, as well as intertidal sediments.

3.2. Material and methods

Sample collection

Microbial mat samples were collected in Schiermonnikoog island (The Netherlands, 53° 29' 24.29"N, 6° 8' 18.02"E), during March 2011 and July 2012. A detailed description of the coastal microbial mats in this area is provided in Bauersachs and colleagues (2011).

Ten samples of 15 x 8 x 4 cm (L x W x H) were individually collected and placed in clean plastic boxes at *in situ* temperature and taken to the laboratory within 3 - 4h. In the laboratory, samples were kept at 8°C in a 16:8h L:D cycle with a low light intensity ($15 \mu\text{mol quanta m}^{-2} \text{s}^{-1}$) until sampled for viral and prokaryotes enumeration.

Subsamples were collected with a core (0.7 cm inner diameter). The top 1 mm (~100 mg), containing the photosynthetic microorganisms, was sliced with a knife and placed in a sterile 2 ml Eppendorf tube and fixed with 800 μl of 25 % glutaraldehyde (Electron microscopy grade, Merck; 2 % final concentration) diluted in sterile seawater. Samples were kept for 15 min at 4°C in the dark. Tests were performed with four replicate samples each obtained from an individual core. As the various tests were not always performed with the same natural microbial mat samples, the obtained viral and prokaryotes abundances in the individual tests may show some variation.

Extraction of viruses and prokaryotes from photosynthetic microbial mats

The extraction efficiency of viruses (and prokaryotes) from the top layer of the photosynthetic microbial mat was tested using a combination of chemical and physical treatments (Table 3.1). Solutions used for extraction were made with MilliQ water (18.2 M Ω) and only added after fixation of the sample, therefore avoiding

osmotic shock. To promote the release of particle-associated viruses (and prokaryotes) in the microbial mat samples, chemical treatment was first tested with tetrasodiumpyrophosphate (TSPP; Danovaro & Middelboe 2010) and Ethylenediaminetetraacetic acid (EDTA; Helton et al. 2006, Garren & Azam 2010) in combination with water bath sonication (Danovaro & Middelboe 2010). TSPP is commonly used to extract viruses and prokaryotes from sediment particles (Danovaro & Middelboe 2010). EDTA was chosen because it destroys cation links between EPS polymers and sediment particles, thus releasing EPS-bound viruses, and because it is known to permeabilise outer membranes, thereby facilitating dye uptake (Zhang et al. 2000). Both tests were performed with water bath sonication as described by Danovaro and Middelboe (2010).

The most efficient release of viruses was obtained by addition of 0.1 mM EDTA and this addition was then applied in the following comparison of the efficiency of water bath sonication versus probe sonication. Probe sonication resulted in a visual destruction of the microbial mat and showed improved extraction efficiency compared with the sonication water bath treatment. From this comparison, probe sonication was then applied in a series of 10 s sonication cycles (0, 2, 3, 4, 6 and 8) using an ultrasonic probe (Soniprep 150; 50 Hz, 4 μm amplitude, exponential probe) with 10 s intervals while keeping the sample tubes on ice-water. Finally, different EDTA concentrations (no addition, 0.01, 0.1, and 1 mM, final concentrations) were tested specifically in combination with probe sonication. Viruses and prokaryotes in the treated samples were enumerated using epifluorescence microscopy (EFM), as is standard for benthic microbial ecology (Danovaro & Middelboe 2010).

One of the challenges of quantification of fluorescently stained viruses in sediment samples is the large background fluorescence due to the staining of free nucleic acids. To reduce this background fluorescence in the sample, three nucleases were tested: DNase I from bovine pancreas (~ 4000 Kunitz units mg^{-1} ; final concentration $5 \mu\text{g ml}^{-1}$; Sigma-Aldrich), RNase A from bovine pancreas (≥ 70 kunitz units mg^{-1} ; final concentration $10 \mu\text{g ml}^{-1}$; Sigma-Aldrich) and benzonase endonuclease from *Serratia marcescens* (final concentration $> 250 \text{ U } \mu\text{L}^{-1}$; Sigma-Aldrich). Benzonase degrades both free DNA and RNA in several forms (single-stranded, double-stranded, linear, circular and supercoiled) and has been found to

leave adenoviruses intact (Huyghe et al. 1995). A subsample of 1 μl from the extracted samples was diluted in 1 ml of sterile MilliQ water, after which the enzyme was added and the sample incubated for 30 min at 37°C (optimal conditions provided by the manufacturer). Three enzyme combinations were tested: 1 μL of DNase I, a mixture of 1 μL DNase I and 1 μL RNase A, and 1 μL of benzonase. EDTA concentrations above 1 mM can partly inhibit benzonase activity (conditions provided by the manufacturer), however the final concentration of EDTA after the addition of benzonase was much lower (0.1 μM), and did not appear to inhibit nuclease activity in our test.

As the viral abundances in the microbial mats were high, small sample volumes were used. To test if this small sample size affected the results, subsamples of 1 μl and 10 μl were compared. Also, the effect of sample storage conditions and time, on viral and prokaryotes abundances were examined. Fixed subsamples were directly snap frozen with liquid nitrogen and stored at -80°C either before or after extraction, and subsequently stored for 2 h, 1 - 2 weeks, 4 - 5 weeks and 10 - 14 weeks before analysis. Lastly we tested counting variability by analysing four replicate subsamples of the same original sediment sample. A schematic overview of the procedure is given in Figure 3.1.

Table 3.1. Chemical and physical treatment parameters of the five extraction methods (four previously published and the present study), used to extract and count viruses and prokaryotes from photosynthetic microbial mat samples. n.a. – not applicable.

Reference	Chemical treatment	Physical Treatment
Lunau et al. 2005	MeOH (10 - 30 %) at 35°C	ultrasonic bath (15 min)
Kallmeyer et al 2008	Acetate buffer (pH 4.6 in NaCl) 2 h	vortex 30 - 60 min; ultrasonic probe (outside sample) (10 sec x 3)
	MeOH (10 %) + EDTA (10 mM) + Tween 80 (0.1 % v/v) + Na ₄ O ₇ P ₂ (10 mM)	
	50 % Nycodenz	
Danovaro & Middelboe 2010	Na ₄ O ₇ P ₂ (5 - 10 mM) 15 min on ice	ultrasonic bath (1 min x 3)
	DNase (1 µL) + RNase (1 µL) 15 min at room temperature	
Garren & Azam 2010	EDTA (0.01 mM) 30 min on ice	n.a.
	Trypsin (0.4 %) 15 min at 37°C	
Present study	EDTA (0.1 mM) 15 min on ice	ultrasonic probe (10 sec x 3)
	Benzonase (1 µL) 30 min at 37°C	

Epifluorescence microscopy

Filtration and staining procedures were performed according to Noble and Fuhrman (1998). Samples were filtered onto 0.02 µm pore size filters (Anodisc 25, Whatman), stained with a green fluorescent nucleic acid-specific dye (400 x dilution of commercial stock in MilliQ water) and washed with sterile MilliQ water (3 times). After staining, the filters were placed in glass slides with an anti-fade solution consisting of 50:50 % (v/v) glycerol:PBS (0.05 M Na₂HPO₄, 0.85 % NaCl, pH 7.5) with 1 % *p*-phenylenediamine (Sigma-Aldrich, The Netherlands). Two different nucleic acid specific fluorescent dyes, SYBR Gold and SYBR Green I (Suttle & Fuhrman 2010) (Life Technologies™, NY, USA), were tested. Slides were stored at -20°C and viruses and prokaryotes were counted within a 1 - 3 week period using a Zeiss Axiophot EFM (x 1150 magnification). At least 10 fields and 400 viruses and prokaryotes were counted per sample and quantified per gram (wet weight).

Comparison to other methods

To assess the validity of our methodology, we compared the results of our optimized protocol with results obtained using previously published protocols: Lunau et al. (2005); Kallmeyer et al. (2008); Danovaro and Middelboe (2010) (extraction from sediments); and Garren and Azam (2010) (extraction from coral mucus). Further to testing existing methods, we also tested if the combination of each method with probe sonication yield a better extraction of viruses (and prokaryotes). The details of each method and the physical treatment used are presented in Table 3.1.

Viral and prokaryotes abundance in sediment

As intertidal photosynthetic microbial mats are also closely associated with the underlying sediments beneath the layer of photosynthetic microorganisms, we examined the suitability of our method to extract viruses and prokaryotes from sediments and compared these results with those of the Danovaro and Middelboe (2010) method, using intertidal sediment (Mokbaai, Texel, The Netherlands).

Sediment samples were collected using a sediment core (5 cm, internal diameter) and kept for about 1 h at *in situ* conditions, prior to processing in the laboratory. The top 1 cm was sliced and homogenized and eight subsamples of 100 mg of sediment were used for viral and prokaryotes extraction. All samples were fixed with 2 % glutaraldehyde (final concentration) for 15 min at 4°C. After fixation, four samples were treated according to our method (see schematic overview in Fig. 3.1), and the remaining four samples were treated according to Danovaro and Middelboe (2010). Briefly, the second set of samples received TSPP (10 mM final concentration) for 15 min in the dark after which they were sonicated (water bath sonicator; Pleuger, 50-60 Hz, Sonicor) in three cycles of 1 min with 30 s of manual shaking in an ice bath. One microliter of DNase I from bovine pancreas (~4000 Kunitz units mg⁻¹) and 1 µl of RNase A from bovine pancreas (≥ 70 kunitz units mg⁻¹) were added and the samples were incubated for 15 min in the dark. Filtration and staining was conducted as described above for all samples.

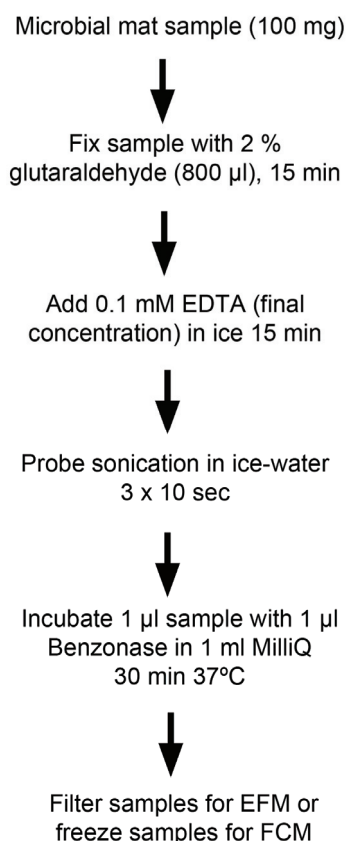


Figure 3.1. Flow diagram of the method established in the present study to extract viruses and prokaryotes from microbial mat samples and sediment.

Flow cytometry counting of viruses

To examine if our extraction method could be used to count viruses by FCM, the sample extracts from the microbial mats and sediment beneath were either filtered, stained and frozen for EFM analysis, or flash frozen in liquid nitrogen and stained for FCM according to Brussaard et al. (2010). Flow cytometric enumeration of viruses was carried out using a standard bench top Becton-Dickinson FACSCalibur flow cytometer, equipped with an air-cooled argon laser (excitation 488 nm, 15 mW power). Samples were diluted (10 - 50 x) in TE buffer (Tris 10 mM, EDTA 1 mM, pH 8.0), stained with SYBR Green I (Molecular Probes®, Invitrogen Inc., Life Technologies™, NY, USA) to a final concentration of 10^{-4} of the commercial stock solution, and incubated for 10 min in the dark at 80°C. The trigger was set for green

fluorescence and the data was analysed using CYTOWIN 4.31 freeware (Vaulot 1989).

Statistical analysis

Prior to statistical analysis, normality was checked. All statistical analyses were performed in SigmaPlot 12.0 (SYSTAT Software) with a confidence level set at 95 %. To determine differences between the different extraction methods, a one-way ANOVA with a post hoc Tukey HSD test was performed. Linear regression analyses were performed to obtain the best-fitting coefficients between pairs of variables of the regression model II (Sokal & Rohlf 1995), when comparing the EFM versus FCM viral counts.

3.3. Results

Chemical and physical dispersion

The extraction of viruses from the photosynthetic layer of microbial mats was initially tested using a water bath sonication treatment in combination with the addition of EDTA (0.1 and 10 mM) or TSPP (5 and 10 mM; as used by (Danovaro & Middelboe 2010)). Results showed a statistically significant ($p < 0.05$) increase after addition of 0.1 mM EDTA compared to TSPP or 10 mM EDTA, with a 2 - 2.5 fold increase in viral abundance compared to the other treatments (Fig. 3.2).

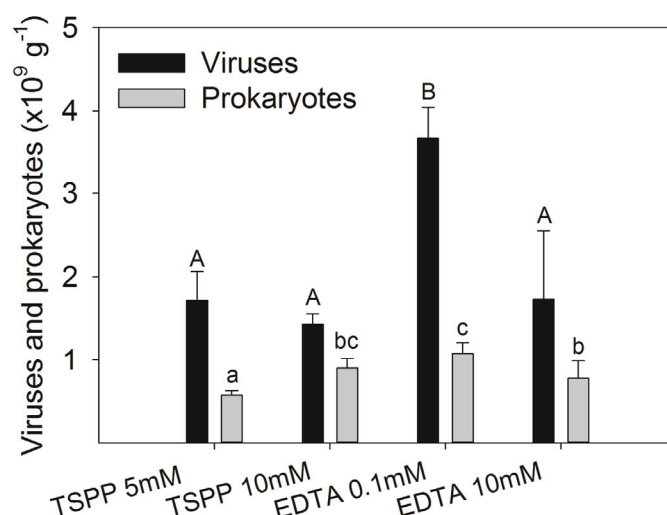


Figure 3.2. Viral and prokaryotes abundance ($\times 10^9 \text{ g}^{-1}$) in the top 1 mm of photosynthetic microbial mat samples using water bath sonication combined with the Danovaro and Middelboe 2010 extraction method (5 and 10 mM TSPP), and the present method (0.1 and 10 mM EDTA). Standard deviations are shown ($n = 4$). Significant differences ($p < 0.05$) are noted by upper case letters for viral abundance and lower case for prokaryotes abundance.

Comparison of water bath sonication versus probe sonication showed a 4.5-fold increase in the viral abundances ($p < 0.001$) and 7.7-fold increase in the prokaryotes abundances ($p < 0.01$) when using probe sonication (data not shown). Moreover, probe sonication was less dependent on addition of EDTA for optimal extraction of the viruses from the photosynthetic mat as there were no statistical differences between the concentrations of EDTA tested (0.01, 0.1, and 1 mM final concentration). However, the addition of 0.1 mM EDTA improved microscope images (ease of counting) and the EDTA treatment was, therefore, maintained in subsequent tests. The ultrasonic probe disrupted the microbial mat (visible by eye) and significantly ($p < 0.001$) increased the extraction efficiency up to 15- and 34-fold for viruses and prokaryotes after three cycles of 10 s compared to no sonication (Fig. 3.3). Although the statistical analysis showed that the number of probe sonication cycles did not significantly affect the viral and prokaryotes abundances, we observed by light microscopy that 20 s of probe sonication did not completely disrupt the mat, and that 60 s induced cell disruption. Therefore, three cycles of 10 s were chosen.

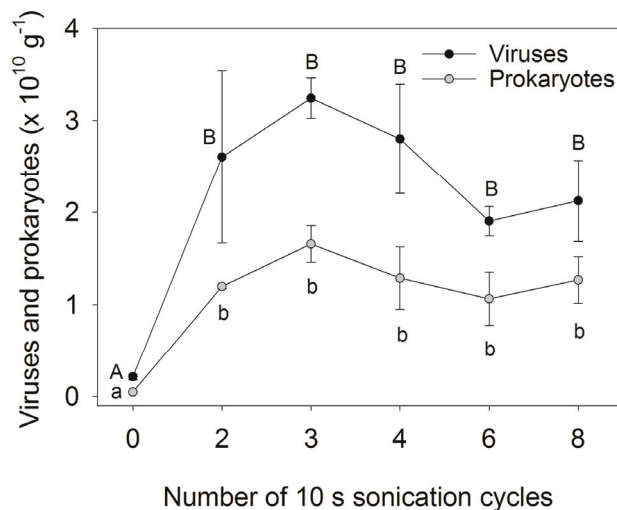


Figure 3.3. Effect of sonication cycles (10 s) on viral and prokaryotes abundance ($\times 10^{10} \text{ g}^{-1}$), after the addition of 0.1 mM EDTA. Standard deviations are shown ($n = 4$). Significant differences ($p < 0.01$) are noted by upper case letters for viral abundance and lower case for prokaryotes abundance.

The addition of different combinations of enzymes (DNase I, DNase I + RNase A, and benzonase) resulted in comparable counts of viruses and prokaryotes without significant differences (data not shown). Nonetheless, the addition of benzonase helped to produce substantially clearer images (lower background noise; Fig. 3.4). Moreover, as benzonase is able to digest both DNA and RNA, the addition of only benzonase is more practical than using a combination of different enzymes.

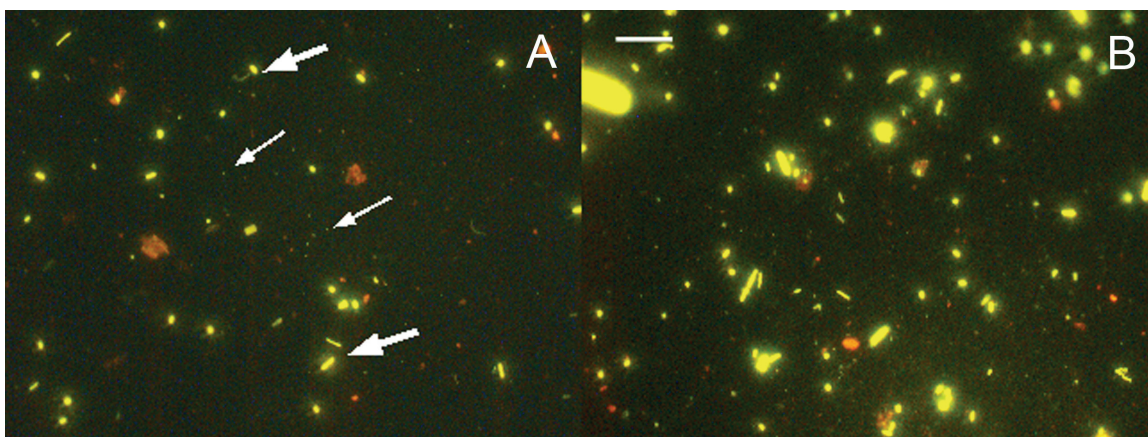


Figure 3.4. Epifluorescence microscopy images of viruses and prokaryotes from the top 1 mm of photosynthetic microbial mat samples (A) with and (B) without benzonase. Scale bar indicates 5 μm . Small and big arrows indicate viruses and prokaryotes, respectively.

Subsampling a volume of 1 or 10 μl from the extracted sample to count showed comparable viral and prokaryotes abundances without any significant statistical differences. The reproducibility of EFM counts of viruses and prokaryotes in the extracts was also tested by counting four subsamples of the same original sample. The coefficient of variation for viral and prokaryotes counts was 1.5 and 10 %, respectively. This means that the standard deviation observed for viral and prokaryotes abundances in the various tests was the result of spatial heterogeneity in the distribution of viruses and prokaryotes among the collected subsamples rather than variability in the actual counting analysis. Counting viruses and prokaryotes by EFM using SYBR Gold showed 1.3-fold higher counts for viruses ($p < 0.05$), but no differences for prokaryotes as compared to SYBR Green I stained samples (data not shown).

Freezing of the fixed microbial mat sample before extraction resulted in a rapid statistical significant loss of viruses and prokaryotes (Fig. 3.5), i.e. the abundance of viruses after one week storage was reduced ($p < 0.05$). However, when samples were stored frozen after the chemical and physical extraction there was no significant loss, even after several months of storage.

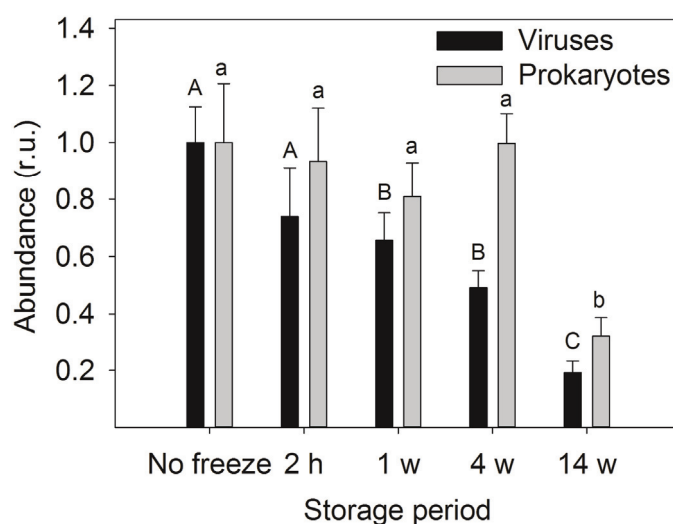


Figure 3.5. Effect of storage period on viral and prokaryotes abundance before extraction from top 1 mm of photosynthetic microbial mat samples (relative units). Samples were snap frozen in liquid nitrogen (-80°C) before storage. Standard deviations are shown ($n = 4$). Significant differences ($p < 0.05$) are noted by upper case letters for viral abundance and lower case for prokaryotes abundance.

In summary, the optimal protocol for extraction of viruses and prokaryotes from photosynthetic microbial mats (Fig. 3.1) comprised the fixation with 2 % glutaraldehyde (final concentration) for 15 min at 4°C, followed by incubation with 0.1 mM EDTA (final concentration) on ice and in the dark for another 15 min. Thereafter, probe sonication is applied in three cycles of 10 s with 10 s intervals, while keeping the samples in ice-water. A subsample of 1 µL is diluted in 1 ml sterile MilliQ water and incubated with 1 µl of benzonase in the dark for 30 min at 37°C. Finally, the sample is placed on ice until filtration for EFM analysis, or frozen in liquid nitrogen and kept at -80°C for EFM or FCM analysis.

Comparison to other methods

The selected existing procedures (Table 3.1) showed a significantly lower extraction efficiencies of viruses and prokaryotes from photosynthetic mat samples when compared to the current protocol (5 - 33 and 14 - 21-fold lower abundances for viruses and prokaryotes, respectively; Fig. 3.6). Addition of a probe sonication step to the published protocols resulted in a statistically significant increase ($p < 0.001$) in viral and prokaryotes abundances compared to the original protocols. Still, our method showed an additional improvement as illustrated by the significant increase in viral ($p < 0.001$) and prokaryotes ($p < 0.05$) abundances compared to the published protocols even with the additional probe sonication step (Fig. 3.6). On average, our method gave 2.5 and 2.2-fold higher viral and prokaryotes abundances, respectively, compared to the other methods performed with probe sonication.

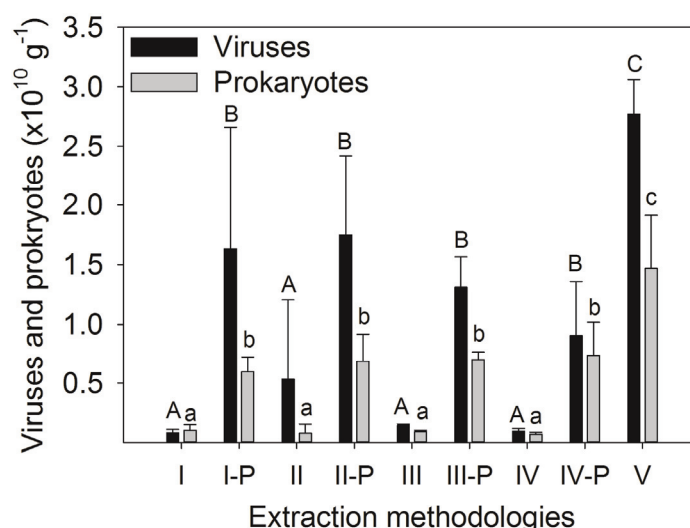


Figure 3.6. Viral and prokaryotes abundances ($\times 10^{10} \text{ g}^{-1}$) in the top 1 mm of photosynthetic microbial mat samples, using the extraction methods of Lunau et al. 2005 (I), Kallmeyer et al. 2008 (II), Danovaro & Middelboe 2010 (III), Garren & Azam 2010 (IV), and the method from the present study (V). P indicates probe sonication step added. Standard deviations are shown ($n = 4$). Statistical analysis showed a significant difference between the original and the combined method with probe sonification ($p < 0.001$ for viral and prokaryotes abundance), and significant differences between the present method and the other four methods ($p < 0.001$ and $p < 0.05$ for viral and prokaryotes abundance, respectively). Significant differences are noted by upper case letters for viral abundance and lower case for prokaryotes abundance.

Sediment counts of viruses and prokaryotes

When applying our microbial mat extraction protocol to intertidal sediment samples, a 4.5 and 4-fold increase ($p < 0.001$) in both viral and prokaryotes abundance, respectively, was obtained as compared to the Danovaro and Middelboe (2010) method, *i.e.* $3.08 \pm 0.63 \times 10^9$ vs. $0.75 \pm 0.12 \times 10^9$ viruses g^{-1} , and $3.36 \pm 0.68 \times 10^9 \text{ g}^{-1}$ vs. $0.75 \pm 0.28 \times 10^9$ prokaryotes g^{-1} , respectively. Consequently, no change in average viruses to prokaryotes ratio was found for the two methods.

Flow cytometry

The present method allowed an easy analysis of viruses using FCM (Fig. 3.7). Two virus clusters with different green fluorescence intensities (V1 with lowest and

V2 with highest intensity) could be distinguished. Comparing virus quantification from microbial mats using EFM and FCM (Fig. 3.8) showed a good correlation ($r^2 = 0.74$; $p < 0.0001$) with FCM giving higher counts. As also observed for pelagic samples (Monfort & Baleux 1992), prokaryotes abundances obtained by EFM and FCM matched well ($r^2 = 0.88$; $p < 0.0001$; $y = 1.02x$).

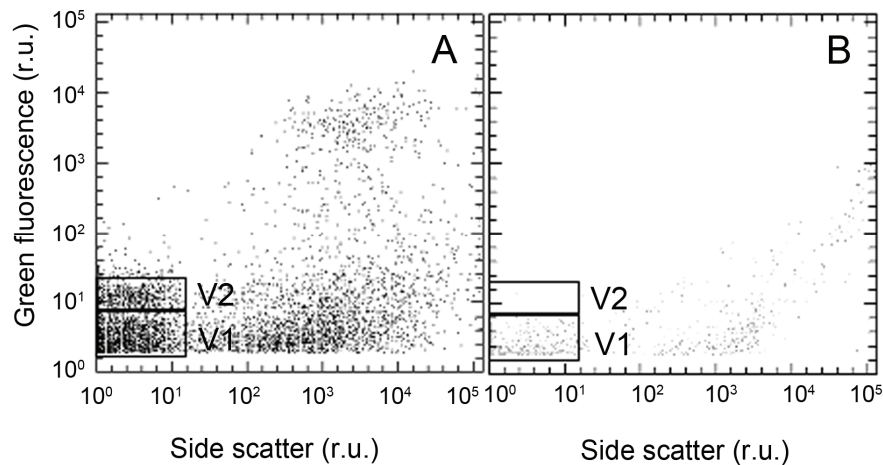


Figure 3.7. Cytogram (A) of viruses from photosynthetic microbial mat samples using flow cytometry after staining with nucleic acid-specific dye SYBR Green I, and (B) from control sample without viruses. Green fluorescence (V1 and V2) allows the distinction of two virus clusters. R.u. stands for relative units.

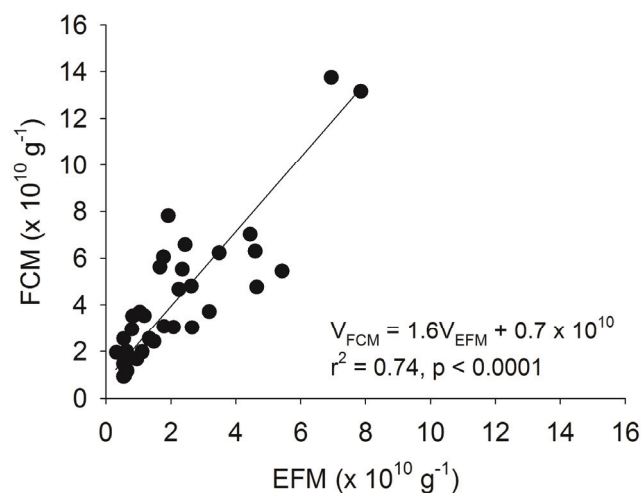


Figure 3.8. Comparison of viral counts ($n = 40$) using flow cytometry (FCM) and epifluorescence microscopy (EFM) after extraction with the present method.

3.4. Discussion

Intertidal photosynthetic microbial mats are mainly composed of intertwined filamentous cyanobacteria and microalgae, glued together in a biofilm composed of EPS, sediment particles, prokaryotes and viruses (Stal 1994, Decho 2000). To extract viruses and prokaryotes from such mats a combination of chemical and physical treatments is necessary. This is most likely related with the need to disrupt the strong links between cyanobacterial filaments and EPS structures. The combination of probe sonication with a low EDTA concentration (0.1 mM) and a nuclease treatment provided an efficient method for the extraction of viruses and prokaryotes from microbial mat samples, as well as optimized conditions for subsequent counting by EFM or FCM.

EDTA has been widely used to extract EPS from both intertidal sediments (Underwood et al. 1995) and microbial mats (Decho et al. 2005) because it chelates bivalent ions (Ca^{2+} and Mg^{2+}) destroying the links between the EPS polymers and between EPS and sediment particles, thereby releasing attached viruses and prokaryotes. EDTA has also been used earlier for the extraction of prokaryotes from coral mucus (Garren & Azam 2010), and in combination with other chemicals for prokaryotes and viral extraction from sediments and biofilms (Hewson & Fuhrman 2003, Helton et al. 2006, Kallmeyer et al. 2008, Garren & Azam 2010). In these studies, EDTA was used in concentrations ranging from 0.01 to 10 mM and showed good results in the extraction of viruses and/or prokaryotes.

Our study is the first comprehensive study comparing water bath sonication with the effects of a probe sonication directly on microbial mat samples. Probe sonication had been previously used in a few studies for the extraction of viruses in marine sediments (e.g. Middelboe et al. 2011). In the current study, the application of probe sonication visually disrupted the microbial mats, significantly increasing the viral and prokaryotes abundances and strongly improving the counting yield of viruses and prokaryotes. Probe sonication proved more effective in viral extraction from microbial mat samples than water bath sonication, the methodology routinely used in sediments. Moreover, we did not observe cell disruption with the sonication times proposed in the current protocol, contrary to previous studies, where probe

sonication had disrupted prokaryotes cells during extended treatment (1 - 22 min) at high energy levels (Holm et al. 2008). Nonetheless, we recommend initial visual inspection of the material when utilizing our protocol.

The effect of nuclease addition on the extraction methodology has shown contradictory results in previous studies. Danovaro et al. (2001) claimed that the increase in viral counts after nuclease addition was due to the disruption of bulks of matter where viruses could be found, and thus a release of attached viruses. Maruyama et al. (1993) ascribed the decrease in the viral fraction to the degradation of uncoated DNA (or extracellular DNA; eDNA) by DNase. Finally, Fischer et al. (2005) showed no differences in viral counts after nuclease addition because of insignificant amounts of eDNA in the analysed samples. In our study, the endonuclease benzonase helped to optimize the counting efficiency by reducing the background fluorescence likely derived from staining free nucleic acids (eDNA). This is supported by previous measurements of high eDNA concentrations in marine sediments ($3.5 - 55.2 \mu\text{g g}^{-1}$; Danovaro et al. 1999) and in activated waste water biofilms ($4 - 52 \text{ mg g}^{-1}$ of volatile suspended solids; Dominiak et al. 2011), where it has been suggested to have an important structural role in prokaryotes microcolonies by binding prokaryotes cells together (Dominiak et al. 2011). Microbial mats are highly active biofilms (Decho 2000) and most probably also contain high concentrations of eDNA. Nucleases have been shown not to degrade viral particles (Jiang & Paul 1995), therefore, the addition of nucleases does not have negative implications on viral abundance.

Clearly probe sonication contributed most to the method improvement, however, the addition of EDTA (viruses and cells are shown brighter) and nuclease (cleaner samples) allowed easier counts. The extraction protocol presented in this study is an effective extraction method for recovery of viruses and prokaryotes from photosynthetic microbial mats. Using this method, viral and prokaryotes abundances obtained from intertidal microbial mats were 1.7 - 2.8 and 2 - 2.5-fold, respectively, higher than those found using other published methods for extraction of viruses and prokaryotes from sediments (Lunau et al. 2005, Kallmeyer et al. 2008, Danovaro & Middelboe 2010) and coral mucus (Garren & Azam 2010), even after adding probe sonication to these protocols.

The microbial mat extraction protocol was shown to improve also the extraction efficiency of viruses and prokaryotes from bulk intertidal sediment underlying the photosynthetic microbial mat when compared to previous published methods (4.5 and 4-fold higher viruses and prokaryotes, respectively). With this method it is thus possible to count viruses and prokaryotes in both microbial mats and sediments, allowing a direct comparison of viral and prokaryotes abundances without biases derived from the use of different extraction methods.

Application of the assay to sediment samples in combination with FCM analysis showed two clear virus clusters as has been observed also for pelagic samples (Brussaard et al. 2010). The higher virus counts using FCM compared to EFM was most likely due to the reduced quenching of the green fluorescent signal using FCM in combination with sensitive detection of the green fluorescent signal (thus overall improved FCM counts of low fluorescent viruses). Our method resulted in less background noise and an improved correlation between EFM and FCM virus counts ($r^2 = 0.74$) as compared to what is published thus far (freshwater sediment; $r^2 = 0.55$; Duhamel & Jacquet 2006). Compared to EFM counting of viruses, FCM has the advantage of being faster and more accurate.

The application of our method to natural photosynthetic microbial mats showed that viral abundances in these environments are among the highest recorded in natural aquatic systems ($2.8 \pm 0.3 \times 10^{10} \text{ g}^{-1}$). Higher viral numbers have been reported only in eutrophic sediments with large anthropogenic influence, e.g. Chesapeake Bay ($1.5 \times 10^{11} \text{ ml}^{-1}$; Helton et al. 2012) and Brisbane River ($2.2 \times 10^{11} \text{ ml}^{-1}$; Hewson et al. 2001). Furthermore, the presented extraction procedure may also be beneficial for capturing genetic information (e.g. next-generation sequencing) from the recovered microbes, thereby coupling quantitative abundance analysis to biodiversity information. However, for DNA extraction, we advice on testing the protocol without the use of a fixative, as this might inhibit good DNA extraction. Alternatively, heat treatment has been suggested to reverse the cross linking of DNA/RNA to proteins caused by fixatives (Gilbert et al. 2007). We anticipate that the methodology here presented will stimulate a systematic and quantitative exploration of viral ecology in benthic microbial mat systems.

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Chapter 4

Autofluorescence imaging system to discriminate and quantify the distribution of benthic cyanobacteria and diatoms

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Abstract

Observation of benthic oxygenic photoautotrophs on sediment surfaces shows a single photoautotrophic layer without distinction between photosynthetic groups. Until now it has not been possible to distinguish between benthic photosynthetic microorganisms, i.e. cyanobacteria and diatoms, at μm to mm scales using a single non-disruptive system. Chlorophyll autofluorescence can be used to distinguish different oxygenic photoautotrophic groups if the correct excitation light is applied. Using this principle a non-intrusive technique was developed to study the spatial distribution of benthic cyanobacteria and diatoms. By means of a charge coupled device (CCD) camera, diatoms and cyanobacteria could be identified by blue light (470 nm) excitation and amber light (600 nm) excitation, respectively. By this approach, diatom or cyanobacterial dominance could be easily distinguished using the blue over amber ratio (BAR). We applied this method successfully to (mixed) laboratory cultures as well as natural photosynthetic microbial mats. Cultures of the diatom *Nitzschia capitellata* and the cyanobacterium *Geitlerinema* sp. showed close correlation between autofluorescence and cell abundance. This simple and cheap imaging system allows fast observations of the microscale (μm to mm) spatial heterogeneities of live benthic oxygenic photoautotrophs both in culture and natural photosynthetic biofilms structure (e.g. microphytobenthos, photosynthetic microbial mats).

Key words: autofluorescence, imaging, cyanobacteria, diatoms, distribution

4.1. Introduction

Photosynthetic microbial mats are productive ecosystems (Canfield et al. 2005), with a photosynthetic layer composed of a mixture of cyanobacteria and diatoms (Jørgensen et al. 1983, Mir et al. 1991). Using imaging pulse amplitude modulation (PAM) it is possible to detect the presence and distribution of oxygenic photoautotrophs, but the technique does not distinguish between different oxygenic photoautotrophic groups. Although high performance liquid chromatography (HPLC) (Brotas et al. 2003) and microscopy allow the identification of oxygenic photoautotrophic groups, both techniques are relatively time consuming. Combining these analysis techniques with core slicing at high resolutions, e.g. 100 μm , can give an indication of the vertical structure of the oxygenic photoautotrophic groups. However, this approach is not very practical and prone to errors (due to small sample size). Moreover, because of the need for subsamples, it takes away the possibility for temporal analysis. There is thus need for an alternative fast and non-disruptive method. This method should discriminate between major benthic photosynthetic groups, while showing spatial distribution and temporal development.

Cyanobacteria and diatoms are the most common oxygenic photoautotrophic microorganisms in benthic systems (Mir et al. 1991, Brotas & Plante-Cuny 1998) with different compositions of accessory pigments (Campbell et al. 1998, Jeffrey et al. 2005). Although autofluorescence always occurs via chlorophyll *a* (chl *a*), different wavelengths can be used to excite the accessory pigments, thus allowing discrimination of different algal groups based on the excitation light applied. Cyanobacteria absorb light in the yellow, orange and red area of the visible spectrum using a combination of phycobiliproteins (Campbell et al. 1998). The most common phycobiliproteins are phycocyanin and phycoerythrin. Phycoerythrin occurs in cyanobacteria as well as in red algae and has a wide absorption peak ranging from 500 to 575 nm, while phycocyanin are exclusively found in cyanobacteria absorbing in the orange range (590 - 640 nm) (Van den Hoek et al. 1995). Diatoms absorb blue and red light with chl *a*, chl *c* and fucoxanthin (~450 nm) (Van den Hoek et al. 1995, Jeffrey et al. 2005). Green microalgae can also use blue light and thus compete with diatoms. However, although green microalgae have been found in

benthic systems (Barranguet et al. 1997), diatoms are generally more common in intertidal photosynthetic microbial mats (Mir et al. 1991).

Here we present a fast, simple and non-intrusive method based on the difference in fluorescence excitation of the different oxygenic photoautotrophic groups that allows determining microscale variability in spatial distribution and quantification of the two major primary producers of photosynthetic microbial mats, i.e. cyanobacteria and diatoms.

4.2. Material and methods

An imaging system was built using a cooled charge coupled device (CCD) 16 bits camera (1360 x 1024 pixels; TCC-1.4LICE Monochrome, Tucsen Imaging Technology Co. LTD, China), cooled to -30°C (below ambient temperature) with a ring of 3 blue (peak emission at 470 nm; LXHL-MB1D, Lumileds, The Netherlands) and 3 amber (peak emission at 597 nm; LXHL-ML1D, Lumileds, The Netherlands) 1 W LEDs positioned around the lens (Fig. 4.1). The near infrared filter in front of the code chip was removed by unscrewing the glass in front of the code chip. Blue and amber lights were chosen as these excite fucoxanthin and phycocyanin pigments, characteristic of diatoms and cyanobacteria, respectively. A long-pass infra red filter (695 nm, IF092, Schneider-Kreuznach, Germany) was placed on top of the lens (Xenoplan 1.4/17cm, 17 mm focal length, C-mount, Schneider-Kreuznach, Germany) allowing chl *a* autofluorescence (~685 nm) to be captured. The camera was connected to a computer, and the software TSView 7.1.1.5 (Tucsen Imaging Technology Co. LTD., China) was used to capture the images. The camera was connected to a manual one dimensional vertical stage allowing the camera to be accurately moved up and down. The method was initially tested on cultures of the diatom *Nitzschia capitellata* and the cyanobacterium *Geitlerinema* sp. Single and mixed populations were exposed to blue and amber light excitation. A rack was built to support the plate with organisms under observation. It consisted of a “table type” of setup (4 legs and a top) with a whole in the middle allowing the passage of light and to capture the images (Fig. 4.1B). It also allowed up and down adjustment. The focal distance of the lens was between 5 and 20 cm. With a focal distance of 5 cm, the

observation area was 4.5 x 3.4 cm (L x W) resulting in a resolution of 30 μm per pixel, and if 20 cm was the focal distance, the monitored area was 7 x 5.3 cm with a resolution of 51 μm per pixel. Exposure times were chosen so that there were no over-saturated pixels or photobleaching and were typically around 400 ms. Samples were adapted to the dark for 5 min before excitation light was applied. Two autofluorescence photos were taken per sample. One photo with blue excitation light and one photo with amber excitation light to distinguish between diatoms and cyanobacteria, respectively. The relative dominance of the different oxygenic photoautotrophic groups was obtained by dividing the blue autofluorescence image over the amber autofluorescence image (blue amber ratio; BAR). Thereafter, the ratio image was log transformed to make the scale linear. The calculated BAR indicates cyanobacterial dominance when negative, and diatom dominance when positive. Image analysis and calculations were performed using the freeware imaging software Image J 1.47m (<http://imagej.nih.gov/ij/>).

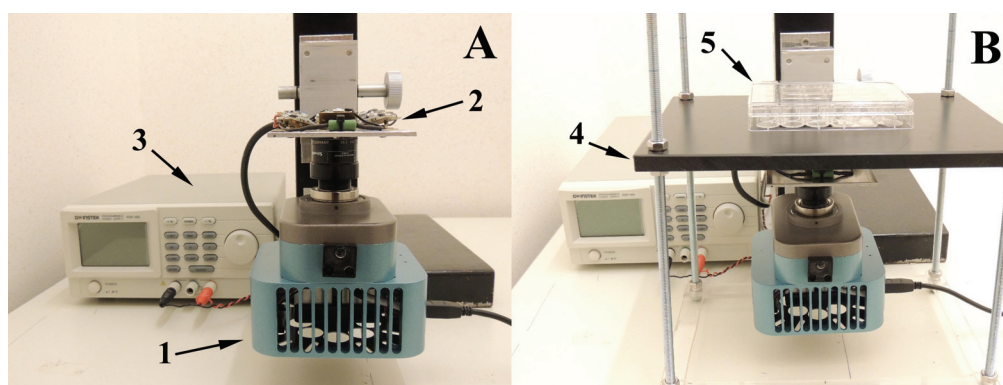


Figure 4.1. Autofluorescence imaging system with CCD cooled camera set-up (A) showing the (1) CCD camera (2) LEDs and (3) LED power supply. The imaging system (B) shows the (4) support structure for oxygenic photoautotrophs culture measurements and (5) the microwell plate with oxygenic photoautotrophs culture samples.

To test the relation between abundance and autofluorescence intensity of oxygenic photoautotrophs, growth curves were made with cultures of the diatom *N. capitellata* and the cyanobacteria *Geitlerinema* sp. The oxygenic photoautotrophs were kept in f/2 media, at 100 $\mu\text{mol quanta m}^{-2} \text{ s}^{-1}$ in a 16:8h L:D cycle at 20°C. *N. capitellata* and *Geitlerinema* sp. were grown separately in 24-microwell plates

(Greiner Bio-one) for 9 - 15 days (until stationary phase). Blue and amber autofluorescence photos of 8 wells (each containing 1 mL) were taken daily at ambient temperature in a dark room. Every day, images were taken of the same four wells, and to four extra wells (in a total of 8 wells per day) that were daily destructively sampled, fixed with Lugol and kept in the dark, until counts within 1 - 3 weeks. Counts were performed in a Sedgewick-Rafter chamber using a light microscope (Zeiss Axiophot) at x 200 - 400 magnification. At least 400 cells were counted per sample. To determine the autofluorescence in the wells we excluded all pixels that were not fluorescent by setting a threshold. We then selected a circle of 660 pixels in the centre of the well and measured the number of fluorescent pixels and their intensity.

Application of the method to natural samples was tested using photosynthetic microbial mat samples from the Wadden Sea Island Schiermonnikoog, The Netherlands. With a knife, squares of 15 x 8 x 4 cm (L x W x H) were cut out from the sediment and placed inside plastic boxes and taken to the laboratory within 3 - 4 h. Samples were kept under *in situ* conditions until use within 1 - 2 days. The photosynthetic microbial mats were exposed to blue and amber excitation light and the images analysed as described above.

Linear regression analyses were performed to obtain the best-fitting coefficients between counts and autofluorescence, and prior to statistical analysis, normality was checked and the confidence level was set at 95 % (Sokal & Rohlf 1995). The statistical analysis was conducted in SigmaPlot 12.0.

4.3. Results

Imaging of single populations of *N. capitellata* and *Geitlerinema* sp. after exposure to amber and blue light (Fig. 4.2) showed that *N. capitellata* autofluorescence is only observed with blue light excitation (Fig. 4.2A, B), and *Geitlerinema* sp. is only observed with amber light excitation (Fig. 4.2C, D). Therefore blue light does not excite the cyanobacterium *Geitlerinema* sp., and amber light does not excite the diatom *N. capitellata*, allowing to clearly distinguishing between the two oxygenic photoautotrophic groups.

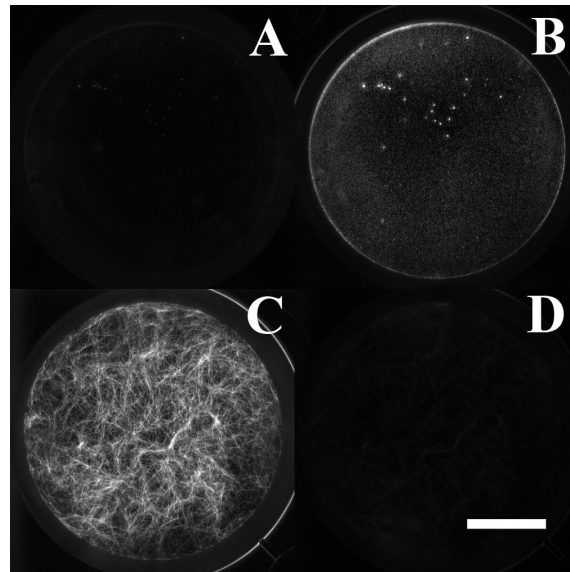


Figure 4.2. Autofluorescence images of single populations of the diatom *N. capitellata* (A, B) and cyanobacterium *Geitlerinema* sp. (C, D) in culture. Autofluorescence after amber light excitation (A, C), and after blue light excitation (B, D). Scale bar is 1 cm.

Simultaneous autofluorescence measurements and cell counts of *N. capitellata* during growth in culture experiments showed comparable temporal dynamics in the two parameters (Fig. 4.3A), and, consequently, a strong linear correlation ($r^2 = 0.98$, $p < 0.05$, Fig. 4.3B). *Geitlerinema* sp. autofluorescence and cell counts showed a similar development until day 8, after which the autofluorescence decreased and the cell abundance eventually reached a plateau (Fig. 4.4A). *Geitlerinema* sp. abundance showed a significant linear correlation with autofluorescence during the first 8 days ($r^2 = 0.99$, $p < 0.0001$, Fig. 4.4B). However, after day 8, the relationship between counted cells and autofluorescence was lost, since the autofluorescence decreased while cell numbers in the wells still increased. In several of the wells *Geitlerinema* sp. filaments got detached from the bottom and floated at the surface out of the camera's focus plane. When fluorescing cells moved out of focal plane it resulted in lower fluorescence intensity. The number of wells in which filaments started to float increased with time. Moreover, an increasing amount of filaments were observed growing on the side walls of the wells after day 9. Both floating filaments and wall growth could not be captured by the 2-dimensional camera (Fig. 4.5) as the setup of the camera was developed for benthic systems.

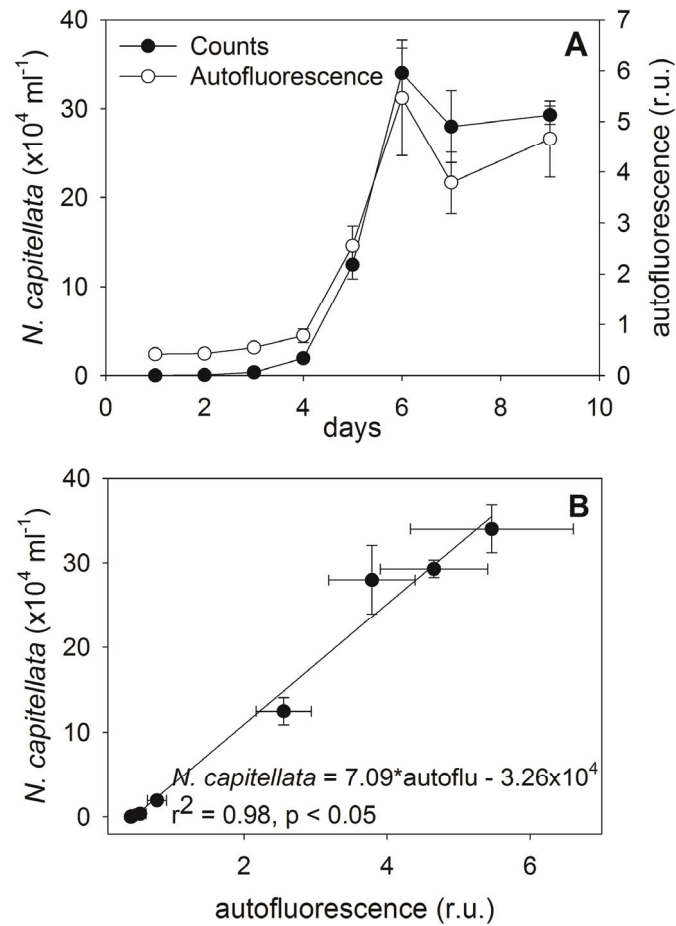


Figure 4.3. Autofluorescence and cell counts of the diatom *N. capitellata*. (A) Temporal dynamics of average abundance (filled circles) and autofluorescence after blue light excitation (open circles), and (B) linear regression of *N. capitellata* autofluorescence with counts ($r^2 = 0.98, p < 0.05$). The standard deviations are shown ($n = 4$).

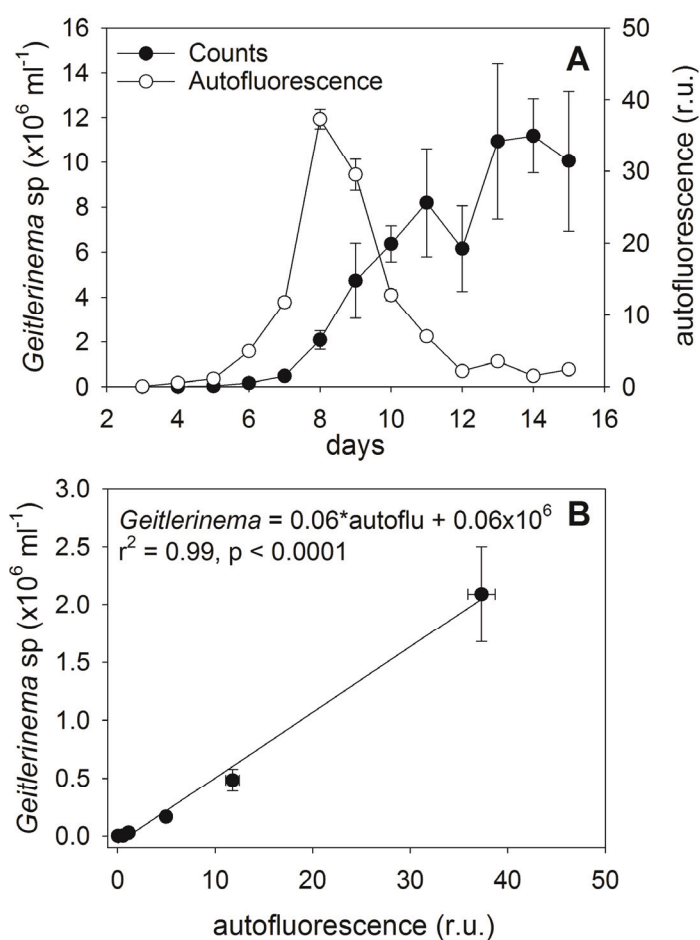


Figure 4.4. Autofluorescence and cell counts of the cyanobacterium *Geitlerinema* sp. (A) temporal dynamics of average abundance (filled circles) and autofluorescence after amber light excitation (open circles), and (B) linear regression of *Geitlerinema* sp. autofluorescence with counts ($r^2 = 0.99$, $p < 0.0001$) of the first 8 days. The standard deviations are shown ($n = 4$).

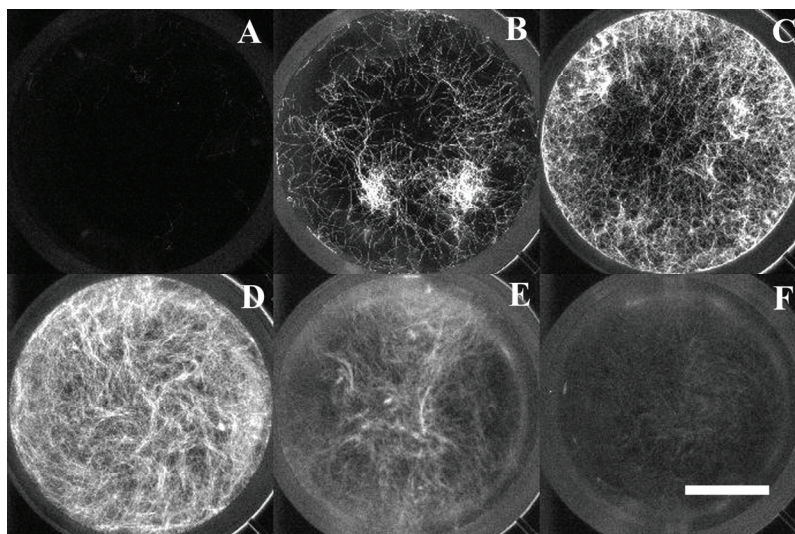


Figure 4.5. Autofluorescence images of *Geitlerinema* sp. growth at days 3, 5, 7, 9, 11, and 13 (A - F). After a week, the cells detached from the bottom which negatively affected the quality of the autofluorescence images as the floating cells were out of the plane of focus of the camera. Scale bar is 1 cm.

Standard colour photo observations of the mixed cultures (Fig. 4.6A) did not allow discrimination between *Geitlerinema* sp. and *N. capitellata*. When using blue excitation light the diatom *N. capitellata* fluoresced showing a spatial distribution in the edge of the well it grew in (Fig. 4.6B). The cyanobacterium *Geitlerinema* sp. fluoresced when using amber light and showed a more patchy distribution (Fig. 4.6C). The BAR image displayed the distinct distribution pattern of both diatom and cyanobacterium (Fig. 4.6D). BAR values < 0 indicate dominance of *Geitlerinema* sp., while values > 0 indicate diatom dominance. BAR values ranged from -1.83 to 1.98, with an average of 0.16 ± 0.45 . Although there was on average no overall dominance of either species, on microscale, distinct dominance of either species could be clearly detected (Fig. 4.6E).

Observation of the colour image of a photosynthetic microbial mat showed a greenish surface without obvious spatial patterns in oxygenic photoautotrophs distribution (Fig. 4.7A). However, after blue (Fig. 4.7B) and amber (Fig. 4.7C) light excitation strong microscale heterogeneity in spatial distribution of diatoms and cyanobacteria could be discriminated. BAR imaging showed a detailed distribution pattern of dominance of either photosynthetic group (Fig. 4.7D). The BAR along a cross section of the microbial mat (Fig. 4.7D) showed a distinct patchy distribution of

these two key benthic oxygenic photoautotrophs. The microscale spatial heterogeneity in the dominance of either cyanobacteria or diatoms was observed at pixel level (Fig. 4.7E).

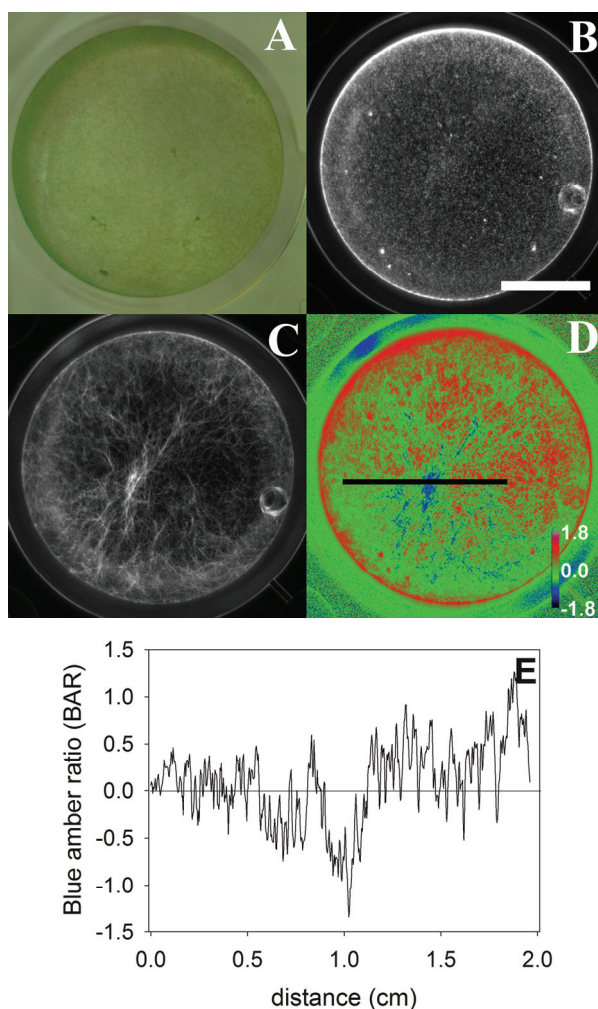


Figure 4.6. Mixed populations of the diatom *N. capitellata* and cyanobacterium *Geitlerinema* sp. in culture: (A) Standard colour camera image; (B) autofluorescence after blue light exposure, (C) autofluorescence after amber light exposure; (D) Blue amber ratio (BAR), and (E) BAR values along a cross section indicated by the 2 cm black line in figure D. Scale bar is 1 cm.

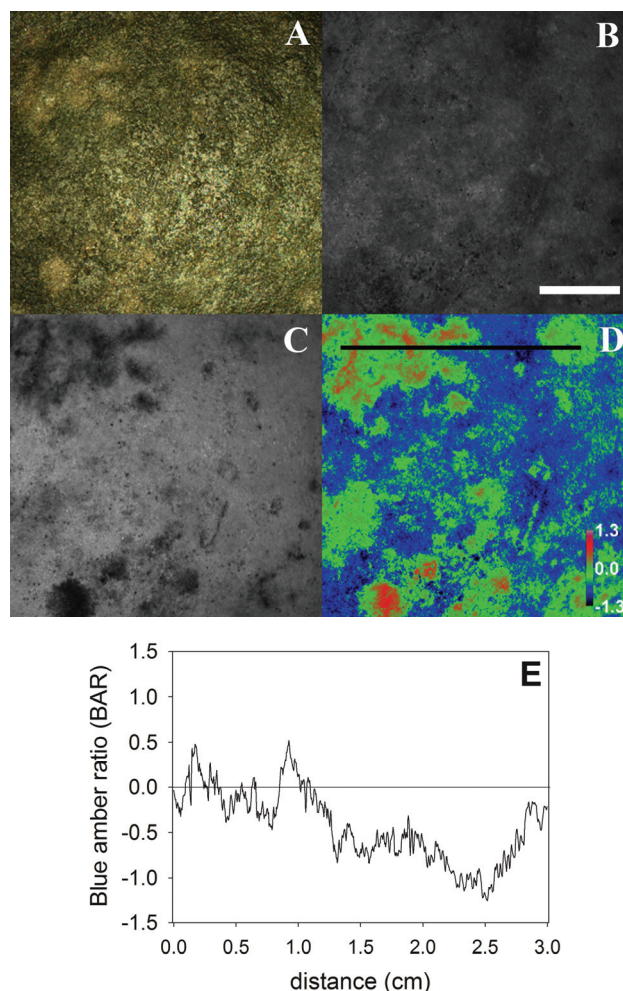


Figure 4.7. Photosynthetic microbial mat from the tidal Wadden Sea island Schiermonnikoog, The Netherlands by (A) Standard colour image; (B) autofluorescence after blue light exposure, (C) autofluorescence after amber light exposure; (D) Blue amber ratio (BAR), and (E) BAR values along a cross section indicated by the 3 cm black line in figure D. Scale bar is 1 cm.

4.4. Discussion

Using the blue and amber lights as excitation colours in combination with a monochromatic camera we were able to discriminate between cyanobacteria and diatoms in mixed cultures and natural populations even with a relative low resolution of the camera CCD chip. A CCD camera, rather than a photodiode, was used to investigate the spatial component of the distribution of the different oxygenic photoautotrophic groups. Several imaging systems (i.e. Waltz, PSI) for imaging of photosynthetic performances of plants, photosynthetic biofilms and unicellular algae

are commercially available (Oxborough et al. 2000, Ralph et al. 2005, Aldea et al. 2007). However, the macro-imagers of these types have only one excitation colour and cannot be used for spatial differentiation of photosynthetic groups in mats, and only measure the photosynthetic performance of one of the oxygenic photoautotrophic groups. In heterogeneous systems like photosynthetic microbial mats differences in photosynthetic performance of selected areas can be caused by physiological factors or by a variation in dominance of different oxygenic photoautotrophic groups. To discriminate between these two factors it is necessary to separate the individual algal groups which require the use of different excitation colours within a single imaging system. Beutler et al. (2002) have used a submersible probe with different excitation colours to distinguish between phytoplankton groups, but without visual images. Other imaging systems (Polerecky et al. 2009, Trampe et al. 2011) have been built that can be mounted on a microscope which can excite benthic oxygenic photoautotrophs at different colours. However, although such microscopy systems allow both visualising and quantifying the autofluorescence in mixed population systems, the field of view in our system is much wider (15 - 37 cm²) than what can be obtained in a microscope (0.01 - 0.96 mm²), while still acquiring fine resolution (μ m) information. Furthermore, the transport of a microscope with an attached camera is less practical and more costly than our system, which only needs a camera and light source (~\$2500) (Table 4.1). The objective of this system is to determine population distribution of different benthic oxygenic photoautotrophs which is not possible under the microscope, and without destroying the samples (both in field and in laboratory experiments). Finally, as our imaging system uses few optical elements it has high transmission of light, increasing sensitivity and may allow visualisation of single filaments that may not be visible using a standard compound microscope. The system described in this study can be easily converted to suit field studies just by mounting the set up in a dark box with an opening of the field of view on the sediment site and the addition of a portable power supply. Quantification of the two ecologically important oxygenic photoautotrophs was tested by comparing their specific autofluorescence with their biomass in the form of cell abundance. Culture experiments showed that autofluorescence was a good indicator of abundance for both the diatom *N. capitellata* and the cyanobacterium *Geitlerinema*

sp., as long as cells stayed attached to the bottom of the wells (in the plane of focus). This shows that the camera system is a good indicator of cell abundance in systems where all biomass is present in the focal plane. We used a lens with a short focal distance (5 cm, with a macro ring) that resulted in a relatively thin focal plane. With our settings, the 16 bit grey value increased by 1600 for the *Geitlerinema* sp. filaments, corresponding to the 4 μm thickness of the filaments. The highest grey values were around 62 000 (found at day 8), indicating that layers of up to 35 filaments thick can be measured with this method, equivalent to at least 140 μm depth. It is expected that excitation levels decrease with depth in the biofilm layer due to absorbance of the excitation light. By using a linear extrapolation the thickness of the layer will likely be underestimated. Usually the photosynthetically active microbial mat layer is on the order of a few hundred μm , however in sandy sediments there may be substantial light at deeper layers (order of mm; Kühl & Jørgensen 1992, Al-Najjar et al. 2012).

Table 4.1. Brand, series and price (USD) information of the material used to set-up the autofluorescence imaging system.

Material	Brand/series	Price (USD)
CCD camera	Tucsen Imaging Technology Co. LTD, TCC-1.4LICE mono cooled	1085
Lens	Schneider-Kreuznach, Xenoplan 1.4/17cm	850
Long pass filter	Schneider-Kreuznach, IF 092, 695 nm	92
LED	Luxeon 1watt, x3 blue (470 nm) and x3 Amber (597)	2.5 - 5 each
Power supply	GW Instek programmable power supply PSP-405	400
Software		
TSView	7.1.1.5	Provided with the camera
ImageJ	1.47m	Freeware

Although we did not observe significant amounts of green microalgae in the studied microbial mats, the possible interference from green microalgae when obtaining the diatom autofluorescence may be solved by using an additional green colour to excite chlorophyll *b* present in green microalgae but not in diatoms (Van den Hoek et al. 1995). Thus by using a ratio of blue over green autofluorescence it is possible to distinguish between diatoms and green microalgae distribution.

This study used the well known phenomenon by which specific excitation colours of algal pigments can distinguish different photosynthetic groups in a new imaging approach to quantify the spatial distribution of cyanobacteria and diatoms in photosynthetic mats. Furthermore, the use of the BAR allows the rapid identification of areas of dominance of either cyanobacteria or diatoms in mixed laboratory culture and in natural photosynthetic microbial mats.

Composition, distribution and quantification of the biomass of specific oxygenic photoautotrophic groups are important parameters in most ecological and ecophysiological studies. Until now, the horizontal spatial distribution of the different groups of oxygenic photoautotrophs in microbial mats have been inferred from vertical profiles (Jørgensen et al. 1983, Mir et al. 1991) and the maximum spatial resolution was limited to the centimetre scale at best. Using different light excitations and collecting the autofluorescence we could resolve the spatial heterogeneity and quantify the distribution of living diatoms and cyanobacteria in culture and in field samples at the μm to mm scale. Since this technique is non-intrusive it has a high potential for studies on population dynamics on spatial and temporal scales which are relevant for microbial communities. Furthermore, it is easy to build a mobile system that can be applied to field studies for *in situ* measurements.

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Chapter 5

Microscale spatial distributions of microbes and viruses in intertidal photosynthetic microbial mats

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Abstract

Intertidal photosynthetic microbial mats from the Wadden Sea island Schiermonnikoog were examined for microscale (millimetre) spatial distributions of viruses, prokaryotes and oxygenic photoautotrophs (filamentous cyanobacteria and benthic diatoms) at different times of the year. Abundances of viruses and prokaryotes were among the highest found in benthic systems ($0.05 - 5.43 \times 10^{10}$ viruses g^{-1} and $0.05 - 2.14 \times 10^{10}$ prokaryotes g^{-1}). The spatial distribution of viruses, prokaryotes and oxygenic photoautotrophs were highly heterogeneous at mm scales. The vertical distributions of both prokaryotic and viral abundances were related to the depth of the oxygenic photoautotrophic layer, implying that the photosynthetic mat fuelled the microbial processes in the underlying layer. Our data suggest that viruses could make an important component in these productive environments potentially affecting the biodiversity and nutrient cycling within the mat.

Key words: spatial distribution, viruses, prokaryotes, oxygenic photoautotrophs, photosynthetic microbial mats

5.1. Introduction

Microbial mats are laminated microbial communities growing in a variety of environments, including extreme habitats such as sea ice, hot springs and environments with high salinity or periodic desiccation (Castenholz 1994, Fenchel et al. 2012). Marine intertidal flats can sustain microbial mats under specific conditions, like occasional flooding and low sand deposition (Bolhuis et al. 2015). Microbial mats are characterized by large chemical gradients and densely packed biomass within a few cm depth, growing an average of 1 - 3 mm per year, and covering sandy sediments in intertidal flats (Fenchel 1998, Fenchel & Kühl 2000, Bolhuis et al. 2015).

Filamentous cyanobacteria are considered the first colonisers in the development of intertidal photosynthetic microbial mats, forming a green layer that releases organic compounds (lysis, excretion) and oxygen, and captures atmospheric nitrogen (Stal 1995). Diatoms settle after, and together with the cyanobacteria make up the oxygenic photoautotrophic surface layer (Fenchel et al. 2012). Below this layer, bands of colourless sulfur and purple sulfur bacteria (purple layer) may develop, that oxidize sulfide generated by the underlying sulfate reducing bacteria (black layer) (Van Gemerden 1993). The intertwined filamentous cyanobacteria in the top layer and the excretion of exopolymeric substances (EPS) by the microbial communities form a matrix which stabilises the microbial mats, making them resistant to wind and wave erosion (De Brouwer et al. 2002). The development of mats is also enhanced by the reduced impact of grazers and bioturbating animals (Fenchel 1998) due to the relatively extreme physico-chemical conditions (e.g. hot springs, sea ice; dry and windy condition) inside and below the mat (Jørgensen et al. 1983, Des Marais 2003).

Studies in water column, biofilms, and sediments have demonstrated that microorganisms are heterogeneously distributed on small spatial scales with microscale hot spots of elevated activity (Neu & Lawrence 1997, Seymour et al. 2006, Stewart & Franklin 2008, Carreira et al. 2013). Moreover, studies have shown that to capture the actual microbial diversity, production, and the micro-environmental drivers of these habitats, it is important to study microbial ecology at microscale (Paerl & Pinckney 1996, Azam & Malfatti 2007). In benthic environments, viruses

were shown to play an important structuring role by driving spatial heterogeneity and temporal dynamics of their host populations (e.g. prokaryote; Siem-Jørgensen et al. 2008, Carreira et al. 2013), and as drivers of organic carbon and nutrient recycling (Danovaro et al. 2008b). Although photosynthetic microbial mats are highly productive habitats (Canfield et al. 2005) that have been intensively studied with regard to their structure, composition, and biogeochemistry (Des Marais 2003, Ward et al. 2006, Bolhuis et al. 2015), to the best of our knowledge, no studies on millimetre scale distribution of the different ecologically relevant groups in photosynthetic microbial mats have been published to date. The aim of the present study was to determine the microscale (mm) vertical and horizontal spatial distributions of viruses, prokaryotes (Archaea and Bacteria, excluding cyanobacteria) and oxygenic photoautotrophic (oxygenic photosynthetic microbes, i.e. prokaryotic filamentous cyanobacteria and eukaryotic microalgae) in intertidal photosynthetic microbial mats during different times of the year (November 2012, April and July 2013).

5.2. Material and Methods

Site description and sampling

The sampling site is situated in the sandy north-western beach of the coastal island Schiermonnikoog (53° 29' 24.29"N, 6° 8' 18.02"E) in the Wadden Sea (The Netherlands). Intertidal photosynthetic microbial mats are found here due to the dry and windy conditions, with occasional flooding. The microbial mats can be found year-round, but storm, flood and ice cover may destroy the mats in winter (Bauersachs et al. 2011). Samples were collected in November 2012, April and July 2013, over a spatial range of 200 metres. Each time, ten samples of 15 x 8 x 4 cm (L x W x H) were collected, placed individually in clean plastic boxes, and transported back to the laboratory, where they were kept at *in situ* conditions until sub-sampling the next day. Three cores of 7 mm diameter were sampled and used for characterisation of water content, organic matter content and particle size. The cores were sliced every 1 mm down to a depth of 10 mm. At each sampling date, four

similar 7 mm diameter cores were taken and sub-sampled for depth profiles of prokaryotes and viruses. Additionally, in November 2012 viruses and prokaryotes were also sampled using the 7 mm diameter cores, in a horizontal grid to obtain the 3-dimensional (3D) distribution. For this 3D-distribution subsamples were taken at 20 mm intervals, both in length and width, at two depths (0 - 1 and 1 - 2 mm), sampling a total volume of 29400 mm³ (210 x 70 x 2 mm).

Sediment characteristics

Water content of the sediment was determined as the weight loss after drying the sediment at 105°C for 12 h. Organic matter content was measured as the weight loss of dried samples after combustion at 540°C for 4 h. Particle-size analyses were performed on the C_{org}-free sediment fraction from the 10 slices from 3 cores. Prior to analysis, 20 mL of demineralised water was added to the samples, which were subsequently treated with 5 mL H₂O₂ solution (35 %) and boiled until the reaction stopped and the H₂O₂ had disintegrated into H₂O and O₂. Particle-size analysis was carried out using the Micro-Liquid Module of a Beckmann Coulter Laser Particle Sizer LS13320 at NIOZ, which resulted in 92 particle-size distributions classes from 0.4 to 2000 µm.

Chlorophyll quantification

Chlorophyll *a* (chl *a*) autofluorescence was recorded with a camera to map oxygenic photoautotrophic biomass distribution and to distinguish between the two major groups of photosynthetic microorganisms: cyanobacteria and diatoms (Bolhuis et al. 2013). Although autofluorescence always occurs via chl *a*, different wavelengths can be used to excite the accessory pigments, thus allowing discrimination of different algal groups based on the excitation light applied. Blue light was used to excite chl *a* and fucoxanthin (~450 nm) found in diatoms (Van den Hoek et al. 1995, Jeffrey et al. 2005). Blue light excitation does not allow the distinction between diatoms and green microalgae, but diatoms tend to dominate over green microalgae in this type of microbial mats (Jonkers et al. 2003, Bolhuis et al. 2013).

Moreover, green microalgae were not observed using light microscopy. Amber light was used to excite phycocyanin (590 - 640 nm) which is exclusively found in cyanobacteria (Van den Hoek et al. 1995).

A cooled CCD 16 bits camera (Tucsen Imaging Technology Co. LTD, China) (1360 x 1024), with a long pass filter (> 685 nm) placed in front of the camera was used to photograph the microbial mats during exposure to blue (470 nm) and amber (600 nm) excitation light (Carreira et al. 2015a). Autofluorescence images of blue and amber (Blue-amber ratio, BAR; log scale), were used as an indicator of cyanobacteria dominance (< 0), or diatoms dominance (> 0). Images were analysed with ImageJ 1.47m. Autofluorescence values were extracted from the same area as the core sampling for viruses and prokaryotes.

Viral and prokaryotes abundances

For enumeration of prokaryotes and viruses, 100 mg subsamples were taken from every sampling depth and placed in sterile 2 mL Eppendorf tubes. The subsamples were fixed with 2 % glutaraldehyde final concentration (25 % EM-grade, Merck, diluted in sterile seawater), for 15 min at 4°C. As storage before extraction results in decreased prokaryotes cell and virus count (Carreira et al. 2015b), subsamples were immediately treated and placed on a filter, after which they were stored. Extraction of prokaryotes and viruses from the sediment samples was performed according to the protocol by Carreira et al. (2015b). In short, subsamples were incubated with 0.1 mM EDTA (final concentration) on ice and in the dark for 15 min, ultrasonicated (Soniprep 150; 50 Hz, 4 μ m amplitude, exponential probe) for three cycles of 10 s with 10 s intervals in ice-water. One microliter of each subsample and 1 μ L of Benzonase Endonuclease from *Serratia marcescens* (Sigma-Aldrich; > 250 U μ L⁻¹) were added to 1 mL of sterile MilliQ water (18.2 M Ω), and incubated at 37°C in the dark for 30 min. The digestion of free nucleic acids was stopped by placing the sample on ice, directly followed by filtration onto a 0.02 μ m pore size filter (Anodisc 25, Whatman) and stained according to Noble & Fuhrman (1998) using SYBR Gold (Molecular Probes[®], Invitrogen Inc., Life Technologies[™], NY, USA). Each filter was rinsed three times with sterile MilliQ and mounted on a

glass slide with an anti-fade solution containing 50 % glycerol, 50 % phosphate buffered solution (PBS, 0.05 M Na_2HPO_4 , 0.85 % NaCl, pH 7.5) and 1 % *p*-phenylenediamine (Sigma-Aldrich) and stored at -20°C. Viruses and prokaryotes were counted using a Zeiss Axiophot microscope equipped for epifluorescence (100 W mercury lamp, excitation with a BP 450 - 490, emission LP 515, beam splitter FT 510). Magnification of the ocular was x 12.5 and the oil lens (Plan-NEOFLUAR) was x100. At least 10 fields and 400 viruses and prokaryotes were counted per sample. Abundances were presented per gram of wet weight (as samples were too small, i.e. 100 mg, to conduct simultaneous measurements of abundance and water content). Counting error was 1.5 and 10 % for viruses and prokaryotes respectively.

Statistical analyses

Linear regression analyses (model II) were performed to obtain the best-fitting coefficients between pairs of variables (Sokal & Rohlf 1995). To determine differences in viral and prokaryotes abundances between months and depths, ANOVA analysis with post hoc Tukey HSD tests were performed. Prior to statistical analysis, normality was checked and the confidence level was set at 95 %. All statistical analyses were conducted using SigmaPlot 12.0.

5.3. Results

The intertidal zone on Schiermonnikoog was covered by photosynthetic microbial mats in an area of about 7 km², and was characterised by periodical immersion during high tide. Typically, the sediment consisted of fairly homogeneous, well-sorted sands with a mean modal grain size between 180 - 220 µm. A 1 to 2 mm thick silty layer (modal size between 20 - 40 µm) was found at 4 - 5 mm depth. The water content of the sediment decreased strongly in the top 2 mm from 42 % in the top 0 - 1 mm, to 26 % in 1 - 2 mm depth layer, to 20 % at 2 - 10 mm deep. Organic matter content also decreased with depth, with 7 % at the top (0 - 1 mm), 2 % at the middle (1 - 5 mm) and 0.6 % at depth (5 - 10 mm).

In general, the photosynthetic microbial mats were dominated by filamentous cyanobacteria and benthic diatoms as shown by BAR analysis (Fig. 5.1) and confirmed by light microscopy (green algae not observed). In the 3D study, cyanobacteria dominated the mat surface and were horizontally distributed in clusters separated by diatom clusters within the mm scale (Fig. 5.2).

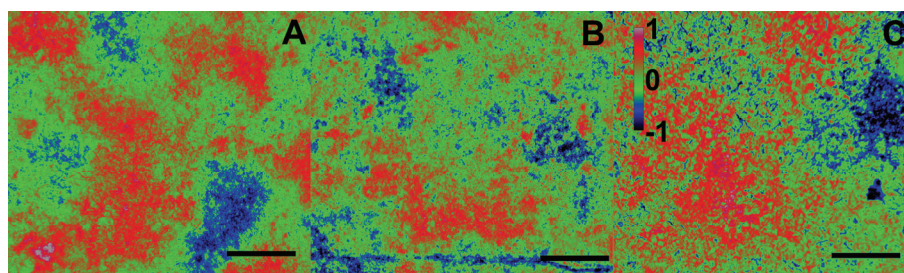


Figure 5.1. Autofluorescence of blue to amber ratio (BAR) images of the top view of the photosynthetic layer of the microbial mat on Schiermonnikoog (The Netherlands). (A) November, (B) April, (C) July. Colour scale indicates that values < 0 are cyanobacteria dominated and > 0 are diatom dominated. Scale bars are 1 cm.

The average prokaryotic abundance (Fig. 5.3) in the November 3D-study was about 2-fold higher in the 0 - 1 mm top layer ($1.7 \pm 0.6 \times 10^{10} \text{ g}^{-1}$) compared to the 1 - 2 mm lower layer ($0.9 \pm 0.4 \times 10^{10} \text{ g}^{-1}$; $p < 0.001$). Viral abundances for the top 0 - 1 mm and the lower 1 - 2 mm, were however, rather similar, i.e. ranged from 1.4 to $5.4 \times 10^{10} \text{ g}^{-1}$ with averages of 3.3 ± 1.1 and $2.9 \pm 1.1 \times 10^{10} \text{ g}^{-1}$, respectively (Fig. 5.4). Because prokaryotic abundance varied more (higher in top) than viral abundance, the average virus to prokaryotes ratio (VPR) was 1.6-fold lower ($p < 0.001$) in the top layer as compared to the bottom layer (3.5 ± 1.3), causing a VPR ‘hotspot’ (VPR = 7.2) in the lower layer (Fig. 5.5). Viral and prokaryotic abundances did not show significant correlation with oxygenic photoautotrophs (Fig. 5.4).

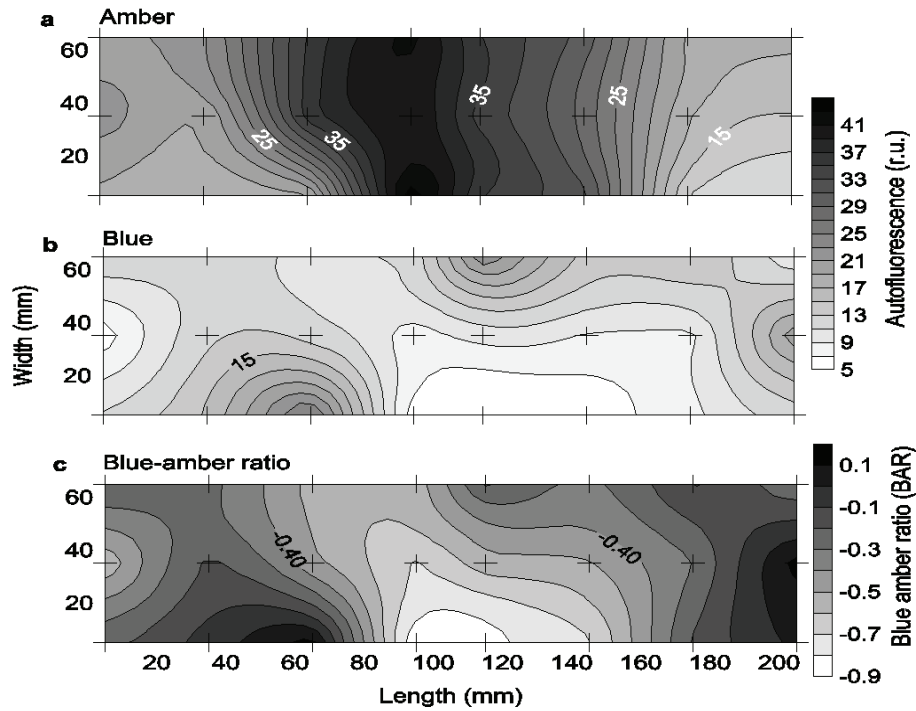


Figure 5.2. Spatial distribution of autofluorescence (relative units) distribution in the photosynthetic microbial mat on Schiermonnikoog in November 2012, after (A) amber (cyanobacteria) light exposure, (B) blue (diatoms) light exposure, and (C) blue to amber ratio (BAR). Area of studied sample: 210 x 70 x 1 mm (L x W x H). Crosses indicate sampling points.

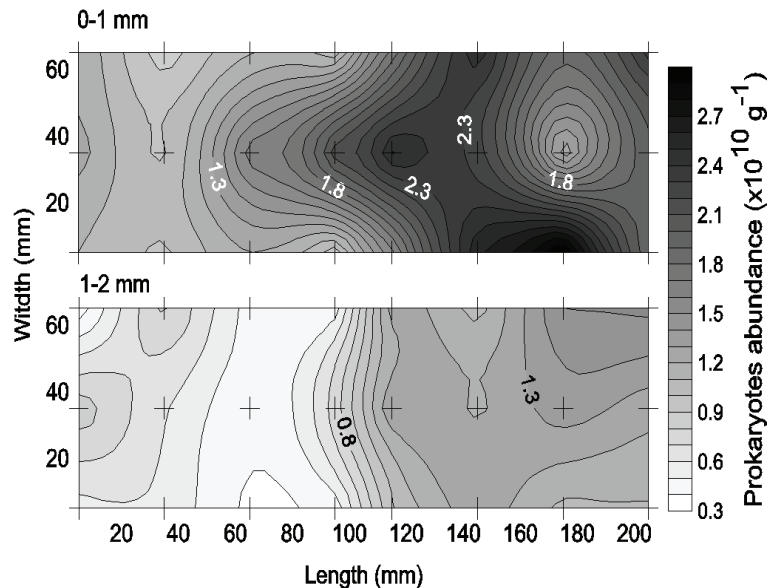


Figure 5.3. Spatial distribution of the prokaryotic abundance in (A) the top 0 - 1 mm, and (B) the lower 1 - 2 mm layer of a photosynthetic microbial mat on Schiermonnikoog (The Netherlands) in November 2012. Area of sampling was 210 x 70 x 2 mm (L x W x H). Crosses indicate the sampling points.

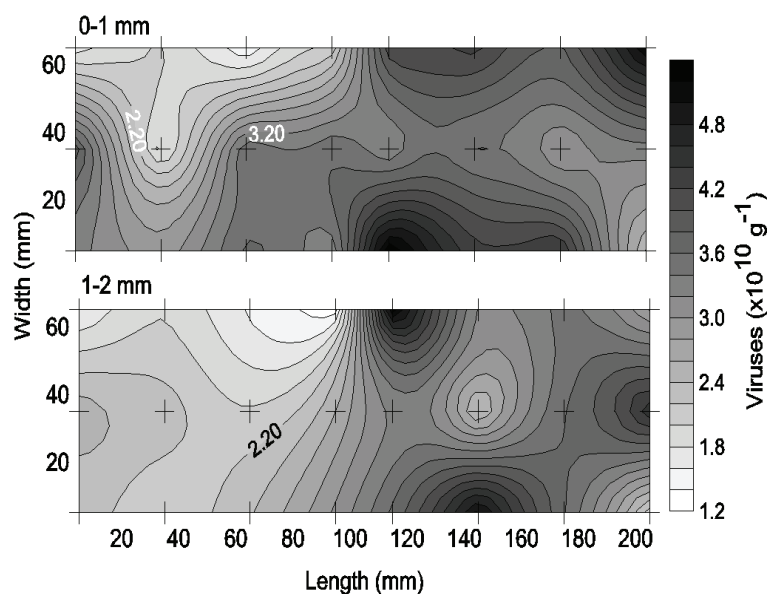


Figure 5.4. Spatial distribution of the viral abundance in (A) the top 0 - 1 mm, and (B) the lower 1 - 2 mm layer of a photosynthetic microbial mat on Schiermonnikoog (The Netherlands) in November 2012. Area of sampling was 210 x 70 x 2 mm (L x W x H). Crosses indicate the sampling points.

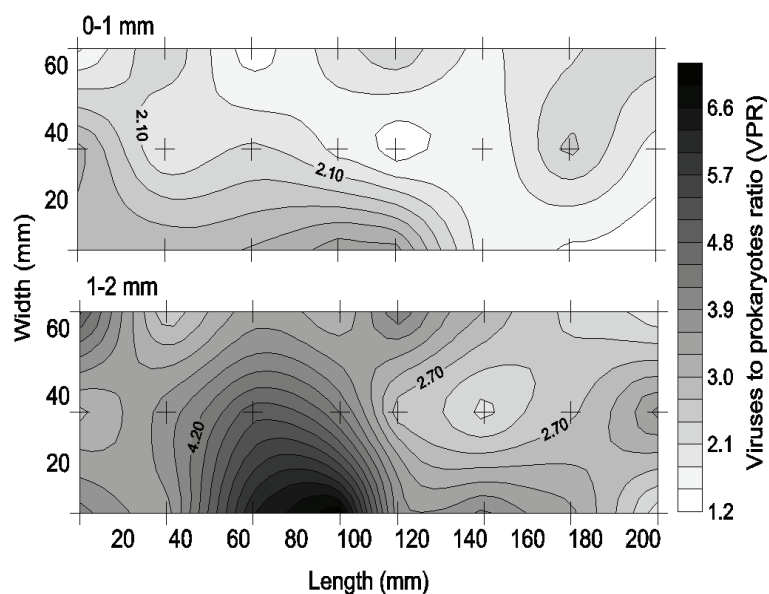


Figure 5.5. Spatial distribution of the viruses to prokaryotes ratio (VPR) in (A) the top 0 - 1 mm, and (B) the lower 1 - 2 mm layer of a photosynthetic microbial mat on Schiermonnikoog (The Netherlands) in November 2012. Area of sampling was 210 x 70 x 2 mm (L x W x H). Crosses indicate the sampling points.

The top photosynthetic layer decreased from 2 mm in November to 1 mm in April, and increased again in July to 1.5 mm (Fig. 5.6A, B). The anoxic black layer characterized by iron sulfide appeared 4 - 5 mm lower in April and July than at the 2 mm depth in November. Independent of season, a distinct purple sulfur bacteria layer of about 1 mm was occasionally observed below the oxygenic photoautotrophs. The vertical profiles in the abundances of prokaryotes and viruses varied with date of sampling (Fig. 5.7). Viral abundances were significantly different in November and April ($p < 0.001$) compared to July, whereas no differences were found between November and April viral abundances. Prokaryotes and VPR showed significantly different abundances in November compared to April ($p < 0.05$ and $p < 0.001$, respectively) and July ($p < 0.001$ and $p < 0.05$, respectively), but no differences were found between April and July. Overall, prokaryotes and viral abundances were highest in November, followed by April, and July. VPR was highest in November (average 2.7 ± 0.7 , range 1.0 - 4.7), followed by April (1.8 ± 1.6 ; 0.5 - 5.4) and July (1.4 ± 0.7 ; 0.4 - 3.6) (Fig. 5.7).

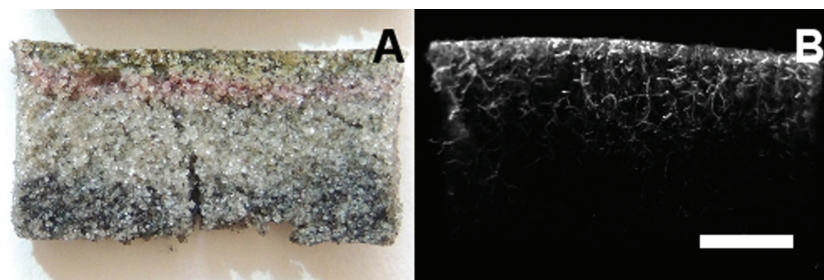


Figure 5.6. Cross section example of a typical photosynthetic mat from Schiermonnikoog (The Netherlands). (A) Colour image, (B) autofluorescence image after amber excitation showing the photosynthetic layer. Scale bar is 0.5 cm.

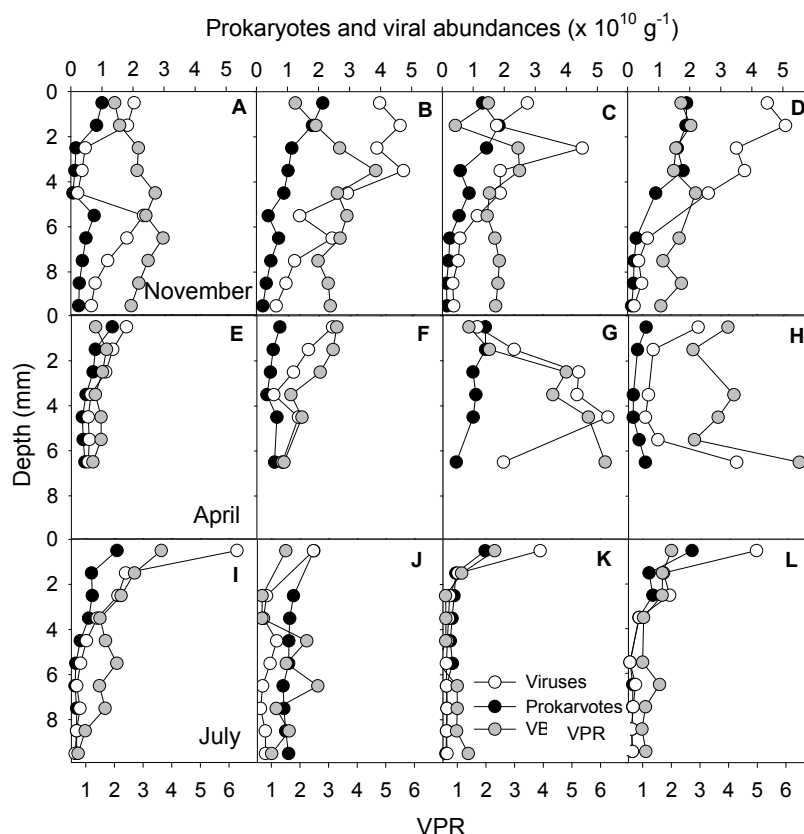


Figure 5.7. Depth profiles of the four replicates showing the abundances of viruses (open symbols) and prokaryotes (black symbols), and virus to prokaryotes ratio (VPR; grey symbols) in November (panel A, B, C, and D), April (panel E, F, G, and H) and July (panel I, J, K, and L) in Schiermonnikoog (The Netherlands).

Viral abundances in the top 0 - 1 mm layer were significantly different from the abundances in the layers just below (1 - 3 mm), and the abundances in the 1 - 2 mm layer were significantly different from all layers below 5 mm (Table 5.1). Prokaryotic abundances was significantly different between the top 0-1 mm and all layers below 2 mm, while the layer 1 - 2 mm was significantly different from all layers below 4 mm (Table 5.1). Overall most differences were found between the top 2 or 3 mm and the layers below (2 - 10 mm) (Table 5.1). Therefore, in order to identify more detailed spatial patterns in the relationship between the distribution of prokaryotes and viruses, we divided the depth profiles into three depth intervals: 0 - 1 mm, 1 - 2 mm and 2 - 10 mm, representing the top and bottom of the photosynthetic microbial mat, and the sediment under the photosynthetic microbial mat (Fig. 5.8). Viral abundance showed significant positive linear correlations with prokaryotes abundance for all

depth intervals and dates sampled (except in the 0 -1 mm layer in April; Table 5.2). The slopes of regression differed only slightly between the different layers and months. Correlations between viral and prokaryotic abundances were generally weaker in the top photoautotrophic layer (Fig. 5.8). Neither bacterial nor viral abundances correlated with oxygenic photoautotrophs autofluorescence.

Table 5.1. Statistical analysis (ANOVA) of viral and prokaryotic abundances for each depth. Numbers in *italic* are the P-values for viral abundances, others are the P-values for prokaryotic abundances. N.s. stands for not significant.

Prokaryotes\Viruses										
Depth (mm)	0.5	1.5	2.5	3.5	4.5	5.5	6.5	7.5	8.5	9.5
0.5		< 0.001	< 0.05	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
1.5	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	< 0.05	< 0.05	< 0.001	< 0.001	< 0.001
2.5	< 0.05	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	< 0.05	< 0.05	< 0.001
3.5	< 0.001	<i>n.s.</i>	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	< 0.05
4.5	< 0.001	< 0.05	<i>n.s.</i>	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
5.5	< 0.001	< 0.05	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
6.5	< 0.001	< 0.001	< 0.05	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>
7.5	< 0.001	< 0.001	< 0.001	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>		<i>n.s.</i>	<i>n.s.</i>
8.5	< 0.001	< 0.001	< 0.001	< 0.05	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>		<i>n.s.</i>
9.5	< 0.001	< 0.001	< 0.001	< 0.05	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	<i>n.s.</i>	

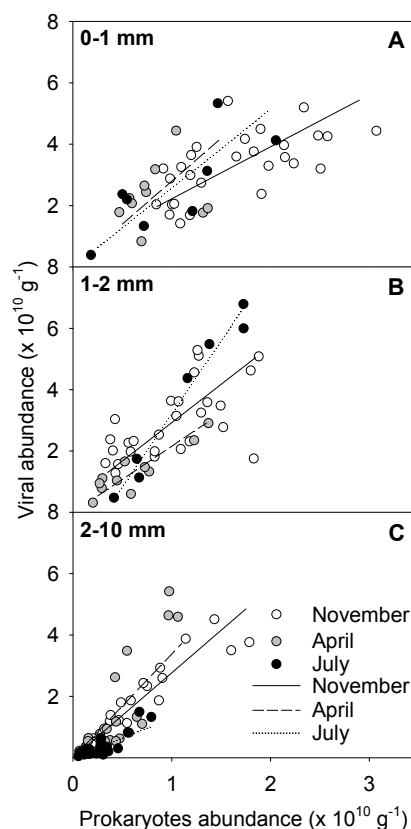


Figure 5.8. Linear regressions between the abundances of prokaryotes and viruses in three depth zones and per sampling month: (A) 0 - 1 mm, (B) 1 - 2 mm, and (C) 2 - 10 mm.

Table 5.2. Linear regression analysis information (R^2 , equation, and significance) between viral and prokaryotic abundances in a photosynthetic microbial mat in Schiermonnikoog (as shown in Fig. 5.8), for the different sampling months (November, April, and July) and depths (0 - 1, 1 - 2, and 2 - 10 mm). N.s. stands for not significant.

Month	Depth (mm)	R^2	Equation	P
November	0 - 1	0.40	Virus = 1.7 Prok + 1.6×10^{10}	< 0.001
	1 - 2	0.38	Virus = 2.5 Prok + 1.4×10^{10}	< 0.001
	2 - 10	0.90	Virus = 2.8 Prok + 0.1×10^{10}	< 0.0001
April	0 - 1	0.02	Virus = 2.8 Prok + 1.9×10^{10}	n.s.
	1 - 2	0.81	Virus = 2.2 Prok + 0.2×10^{10}	< 0.001
	2 - 10	0.70	Virus = 3.4 Prok - 1.0×10^{10}	< 0.0001
July	0 - 1	0.61	Virus = 2.6 Prok + 0.6×10^{10}	< 0.05
	1 - 2	0.97	Virus = 4.8 Prok - 1.5×10^{10}	< 0.0001
	2 - 10	0.75	Virus = 1.3 Prok - 0.1×10^{10}	< 0.0001

5.4. Discussion

Intertidal photosynthetic microbial mats are dynamic laminated heterogeneous systems with a photoautotrophic layer typically composed of cyanobacteria and diatoms (Dijkman et al. 2010). These mats are characterised by high primary production, high biomass concentration, and high rates of remineralisation and nutrient cycling (De Wit et al. 1989, Van Gernerden 1993, Jonkers et al. 1998). Temperature and light have been shown to influence their productivity, but equally important for the mat development is the reduced impact of grazers (Fenchel 1998).

In an environment with reduced grazing, viruses may potentially play a key role in microbial mortality. In addition, as drivers of nutrient recycling, viruses may support mat regeneration and productivity. Our study shows that the intertidal photosynthetic microbial mats in Schiermonnikoog have high viral abundances, reaching densities of 0.05 to 5.4×10^{10} viruses g^{-1} . The observed abundances of viruses in the mats are more than 10-fold higher those typically recorded in marine sediments (Danovaro et al. 2008a). Such high viral abundances have only been reported for eutrophic estuarine sediments with high microbial activity (Hewson et al. 2001, Helton et al. 2012). An earlier study of abundances in lagoon microbial mat also showed high abundances ($\sim 15 \times 10^{10} \text{ g}^{-1}$ dry weight) (Pacton et al. 2014). In this study the data was presented as dry weight specific densities (Pacton et al. 2014). Converting the wet weight specific densities obtained in our study to the corresponding densities per gram dry weight using an average water content (42 and 26 % corresponding to 0 - 1 and 1 - 2 mm layers, respectively; this study), showed equally high viral ($0.2 - 20 \times 10^{10} \text{ g}^{-1}$) and prokaryotes ($0.2 - 7.3 \times 10^{10} \text{ g}^{-1}$) abundances in the top 0 - 2 mm of the Schiermonnikoog mats.

The high viral abundances found in the present study are possibly the result of the high biological activity found in microbial mats (De Wit et al. 1989, Van Gernerden 1993), as reflected by the high densities of both prokaryotes and oxygenic photoautotrophs in the mats (although inputs from other sources such as sand deposition or rain cannot be excluded).

The higher prokaryotic abundances in the first 0 - 1 mm of the mat during the November 3D-experiment were most likely linked to the oxygenic photoautotrophic

dominance in this top layer. As the viral abundance did not show much variation between the top layer and the 1 - 2 mm below, our data indicate that prokaryotes were more actively growing in the top layer, or that viral decay was higher in the top than in the 1 - 2 mm layer below. Prokaryotic and viral activity in coastal marine sediments are generally positively correlated with organic carbon loading (as indicated from carbon mineralization rates), resulting in gradients of increasing viral and bacterial production along trophic gradients (Middelboe & Glud 2006, Middelboe et al. 2006). Moreover, microbial metabolic activity has been found to be highest in the top of the photosynthetic microbial mats and indeed directly linked to organic matter load (Jonkers et al. 2003). Much of the organic matter load that fuels prokaryotic production, is excreted by photosynthetic microbes as exopolymeric substances (Decho et al. 2005). Higher growth rates of prokaryotes have been shown to result in higher viral burst sizes (Middelboe & Glud 2006). However, we found that the VPR in the upper 1 mm layer was mostly lower than in the layers below. Since the surface layer receives higher UV doses and higher radical concentrations due to O₂ oversaturation (non published data), it seems reasonable to assume that viral decay is higher in the upper layer than the lower layers. This may explain the low average VPR found in the photosynthetic microbial mat as compared to other marine sediment environments (Danovaro et al. 2008a).

The large spatial heterogeneity in oxygenic photoautotrophs distribution and viral and prokaryotic abundances emphasizes that the microbial mats are dynamic structures. This is, to our knowledge, the first study showing distinct spatial separation of cyanobacterial and diatom populations at the microscale. Until now, only patchy spatial dispersal of all oxygenic photoautotrophs combined (inferred from chl *a*) had been observed for the intertidal sediment surface (Seuront & Leterme 2006).

The distribution of prokaryotes and viruses in the mat was also heterogeneous at the microscale, both horizontally and vertically. Such high heterogeneity was previously demonstrated in studies in the water column, biofilms, and sediments (Neu & Lawrence 1997, Seymour et al. 2006, Stewart & Franklin 2008, Carreira et al. 2013). While, energy availability is among the most studied driving forces for spatial heterogeneity in microbial communities in sediments (Blackburn & Fenchel 1999,

Fenchel & Glud 2000), other factors such as competition and losses (grazing, viral lysis) may also contribute significantly to spatial heterogeneity. In addition, a recent study has shown that viral activity contributes to the microscale distribution of prokaryotes in intertidal sediments (Carreira et al. 2013). In microbial mats, where grazing is greatly reduced (Fenchel 1998), viruses could constitute the main mortality agent, and consequently hypothesize, that viruses are an important factor driving spatial heterogeneity in prokaryotes and oxygenic photoautotroph distribution patterns in a microbial mats. To test this hypothesis, future studies should focus on measuring viral lysis rates for the different hosts.

In November, at the end of the growth season, the photosynthetic microbial mat was fully developed, with a thick cyanobacterial sheet at the surface and high abundances of prokaryotes and viruses below. As the seasons progressed from November to July, the oxygenic photoautotrophic layer initially decreased with depth (April), but by July it started to increase again. The relatively low microbial abundances at the top in combination with the thin photosynthetic layer in April suggest limited activity of the mat after the winter period, followed by increased activity during summer (July) leading to higher abundances of viruses and prokaryotes in the top layer in July. The high prokaryote and viral abundances observed deeper in the heterotrophic layers in April reflect most likely inter annual variation or ongoing microbial decomposition of mat layers from previous years. We hypothesize that the temporal changes in distribution of viruses and prokaryotes are tightly linked to the photosynthetic activity and extension of the top productive layer of the mat. The general vertical stratification observed agrees with other published works from the same (Bauersachs et al. 2011) or similar areas (Stal et al. 1985, Mir et al. 1991) with different levels of development, due to seasons. This emphasizes the close spatial coupling between autotrophic and heterotrophic processes in these mats.

In general, viruses could be associated with both prokaryotes and oxygenic photoautotrophs in the top 2 mm, as the correlation between viruses and prokaryotes in these layers is weaker than when oxygenic photoautotrophs are absent (2 - 10 mm), independently of sampling period. However, as photoautotrophic microbial mats contain many different prokaryotes and viruses, future studies should be

complemented with more detailed analyses of community composition. This could be accomplished for example, by integrating other methodologies, such as electron microscopy to verify viral morphologies and frequency of infected cells, and metagenomics to verify the microbial diversity. Particularly in such a densely packed, biological and metabolic distinctly diverse ecosystem, molecular techniques are expected to advance our understanding of microbial and viral diversity and host-virus interactions. However, obtaining actual viral mediated mortality rates for this small-scale laminated and spatial heterogeneous mat system will be challenging.

In summary, oxygenic photoautotrophs, prokaryotes and viruses were distributed in microscale (mm-scale) patches both horizontally and vertically in the photosynthetic microbial mat. The abundance of both prokaryotes and viruses increased in the growing season, in accordance with the greater depth of the photosynthetic layer. The very high viral abundances in the microbial mat, the variation in depth profiles at different times of the year, together with the spatial 3D heterogeneity, suggest that viruses are likely active components in these productive environments. An active role of viruses can be expected to have implications for the development and productivity of the mat and the biogeochemical fluxes within the mat. Our results emphasize the importance for further studies on the role of viruses as regulators of community dynamics and productivity in photosynthetic microbial mats.

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Chapter 6

Disruption of photoautotrophic intertidal mats by filamentous fungi

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Abstract

Ring-like structures, 2.0 - 4.8 cm in diameter, observed in photosynthetic microbial mats on the Wadden Sea island Schiermonnikoog (The Netherlands) showed to be the result of the fungus *Emericellopsis* sp. degrading the photoautotrophic top layer of the mat. The mats were predominantly composed of cyanobacteria and diatoms, with large densities of prokaryotes and viruses both in the top photosynthetic layer and in the underlying sediment. The fungal attack cleared the photosynthetic layer, however, no significant effect of the fungal lysis on the prokaryotes and viral abundances could be detected. Fungal mediated degradation of the major oxygenic photoautotrophs could be induced by inoculation of non-infected mat with isolated *Emericellopsis* sp, and with an infected ring sector. Diatoms were the first re-colonisers followed closely by cyanobacteria that after about 5 days dominated the space. The study demonstrated that the fungus *Emericellopsis* sp. efficiently degraded a photosynthetic microbial mat, with potential implications for mat community composition, spatial structure and productivity.

Key words: fungi, photosynthetic microbial mat, cyanobacteria, diatoms, disruption, rings

6.1 Introduction

Photosynthetic microbial mats, found worldwide in a variety of extreme environments (Castenholz 1994), are dynamic laminated microbial communities containing photoautotrophs, micro fauna, fungi, heterotrophic prokaryotes, and viruses. The top layer of these mats is mostly composed of filamentous cyanobacteria and eukaryotic microalgae, which fuel the heterotrophic prokaryote communities inhabiting the underlying sediment (Van Gernerden 1993, Canfield et al. 2005). Due to the reduced grazing activity (Fenchel 1998) and the production of significant amounts of exopolymeric substances (EPS) by oxygenic photoautotrophs and prokaryotes (De Brouwer et al. 2002), the mats often show a well defined laminated vertical structure. Under certain conditions, characterized by occasional flooding and low sand deposition, marine intertidal flats can sustain physical stable microbial mats (Stal 1994). The chemical and biological landscapes of microbial mats are highly dynamic and the biomass and chemical gradients vary drastically on small spatial scales as well as in short time intervals. The chemical gradients are mainly driven by variable production and consumption rates within the mat and the biomass heterogeneity may be the result of variable growth conditions, local grazing and cell lysis caused by chemical compounds, fungi and viruses.

Fungi have previously been observed in hypersaline microbial mats (Cantrell et al. 2006), and in a recent study fungi were suggested to be diverse and quantitatively important components of carbon degradation in photosynthetic mats along with prokaryotes (Cantrell & Duval-Pérez 2013). Furthermore it was shown that the fungal communities were more diverse in the oxic photosynthetic layer. Fungal activity may not be restricted to decomposition of detritus, as some fungi isolated from freshwater, soil and air have been found to predate and lyse cyanobacteria and green algae (Safferman & Morris 1962, Redhead & Wright 1978, Redhead & Wright 1980). Some of these fungi belonged to the genus *Acremonium* and *Emericellopsis* and produced a heat stable extracellular compound thought to be the antibiotic cephalosporin C. Furthermore parasitic microscopic fungi (chytrids) have been associated with bloom control of the diatom *Asterionella formosa* in both lakes (Canter & Lund 1948) and culture studies (Bruning 1991). Also, a bloom of the

cyanobacteria *Anabaena macrospora* has been shown to be influenced by fungal predation (Gerphagnon et al. 2013). Despite the potential significance of fungi for the mortality and degradation of oxygenic photoautotrophs, little is known about the ecological impact of benthic fungi in photosynthetic microbial mats.

Fairy-rings are a phenomenon occurring in terrestrial environments, where fungi grow in large radial shapes and may manifest as necrotic zones (Bonanomi et al. 2011, Caesar-TonThat et al. 2013, Ramond et al. 2014). To the best of our knowledge ring-structures caused by fungi have never been observed before in microbial mats. In the current study we investigated the spatial distribution of oxygenic photoautotrophs, prokaryotes and viruses in ring-like structures that were found in intertidal photosynthetic microbial mats on the Wadden Sea island Schiermonnikoog (The Netherlands). These rings were caused by local cell lysis of filamentous cyanobacteria, caused by associated fungal activity.

6.2. Material and Methods

Intertidal photosynthetic microbial mat samples were collected during autumn (November 2012) and summer (July 2013 and August 2014) from the island Schiermonnikoog, situated in the intertidal Wadden Sea, The Netherlands (53° 29' 24.29"N, 6° 8' 18.02"E). Microbial mats with visible ring structures were cut out of the mat structure and placed inside a box (15 x 8 x 4 cm; L x W x H). The samples were transported back to the laboratory within 3 - 4 h after sampling, where they were kept outside, at *in situ* conditions until use.

Chlorophyll quantification

Chlorophyll autofluorescence images were taken every second day for 10 days to see whether there were changes in the rings over time. The images were obtained according to Carreira et al. (2015a). Briefly, photographs were taken using a cooled CCD 16 bits camera (Tucsen Imaging Technology Co. LTD, China) (1360 x 1024), with a long pass 685 nm filter placed in front of the camera. The microbial mats were exposed to blue and amber light excitation, to distinguish between

diatoms and cyanobacteria, respectively. Images were analysed with Image J (1.47m). Autofluorescence images of blue to amber (BAR) were used as an indicator of cyanobacteria dominance (< 0), or diatoms dominance (> 0). Colour images were also taken using a 12 bits CCD colour camera (Basler Scout, Germany), and in July, images of the fungus were obtained by stereomicroscope (Carl Zeiss, Germany).

Viral and prokaryotes abundances

For enumeration of prokaryotes and viruses, samples of 1 x 0.5 x 0.1 cm (L x W x H) were taken from distinct locations in the ring, at two depths (0 - 1 and 1 - 2 mm). In November samples were taken to the “core” and “outside”, in a total of three samples per area per ring, in 4 rings. In July samples were taken to “core”, “ring in”, “ring out”, “outside”, and to “mat” (control). Two samples were collected per area and per ring in a total of 3 rings.

Extraction of prokaryotes and viruses were done according to Carreira et al. (2015b). Briefly, the samples were placed in sterile 2 mL Eppendorf tubes and fixed with 2 % glutaraldehyde final concentration (25 % EM-grade, Merck) for 15 min at 4°C, after which samples were incubated with 0.1 mM EDTA (final concentration) on ice and in the dark for another 15 min. Thereafter probe ultrasonication (Soniprep 150; 50 Hz, 4 μ m amplitude, exponential probe) was applied in 3 x cycles of 10 s with 10 s intervals, while keeping the samples in ice-water. Then 1 μ L subsample was diluted in 1 mL of sterile MilliQ water (18.2 M Ω) with 1 μ L of Benzonase Endonuclease from *Serratia marcescens* (Sigma-Aldrich; > 250 U μ L⁻¹) and incubated in the dark at 37°C for 30 min. Next the samples were placed on ice until filtration. Each sample was filtered onto a 0.02 μ m pore size (Anodisc 25, Whatman) and stained according to Noble & Fuhrman (1998) using SYBR Gold (Molecular Probes[®], Invitrogen Inc., Life Technologies[™], NY, USA). The filter was rinsed three times with sterile MilliQ after which it was mounted on a glass slide with an anti-fade solution containing 50 % glycerol, 50 % phosphate buffered solution (PBS, 0.05 M Na₂HPO₄, 0.85 % NaCl, pH 7.5) and 1 % *p*-phenylenediamine (Sigma-Aldrich, The Netherlands) and stored at -20°C. Viruses and prokaryotes were counted using a

Zeiss Axiophot epifluorescence microscope at x1150 magnification. At least 10 fields and 400 viruses and prokaryotes each were counted per sample.

Fungal isolation and identification

An isolation procedure was carried out intending to identify the fungal agents involved in the formation of the ring structure on the intertidal photosynthetic microbial mat. A mat sample (15 x 8 x 4 cm; L x W x H) containing several ring structures was collected as previous described and the presence of fungi on this structure was investigated. Ten pieces (0.5 x 0.5 x 0.1 cm; L x W x H) of mat were randomly taken from the “ring in” in different rings and transferred to a tube containing 10 mL of sterilized water. This mixture was vigorously stirred for 2 min and afterwards 100 µL of this suspension was used to inoculate malt extract agar (MEA) plates supplemented with penicillin and streptomycin to avoid prokaryotes growth. After 7 days of incubation at 25°C fungal colonies were observed on all plates. A unique fungal colony was randomly chosen and sub cultured several times in Petri dishes to ensure the obtainment of a pure culture.

Fungal identification was carried out by amplification and sequencing of three nuclear loci including LSU (large subunit of the nuclear ribosomal RNA gene), ITS (including internal transcribed spacer regions 1 and 2, and the 5.8S rRNA regions of the nuclear ribosomal RNA gene cluster) and β -tub (beta-tubulin intron 3).

Fungal genomic DNA of the isolated strain was isolated using the FastDNA® Kit (Bio 101, Carlsbad, USA) according to the manufacturer's instructions. A fragment containing the LSU region was amplified using primers NL1 (GCATATCAATAAGCGGAGGAAAAG) (O'Donnell 1996) and LR5 (ATCCTGAGGGAAACTTC) (Vigalys & Hester 1990). A fragment containing the ITS region was amplified using forward primer ITS5 (GGAAGTAAAAGTCGTAACAAGG) and reverse primer ITS4 (TCCTCCGCTTATTGATATGC) (White et al. 1990). The β -tub fragment was amplified using primers Bt2a (GGTAACCAAATCGGTGCTGCTTTC) and Bt2b (ACCCTCAGTGTAGTGACCCTTGGC) (Glass & Donaldson 1995). PCR and

sequencing procedures were performed as described previously by Summerbell et al. (2011).

The amplified sequences were compared with homologous sequences deposited in Genbank database and Maximum Likelihood phylogenetic trees were constructed using MEGA 5.0. Maximum parsimony analysis was performed for all datasets using the heuristic search option. The robustness of the most parsimonious trees was evaluated with 1000 bootstrap replications.

The procedure for fungal isolation and identification described above was repeated with mat samples collected in August 2014 and the fungal strain obtained in this second isolating process was absolutely, morphologically and genetically, related with the strain *Emericellopsis* sp. 137197 isolated in the year before.

Healthy mat samples were inoculated with *Emericellopsis* sp 137197 to confirm its ability to attach and degrade photosynthetic microbial mats. The fungus was cultivated in liquid media with the following composition (g L⁻¹): NaNO₃ 6.0; KH₂PO₄ 1.5; KCl 0.5; MgSO₄ 0.5; glucose 10 and 200 µL of trace solution (EDTA 1.0 %; ZnSO₄·7H₂O 0.44 %; MnCl₂·4H₂O 0.1 %; CoCl₂·6H₂O 0.032 %; CuSO₄·5H₂O 0.031 %; (NH₄)₆Mo₇O₂₄·4H₂O 0.022 %; CaCl₂·2H₂O 0.15 %; FeSO₄·7H₂O 0.1 %). The cultivation was carried out for 3 days in orbital shaker at 25°C and 200 rpm. The broth containing the mycelial biomass was homogenized in a blender and directly employed for inoculation. A micropipette was employed to inoculate the mat and 50 µL of homogenized broth were applied in each spot test (n = 20). A negative control (killed fungus) was carried out in parallel by inoculation of healthy mat with autoclaved homogenized broth (120°C, 20 min) (n = 17). All samples were incubated outside at ambient temperature to mimic, as close as possible, natural conditions. The development of ring-like structures was followed over 10 days by autofluorescence and colour images.

To examine the effect of the fungus as the degrading agent of the mat and for the development of the ring structures in the oxygenic photoautotrophs, a piece (1 x 0.5 x 0.1 cm; L x W x H) of microbial mat containing “ring in”, “ring out”, and “outside” was transplanted into a non-infected microbial mat. The growth was followed with autofluorescence images taken every day for 7 days.

Statistical analyses

To determine differences in viral and prokaryotes abundances, and virus to prokaryotes ratio (VPR) between seasons, depths, and sampled areas, ANOVA with post hoc Tukey HSD tests were performed. Prior to statistical analysis, normality was checked and the confidence level was set at 95 %. All statistical analysis was conducted in SigmaPlot 12.0.

6.3. Results

Ring-like structures were observed in the photosynthetic microbial mats on the island Schiermonnikoog (The Netherlands). These ring-like structures were examined by a combination of autofluorescence imaging, epifluorescence microscopy and genomics in order to determine the cause of these patterns.

The horizontal distribution of oxygenic photoautotrophs in the non-infected microbial mats was either characterized by a dominance of cyanobacteria or an equal mix of cyanobacteria and diatoms in both seasons, as showed by the blue to amber ratio (BAR) (fig. 6.1A, B). On average the BAR value was -0.4 ± 0.3 , indicating the cyanobacterial dominance. The distribution of cyanobacteria and diatom populations was heterogeneous and the individual clusters were separated by mm distances.

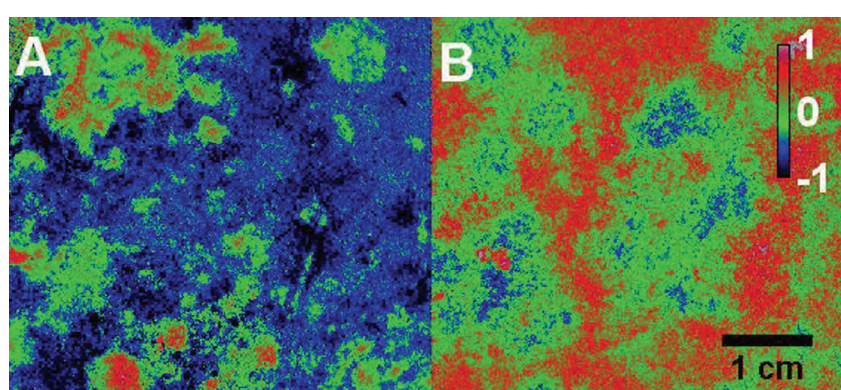


Figure 6.1. Examples of blue to amber ratio (BAR) of the photosynthetic microbial mats. Values < 0 indicate cyanobacteria dominance and values > 0 indicate diatom dominance. (A) exemplifies a microbial mat dominated by cyanobacteria, whereas (B) shows a mat of mixed populations of cyanobacteria and diatoms.

Ring-like structures (2.0 - 4.8 cm diameter) appeared in the photosynthetic microbial mats during summer and autumn (not observed during winter and spring). In November the microbial mat had been recently flooded (Fig. 6.2A, B). Whereas in July and August the mat was dry (Fig. 6.2C, D). To characterize the ring structures, distinct zones were identified. In November, two areas with different structure were identified: the ring core (“core”) and outside the ring (“outside”) (Fig. 6.3A). In July three distinct zones were identified in the ring structures: the ring core (“core”), the ring around the core (“ring”), and outside the ring (“outside”). The “ring” area could usually be divided in two rings: “ring in” and “ring out” (Fig. 6.3A). In July control samples were also taken well away from ring (“mat”).

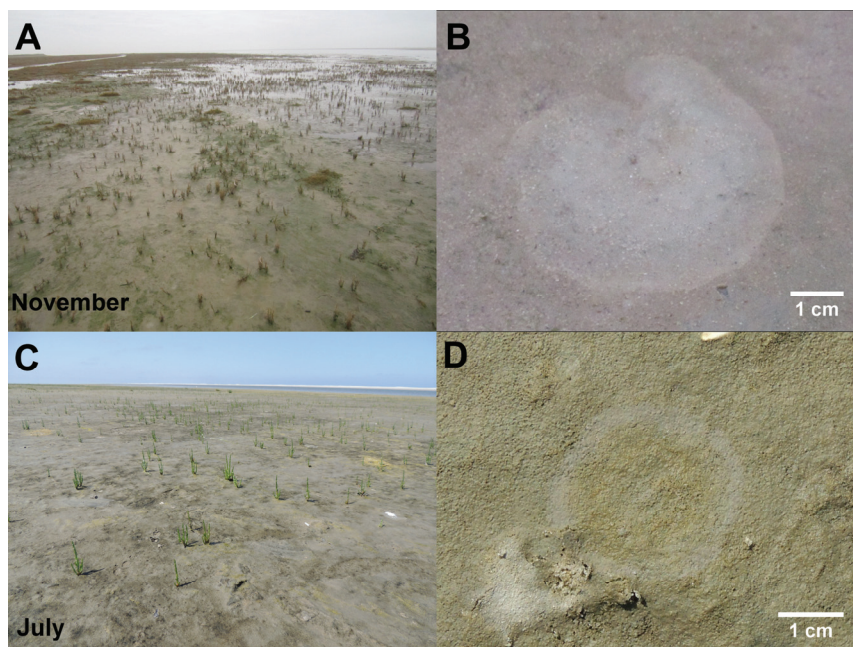


Figure 6.2. View of sampling area and examples of ring-like structures in photosynthetic microbial mats on the Wadden Sea island Schiermonnikoog (The Netherlands), illustrating the different environmental conditions in November (A, B) and July (C, D). Scale bar is the same for B and D.

Examination of 5 rings by stereomicroscope and autofluorescence camera showed the “core” of the ring to be dominated by diatoms, with a minor share of cyanobacteria. The “ring in” was cleared of oxygenic photoautotrophs, thus without autofluorescence, but white of colour. Fungal hyphae were observed in the “ring in” (Fig. 6.3). As the fungi spread towards the outside it formed another ring (“ring out”)

with a light green colour. This ring contained some cyanobacteria filaments, although without autofluorescence, and a few fungal hyphae (Fig. 6.3). The “outside” area was dark green in colour and similar to the control area (“mat”) with a mix of cyanobacteria and diatom (Fig. 6.3E).

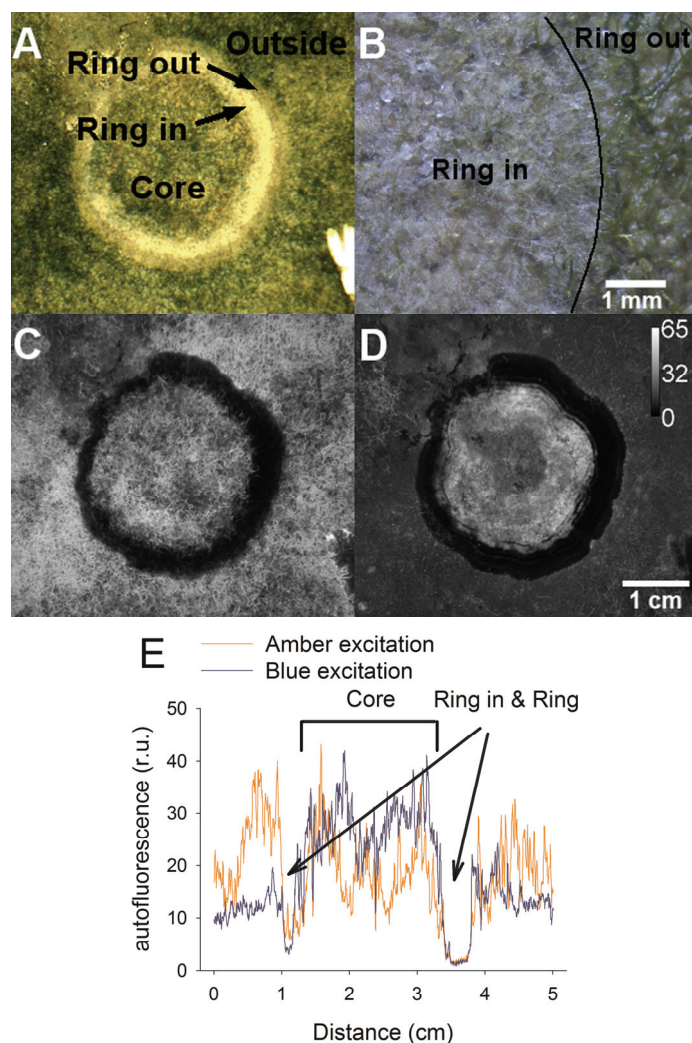


Figure 6.3. Images and plot of autofluorescence across a ring structure. (A) Standard colour camera image of a ring-like structure labelled with the different areas sampled: ring core (core), inner ring (ring in), outer ring (ring out), outside near the ring (outside). (B) Magnified colour image showing the ring-in and ring-out areas (white area contains most fungal biomass), (C) autofluorescence (relative units) after amber excitation, (D) autofluorescence (relative units) after blue light excitation of a ring structure; (E) autofluorescence (relative units) dynamics after amber and blue light excitation across a ring.

The prokaryotes abundances did not vary significantly across the different areas of the ring in any of the samples (Table 6.1). However the top layer always showed higher abundance than the bottom layer, in both seasons. While prokaryotes abundances in the top layer (0 - 1 mm) were similar in November and July ($1.1 \pm 0.4 \times 10^{10} \text{ g}^{-1}$ and $1.3 \pm 0.4 \times 10^{10} \text{ g}^{-1}$, respectively), the bottom layer (1 - 2 mm) showed a significantly ($p < 0.001$) lower prokaryotes abundance in July ($0.4 \pm 0.2 \times 10^{10} \text{ g}^{-1}$) compared to November ($0.9 \pm 0.3 \times 10^{10} \text{ g}^{-1}$). The total average prokaryotes abundances in November and July were similar, i.e. $1.0 \pm 0.4 \times 10^{10} \text{ g}^{-1}$ and $0.9 \pm 0.5 \times 10^{10} \text{ g}^{-1}$, respectively (Table 6.1).

As for the prokaryotes, viral abundances were similar in the different areas of the rings (Table 6.1). Viral abundances in both seasons were higher in the top layer (0 - 1 mm) than in the bottom layer (1 - 2 mm). In November the viral abundance in the 0 - 1 mm layer was similar ($3.4 \pm 1.2 \times 10^{10} \text{ g}^{-1}$) to July ($3.2 \pm 1.2 \times 10^{10} \text{ g}^{-1}$), but the bottom layer (0 - 2 mm) was 3-fold higher in November compared to July ($8.5 \pm 0.5 \times 10^{10} \text{ g}^{-1}$; $p < 0.001$). The total viral abundance did not vary significantly over time and ranged from $2.1 \pm 1.4 \times 10^{10} \text{ g}^{-1}$ (July) to $2.9 \pm 1.3 \times 10^{10} \text{ g}^{-1}$ (November) (Table 6.1).

VPR was not significantly different between the various ring areas. VPR in the bottom layer (1 - 2 mm) was generally lower than the top layer (Table 6.1) and significantly ($p < 0.05$) higher in November than in July for the 0 - 1 mm (3.0 ± 0.8 vs. 2.5 ± 0.7) and the 1 - 2 mm depth (2.8 ± 1.5 vs. 2.0 ± 0.4).

Table 6.1. Average abundances of bacteria and viruses, and the virus to prokaryote ratio (VPR) for the sampled areas (“core”, “ring in”, “ring out”, “outside”, and “mat”) at two depths (0 - 1 and 1 - 2 mm), in November and July. n.d. = not determined. Significant differences between the seasons, depths and sampled areas are noted by different lower case letters for both bacterial and viral abundances, and for the VPR.

	Prokaryotes ($\times 10^{10} \text{ g}^{-1}$)		Viruses ($\times 10^{10} \text{ g}^{-1}$)		VPR	
	November	July	November	July	November	July
Core 0 - 1 mm	1.0 ± 0.5^a	1.5 ± 0.4^a	3.0 ± 0.9^a	3.2 ± 0.5^a	2.9 ± 1.0^a	2.2 ± 0.4^b
Core 1 - 2 mm	0.9 ± 0.2^b	0.5 ± 0.3^c	2.9 ± 0.5^b	1.0 ± 0.7^c	3.2 ± 0.9^a	1.7 ± 0.3^c
Core	1.0 ± 0.4	1.1 ± 0.6	3.0 ± 1.1	2.2 ± 1.3	3.0 ± 1.0	2.0 ± 0.4
Ring in 0 - 1 mm	n.d	1.0 ± 0.4^a	n.d	2.7 ± 1.0^a	n.d	2.8 ± 0.7^b
Ring in 1 - 2 mm	n.d	0.5 ± 0.3^c	n.d	1.0 ± 0.6^c	n.d	2.2 ± 0.5^c
Ring in	n.d	0.7 ± 0.4	n.d	1.8 ± 1.2	n.d	2.5 ± 0.6
Ring out 0 - 1 mm	n.d	1.2 ± 0.4^a	n.d	3.6 ± 1.3^a	n.d	2.9 ± 0.7^b
Ring out 1 - 2 mm	n.d	0.4 ± 0.1^c	n.d	0.8 ± 0.4^c	n.d	2.1 ± 0.5^c
Ring out	n.d	0.8 ± 0.5	n.d	2.2 ± 1.6	n.d	2.5 ± 0.7
Outside 0 - 1 mm	1.3 ± 0.3^a	1.3 ± 0.2^a	3.7 ± 1.3^a	2.5 ± 0.8^a	3.1 ± 0.7^a	2.0 ± 0.4^b
Outside 1 - 2 mm	0.9 ± 0.3^b	0.4 ± 0.1^c	2.0 ± 1.7^a	0.8 ± 0.4^c	2.4 ± 1.8^a	1.7 ± 0.3^c
Outside	1.1 ± 0.3	0.9 ± 0.5	2.9 ± 1.5	1.9 ± 1.2	2.8 ± 1.3	1.9 ± 0.4
Mat 0 - 1 mm	n.d	1.4 ± 0.4^a	n.d	3.7 ± 1.3^a	n.d	2.6 ± 0.8^b
Mat 1 - 2 mm	n.d	0.4 ± 0.2^c	n.d	0.7 ± 0.4^c	n.d	2.0 ± 0.3^c
Mat	n.d	0.9 ± 0.6	n.d	2.3 ± 1.8	n.d	2.3 ± 0.7
Average 0 - 1 mm	1.1 ± 0.4	1.3 ± 0.4	3.3 ± 1.2^a	3.2 ± 1.0^a	3.0 ± 0.8	2.5 ± 0.7
Average 1 - 2 mm	0.9 ± 0.3	0.4 ± 0.2	2.5 ± 1.3^b	0.8 ± 0.5^c	2.8 ± 1.5	2.0 ± 0.4
Total Average	1.0 ± 0.4	0.9 ± 0.5	2.9 ± 1.3	2.1 ± 1.4	2.9 ± 1.2	2.3 ± 0.6

An examination of the fungal morphology and community composition was performed in July, revealing fungus threads in the “ring in” and “ring out” areas. Isolation of the fungi resulted in several colonies all with identical morphological characteristics, suggesting the presence of a single fungal species in the “ring in” and “ring out”. Since all colonies showed the same characteristics, one unique fungal colony was randomly chosen and subcultured several times to warrant a pure culture. The isolated strain presented a radial growth with velvety and white hyphae. Microscopic examination showed that hyphae were septated and hyaline. Sporulation was not observed even after 3 weeks of cultivation on MEA medium, indicating that the fungus requires specific conditions to form reproductive structures.

Fungal identification was carried out by sequence analysis of three loci, LSU, ITS and β -tubulin, and the sequences obtained have been deposited in GenBank database (Accession number: KJ196387, KJ196386 and KJ196385, respectively). A phylogenetic analysis was performed comparing the obtained sequences to available sequences of species of the genus *Acremonium* and *Emericellopsis*. As a first step a one-gene analysis was performed using the LSU sequence, determining the phylogenetic position of the isolated strain in the *Acremonium* clade belonging to the order Hypocreales (Summerbell et al. 2011). The phylogenetic tree 1 (see Fig. S6.1) demonstrated that the isolated strain falls into the *Emericellopsis* clade (94 % bootstrap support), which includes species such as *Acremonium exuviarum*, *Acremonium salmoneum*, *Acremonium potronii* and *Acremonium tubakii*. A second phylogenetic analysis was performed focussing on the *Emericellopsis* clade using a two-gene analysis based on the ITS and β -tub sequences and the dataset generated by Grum-Grzhimaylo et al. (2013). This study suggested that the *Emericellopsis* clade could be split into a terrestrial clade, a marine clade and an alkaline soil clade. The phylogenetic tree (Fig. 6.4) indicated that the fungal strain isolated from “ring in” fell into the terrestrial clade. The strain was most closely related to *Emericellopsis terricola*, *Emericellopsis microspora*, *Emericellopsis robusta* and *Acremonium tubakii*. Based on this analysis we classified the strain isolated from “ring in” as *Emericellopsis* sp. CBS 137197.

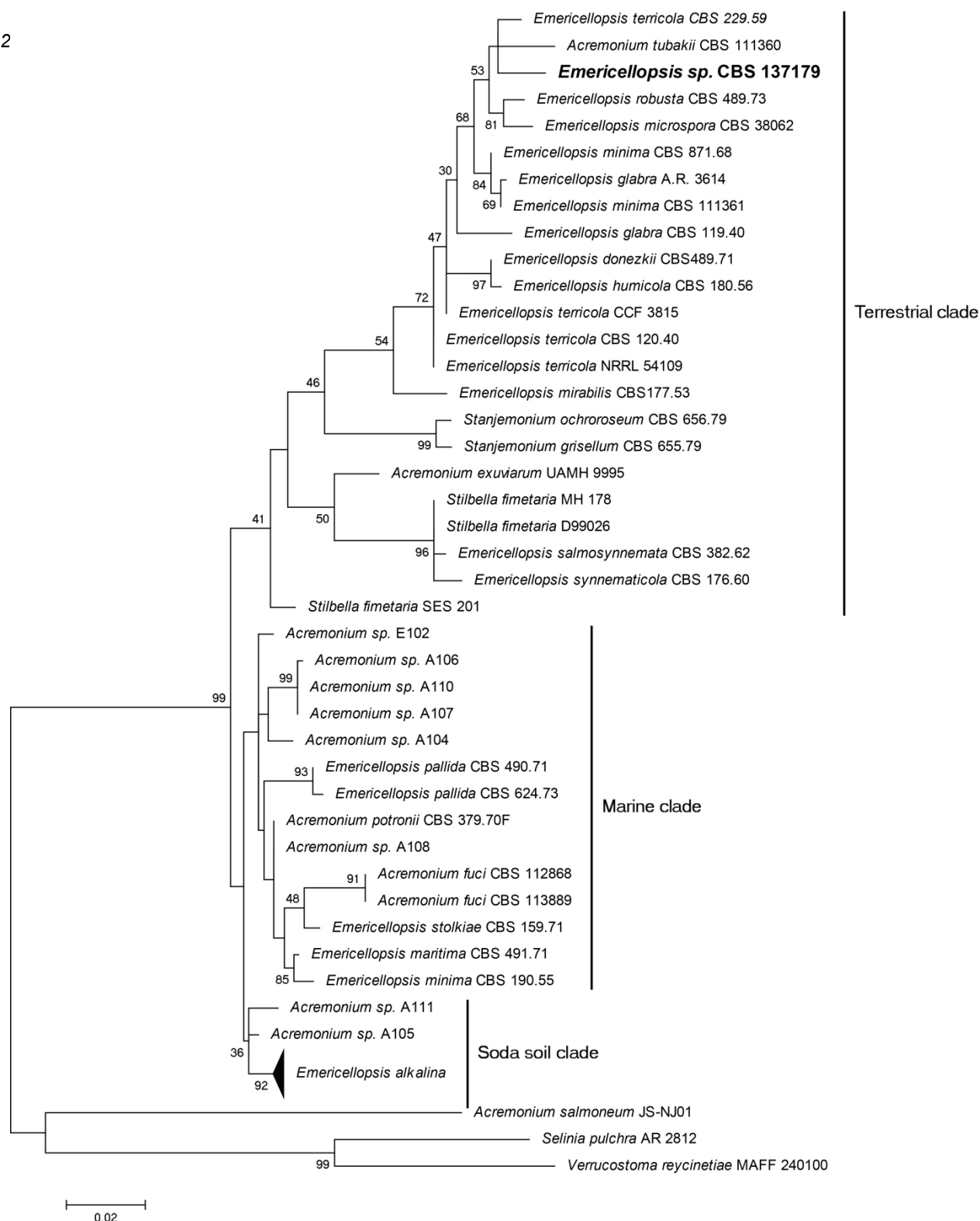


Figure 6.4. The phylogenetic position of strain *Emericellopsis sp. CBS 137197* within Emericellopsis-clade based on partial sequences for ITS and β -tubulin analyzed by maximum likelihood. The classification of Emericellopsis-clade in terrestrial clade, marine clade and soda soil clade was purposed by Grum-Grzhimaylo et al (2013).

Samples of healthy mat were inoculated with the isolated strain *Emericellopsis sp. CBS 137197* (mycelium fragments) aiming to confirm the fungus as the specific

causative for the degradation of the photoautotrophic layers. Autoclaved mycelium was used as a negative control in this experiment. The healthy mat showed rings development already after 3 days in all replicates ($n = 20$), with similar morphology as the natural ring-structures observed in the mats. *Emericellopsis* sp. cleared the infection zone, showing no autofluorescence for cyanobacteria and diatom, and expanding outside while degrading the mat community at an average speed of $0.06 \pm 0.01 \text{ cm d}^{-1}$ (varied between 0.05 and $0.07 \pm 0.01 \text{ cm d}^{-1}$). The total area degraded per ring during the inoculation experiment ranged between 0.5 to 1.3 cm^2 . Addition of killed (autoclaved) mycelium of *Emericellopsis* sp. did not result in ring structures ($n = 17$, Fig. 6.5).

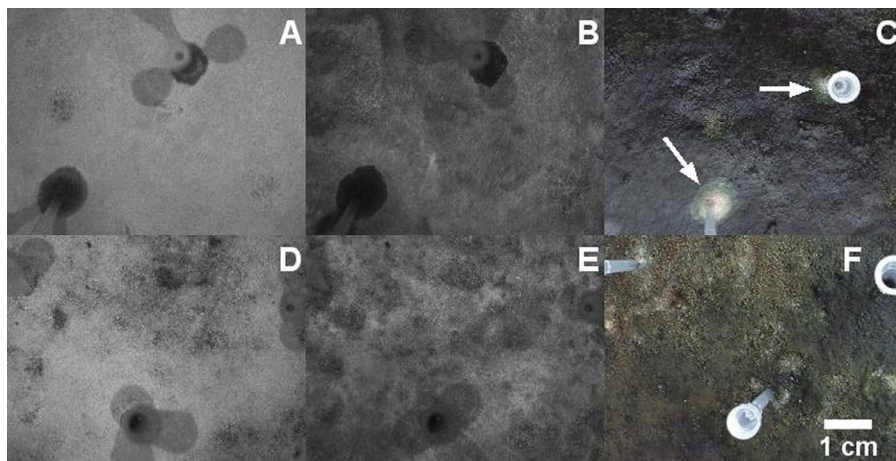


Figure 6.5. Autofluorescence (relative units) (A, B, D, E) images after amber (A, D) and blue (B, E) light excitation, and colour images (C, F) of the infection of microbial mat with live (A - C), and killed (D - F) *Emericellopsis* sp. after 7 days of inoculation. Pipette tips were used to indicate inoculation sites. Arrows indicate the development of ring-like structures in the mat inoculated with live fungus.

The fungal induced lysis of the oxygenic photoautotrophs and the subsequent re-colonization of the main oxygenic photoautotrophs was demonstrated by transferring a piece of microbial mat infected with fungus (“ring in” and “ring out”) to a non-infected microbial mat. The results showed that the fungi in the “ring” area were able to degrade the oxygenic photoautotrophs (Fig. 6.6). The fungi moved from the transplanted area into the new mat while leaving a trail of cleared mat with no autofluorescence (for both cyanobacteria and diatom). This cleared zone was then re-colonised first by diatoms, showing a strong autofluorescence after blue light

excitation, and subsequently, after about 5 days, cyanobacteria showed increasing autofluorescence in the same area (Fig. 6.6).

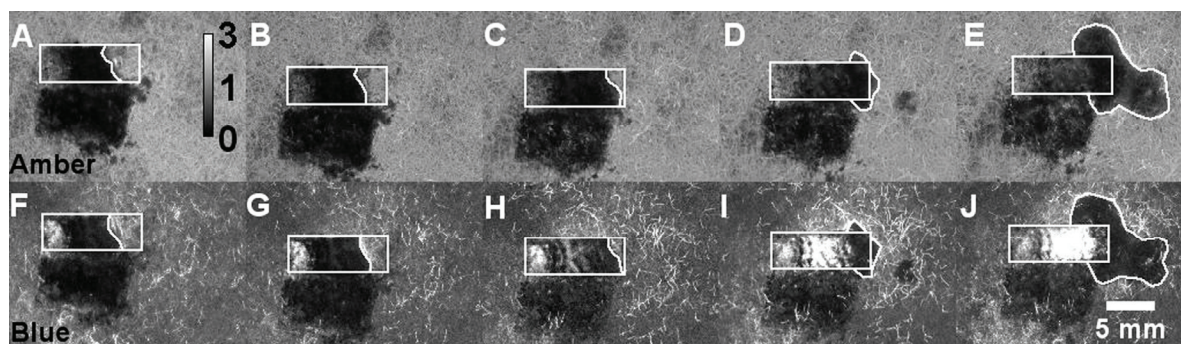


Figure 6.6. Aufluorescence (relative units) images after amber (A - E) and blue (F - J) light excitation of the transplantation of a piece (1 x 0.5 x 0.1 cm indicated by the white square) of infected photosynthetic microbial mat into a non-infected microbial mat. Images collected at day 0 (A,F), 1 (B,G), 3 (C,H), 5 (D,I), and 7 (E,J). White rectangle indicates transplanted part, wherein the black area represents fungus-infected mat. The dark section below the transplanted part was a section without mat (only sediment). The white line (in and outside the rectangle) indicates the expansion of the fungus-infected area. Values (0 to 3) in colour scale indicate increasing autofluorescence of oxygenic photoautotrophs.

The “ring out” area, without autofluorescence, contained fewer fungi than observed in the “ring in” area. In the “core”, “outside” and the “mat” areas the microbial mat did not show visible fungi. The temporal development of the rings due to fungal attack was recorded and measured over a 10 days period by colour and autofluorescence imaging (Fig. 6.7). Autofluorescence images after amber and blue light excitation showed the growth of cyanobacteria and diatoms, respectively, compared to day 0. All 8 rings collected and analysed in November and July were about 2 to 4.8 cm wide, and expanded at an average rate of $0.12 \pm 0.01 \text{ cm d}^{-1}$ (Table 6.2). Despite expected differences in environmental conditions and/or amount of fungus, the range in degradation rates for these natural rings (Table 6.2) as well as the inoculation experiments (Fig. 6.5) is relatively small ($0.05 - 0.17 \text{ cm d}^{-1}$). We estimated that these ring patterns occupied up to 10 % of the microbial mat surface area in the area studied (see Fig. S6.2). The total beach area where we found these ring structures was about 800 x 30 m. Furthermore, we observed different regions, i.e. (i) with clear ring coverings like described here, (ii) with bigger infected regions,

likely representing older infection stages but still with sharp edges of infection, and (iii) with rings grown together (Fig. S6.2).

Table 6.2. Diameter, maximum expansion of rings after 10 days, and rate of expansion for rings 1 - 4 in November, and rings 5 - 8 in July.

Ring	Diameter (cm) day 0	Maximum expansion of infected area (cm)	Expansion rate (cm d ⁻¹)
1	4.20 ± 0.15	0.99 ± 0.18	0.10 ± 0.02
2	4.64 ± 0.32	1.05 ± 0.40	0.12 ± 0.04
3	2.23 ± 0.22	1.45 ± 0.10	0.16 ± 0.01
4	4.82 ± 0.49	1.57 ± 0.26	0.17 ± 0.03
5	3.07 ± 0.12	0.91 ± 0.09	0.09 ± 0.01
6	3.14 ± 0.13	1.08 ± 0.05	0.11 ± 0.01
7	2.58 ± 0.21	1.18 ± 0.22	0.13 ± 0.19
8	2.09 ± 0.30	1.00 ± 0.05	0.10 ± 0.01

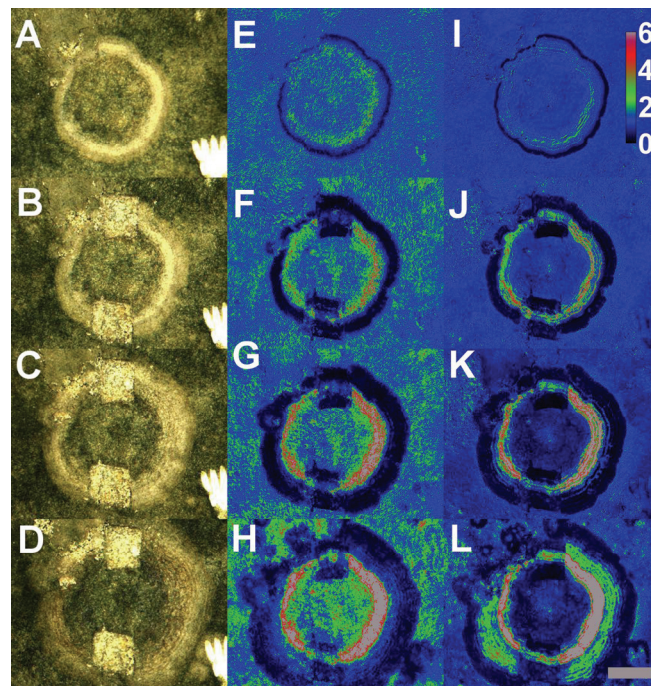


Figure 6.7. Temporal development of a ring by colour imaging (A - D), and autofluorescence imaging after amber (E - H) and blue (I - L) light excitation. Autofluorescence images were made by overlapping autofluorescence image at day 0 with image at days 1 (E, I), 3 (F, J), 6 (G, K), and 10 (H, L). Values above 1 show growth in relation to day 0. Scale bar is 1 cm.

6.4. Discussion

Examination of the ring-like structures and development over time showed clearly that the fungus *Emericellopsis* sp. CBS 137197 efficiently degraded the oxygenic photoautotrophs in the microbial mats, leaving a clear zone of lysed cells. Despite the presence of this fungus in a marine environment, phylogenetic analysis showed that the fungus falls within the terrestrial *Emericellopsis* clade. However, other strains belonging to *Emericellopsis* terrestrial clade have also been isolated from aquatic environments, such as *E. donezkii* CBS 489.71, *E. minima* CBS111361 and *A. tubakii* CBS 111360 (Grum-Grzhimaylo et al. 2013). Even *E. terricola*, a member of the terrestrial clade and representative of a commonly collected species with known marine habitat associations, could undergo conidial germination and growth in sea water (Zuccaro et al. 2004). These examples suggest that some fungi belonging to *Emericellopsis* clade present remarkable adaptive properties and are able to live in both terrestrial and marine biotopes. Fungi are known to control algal blooms in freshwater (Canter & Lund 1948, Kagami et al. 2006), infect marine phytoplankton (Park et al. 2004, Wang & Johnson 2009) and have also been observed in more extreme marine systems such as deep sea hydrothermal systems and hypersaline microbial mats (Le Calvez et al. 2009, Cantrell & Duval-Pérez 2013).

The different areas of the ring structure showed a clear temporal development, with *Emericellopsis* sp. moving from the initial central core towards the outside in a circular shape, thus leaving a trail of recognisable patterns. *Emericellopsis* sp. initially feeds on oxygenic photoautotrophs (“ring in”) and at the same time moves towards non-infected mat (“ring out”) for new supply of resources. This could be facilitated by the release of e.g. toxins or enzymatic activity diffusing out from the fungi, thus creating the characteristic periphery of the ring (“ring out”). The actual mechanism of cell lysis remains unknown. *Emericellopsis* sp. fungal species have been shown to produce the antibiotic Cephalosporin C that lysed cyanobacteria (Redhead & Wright 1978). Quickly after the fungi cleared the mat from oxygenic photoautotrophs, a re-colonisation process took place with diatoms appearing first and cyanobacteria following a few days later and finally dominating the mat again (see schematics in Fig. 6.8).

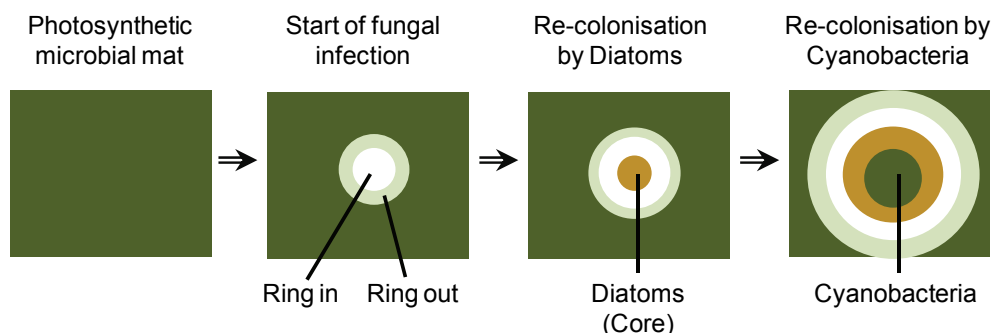


Figure 6.8. Representation of the development of a ring structure. Initially a photosynthetic microbial mat is infected with the fungus and develops the “ring in” area by degrading the photosynthetic mat. The fungus starts to attack the nearest non-infected mat creating the “ring out” area. As the infection spread towards the outside, re-colonisation by diatoms takes place in the newly available areas left behind. Cyanobacteria follow diatoms colonisation and dominate the mat.

It is currently unclear whether the re-colonisation was initiated by the same species (new entry or emerged from deeper subsurface layer) as before the fungal attack, or whether new, perhaps toxin-resistant oxygenic photoautotrophs colonised the area. As fungi were not observed in the core of the ring following lysis, it is likely that their potential toxic effect has disappeared, thus allowing the same oxygenic photoautotrophs to re-colonize the area again. The newly colonised areas with diatoms showed higher autofluorescence compared to outside ring reference mat. Single celled diatoms are known to move fast in sediments (Harper 1969), thus under fungal attack, we speculate that they may have escaped fungal lysis by migrating downwards. Filamentous cyanobacteria glide slower than diatoms (Watermann et al. 1999 and references therein), thus probably becoming trapped in the fungi hyphae, or dying from toxin release. As the fungi moved away from the original attack area, diatoms would re-surface and thrive temporarily without the competing cyanobacteria present.

The direct impact of the fungi on oxygenic photoautotrophs degradation of the mats may also have implications for the cycling of organic matter and nutrients within the mats as fungi have been shown to release labile organic matter and nutrients during degradation of refractory matter (Sigee 2005). Possibly, algal lysate and other organic matter remnants from the fungal degradation support prokaryotes and viral

production in the cleared zones. Overall, the potential increased heterotrophic activity could stimulate the remineralisation of inorganic nutrients sustaining the new oxygenic photoautotrophic production in the mats. Consequently, fungal infections probably drive a local regenerated production that may increase the overall productivity of the mat. The reduction of the oxygenic photoautotrophic biomass due to fungal degradation, however, was not reflected in increased prokaryotes and viral abundances in the infected sections (“ring in” and “ring out”) compared to the non-infected areas (“core”, “outside”, and “mat”). This suggested that the lysed oxygenic photoautotrophic cells were efficiently utilized by the fungi or alternatively, that increased prokaryotes activity did not result in enhanced net abundance. However, more sensitive methods for estimating prokaryotes activity should be applied in future studies to investigate a possible association between the distribution and activity of fungi and prokaryotes.

The rings in November did not show the “ring in” and “ring out” areas compared to July. This could simply reflect that the finer details of the ring structures could not be visually resolved in the more wet sediment in November, although a different type of fungal infection, with different ring morphology, cannot be ruled out. Cantrell et al. (2006) isolated 16 different fungal species from a hypersaline microbial mat, suggesting that fungi are a common feature of microbial mats potentially involved in mat lysis. Nevertheless, we show that *Emericellopsis* sp. was isolated and identified in these mats in two consecutive years. Further study is needed to clarify if also other fungi can cause ring structures and what the exact underlying mechanism is. The ring structures were only found during summer and autumn, suggesting that low temperature and oxygenic photoautotroph biomass limit fungal activity during winter and spring. Gerdes (2007) speculated that other ring-structures (although bigger in diameter) found in microbial mats, may result from gas surfacing from small exit points in the mat causing dispersal of nutrients and stimulation of cyanobacterial growth, although no conclusive studies were followed.

In summary, we showed that a fungus belonging to the *Emericellopsis* clade was able to clear oxygenic photoautotrophs in benthic microbial mats by degradation, resulting in a series of characteristic ring-shaped patterns in the microbial mats, alike smaller versions of necrotic fairy-rings observed in terrestrial systems (e.g. Caesar-

TonThat et al. 2013). The structures were observed during 4 consecutive years (3 of which were sampled) indicating that this is a common feature in intertidal photosynthetic microbial mats. The impact of the fungal lysis of the mat, did not, however, significantly affect the abundance or distribution of prokaryotes and viruses. This loss factor of cyanobacteria and diatoms seems to constitute an important mortality factor for photosynthetic microbial mats, with implications for mat community composition, productivity and spatial structure.

Acknowledgments

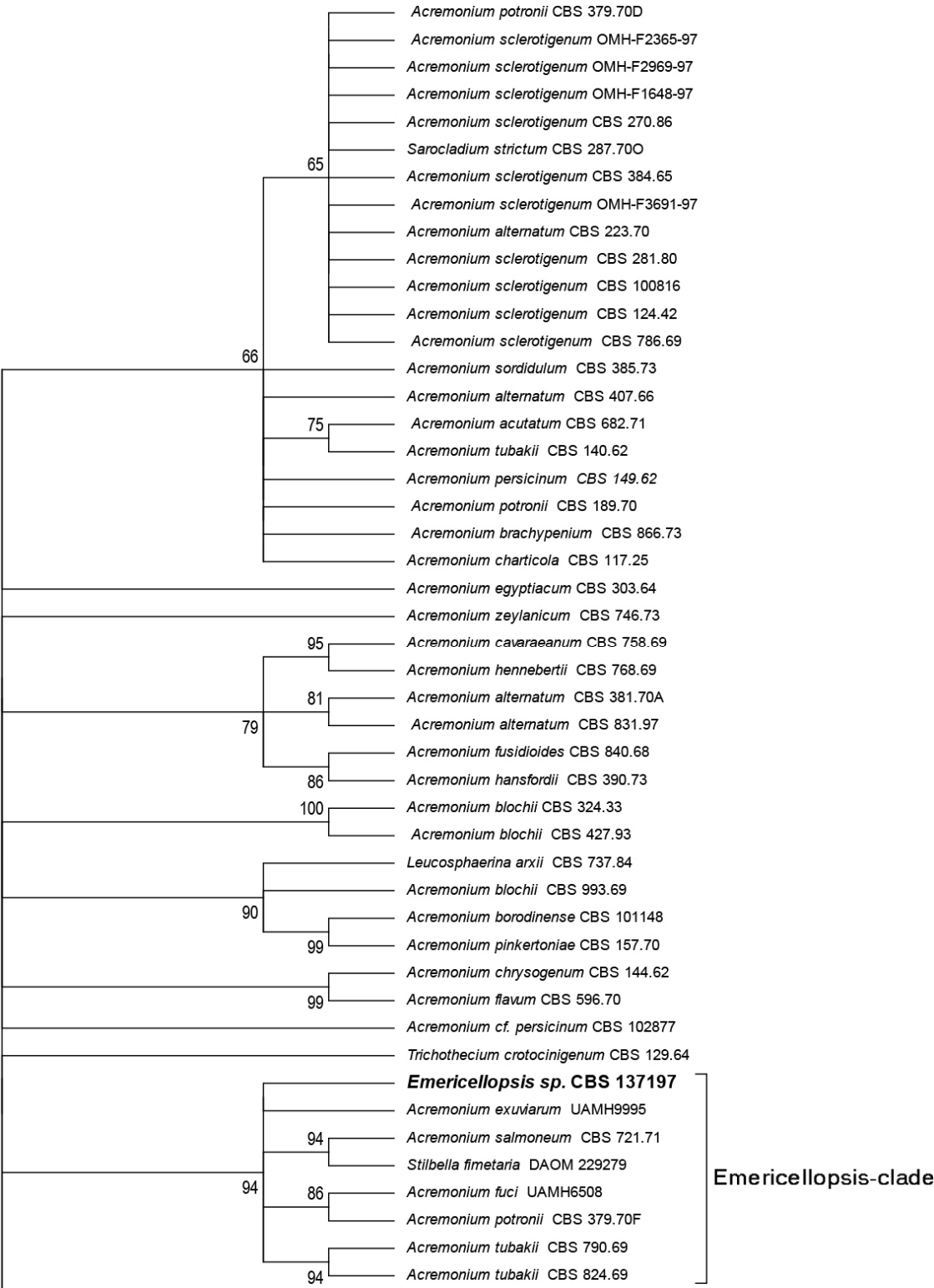
The study received financial support from Fundação para a Ciência e a Tecnologia (FCT) – SFRH/BD/43308/2008, The Royal Netherlands Institute for Sea Research (NIOZ), Conselho Nacional de Pesquisa e Desenvolvimento Científico (CNPq), and the Danish Research Council for Independent Research (FNU). We thank Christian Lønborg, Tim Piel, Robin van de Ven, and Kirsten Kooijman for field and laboratory assistance. We also thank two anonymous reviewers for their constructive comments on the manuscript.

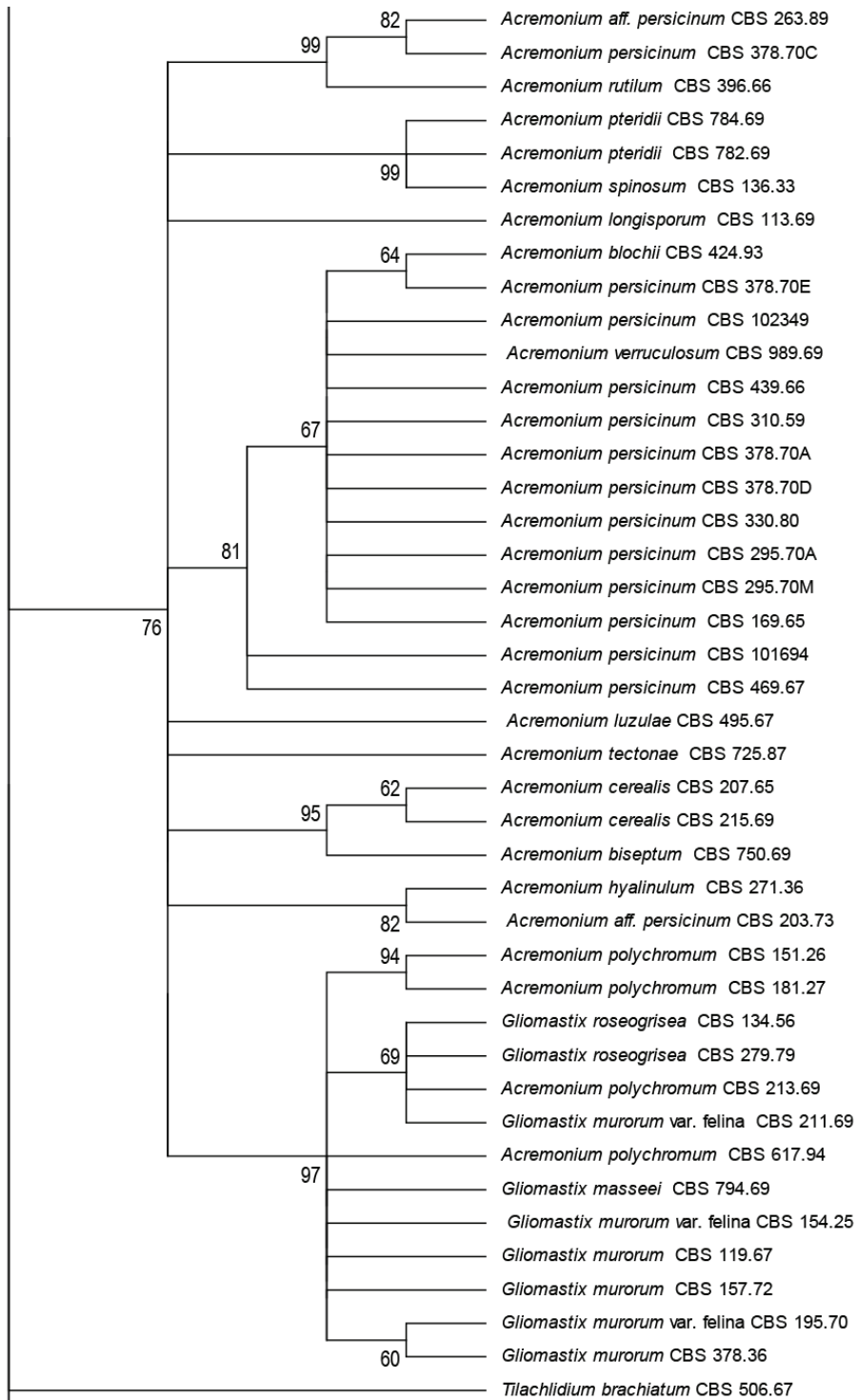
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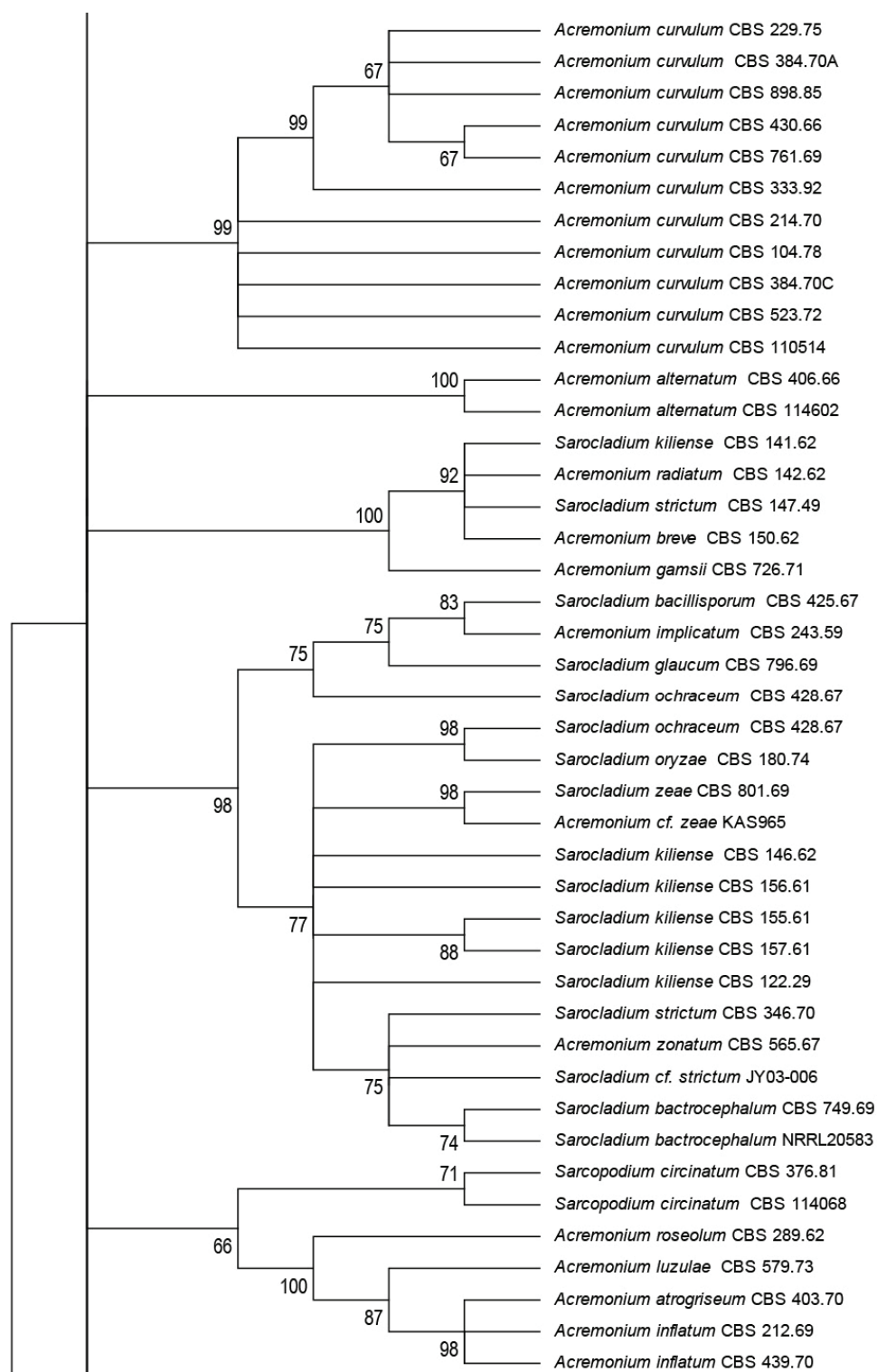
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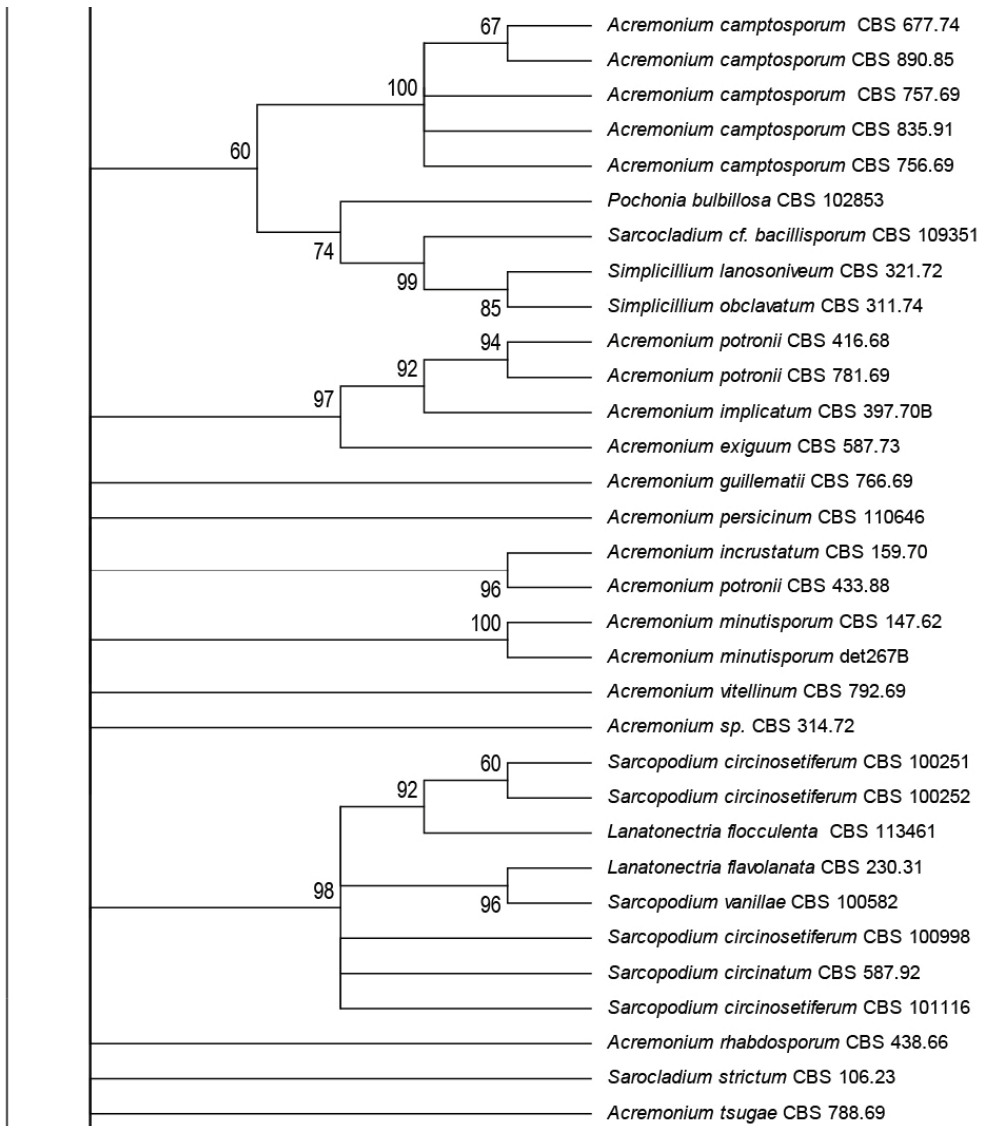
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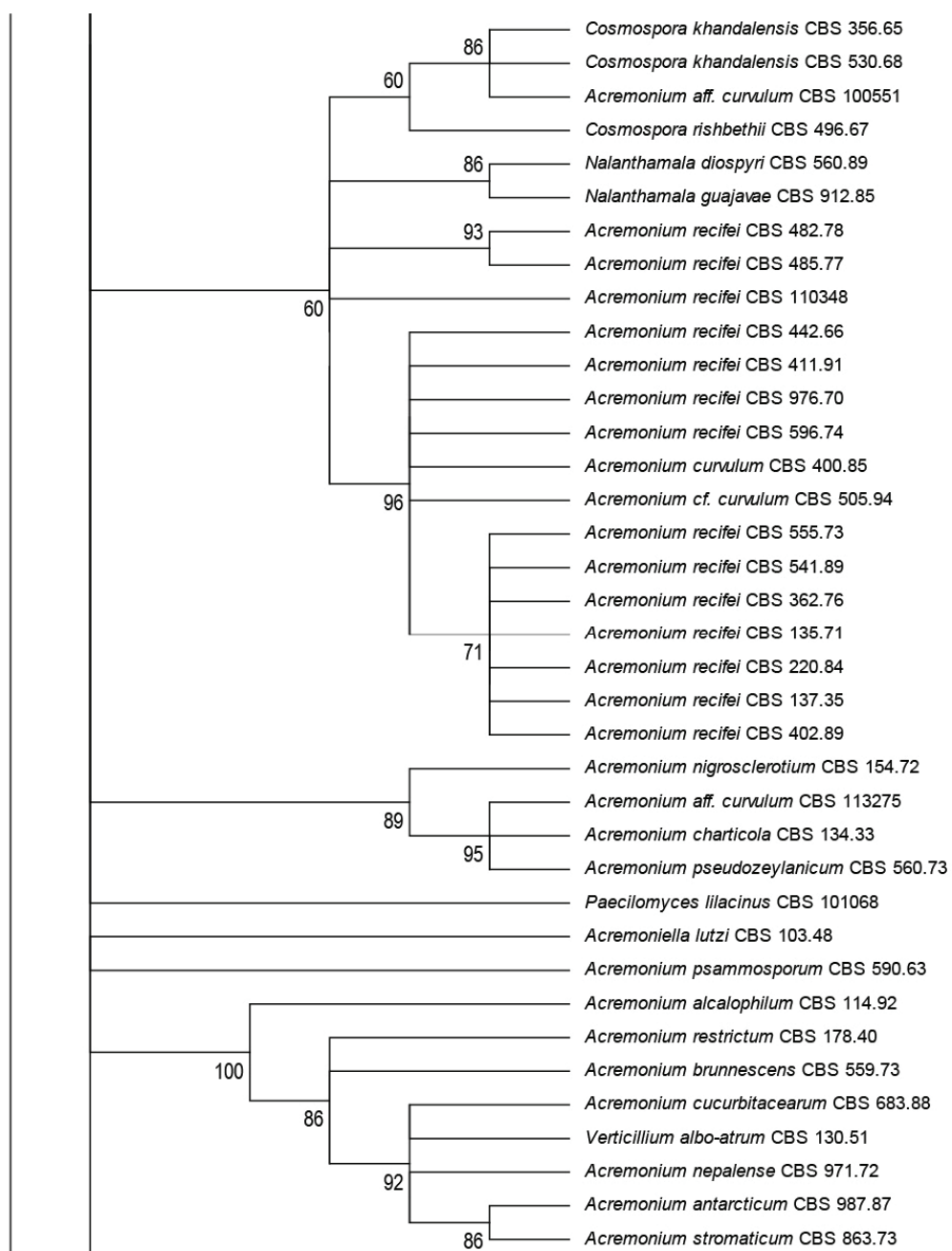
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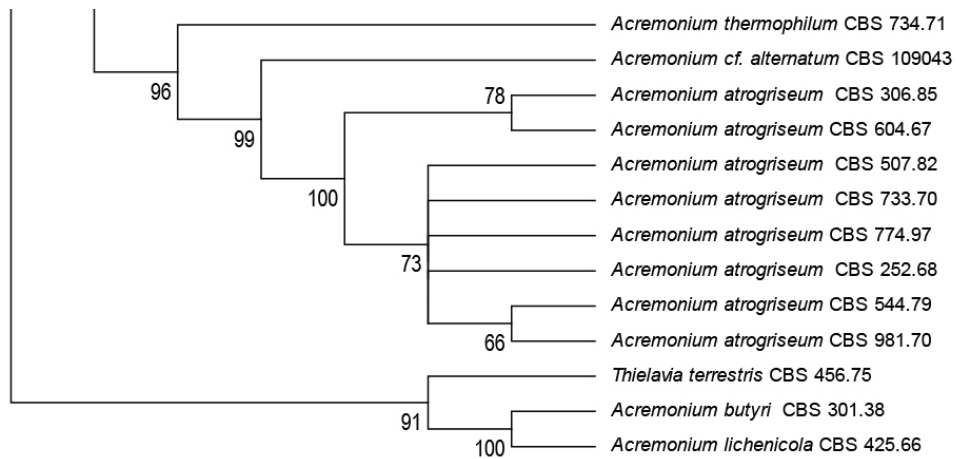


Figure S6.1. The phylogenetic position of strain *Emericellopsis* sp. CBS 137197 within of the order Hypocreales based on partial sequences of Large subunits of Ribossomal RNA (LSU) analyzed by maximum likelihood.

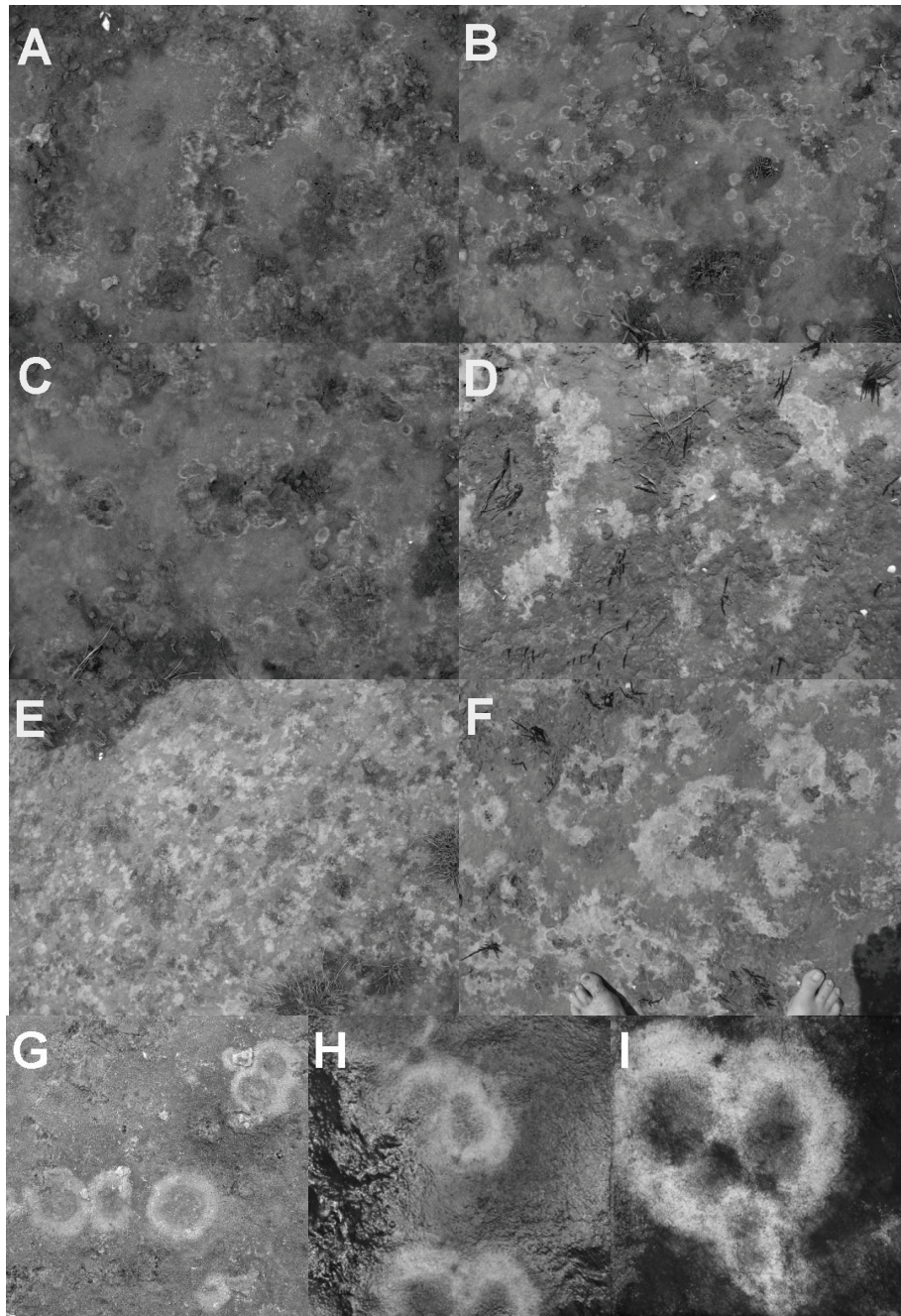


Figure S6.2. The figure shows the range at which the rings occur. Each picture is approximately 1 m^2 . The light areas are infected regions. In the first three pictures (A, B, and C) it shows that there are clear rings covering from 2 to approximately 10 % of the surface. The next three pictures (D, E, and F) show bigger regions with infected photosynthetic microbial mats. There are no clear rings visible in these pictures, as these regions are probably infected earlier and the infection stages are older. Nevertheless, the sharp edges of the infection can be seen. In close-up, one can clearly see examples of rings growing together (area approximately 30 cm^2) (G, H, and I).

Chapter 7

General discussion

General discussion

In this thesis I have presented several studies on the microscale spatial distributions of viruses and microbes in intertidal benthic ecosystems, and mortality factors influencing this distribution (Chapters 2, 5, 6). To be able to study this in microbial mats, I had to modify and develop methods for (I) efficient and reproducible extraction and enumeration of viruses and prokaryotes, and (II) discrimination and quantification of benthic filamentous cyanobacteria from diatoms (Chapters 3 and 4). Firstly, I will discuss the high degree of heterogeneity of viruses and microbes at microscale observed in intertidal benthic habitats (sediments and microbial mats), the potential underlying mechanisms, and the implications of this heterogeneity for studying microbial ecology in these environments. Secondly, I will examine the effects of biological interactions of viruses and fungi with prokaryotes and oxygenic photoautotrophs in structuring benthic microbial ecosystems. Finally, I will expand on future work perspectives within benthic microbial ecology.

7.1. Spatial microscale distributions

The importance of collecting samples at the scale relevant for microbial activity, and how this may impact our understanding of microorganism interaction, has been recognised for pelagic habitats (Blackburn et al. 1998, Azam & Long 2001, Seymour et al. 2004, Azam & Malfatti 2007). Only a few studies thus far looked at the importance of appropriate sampling in benthic habitats, but still without distinction between major oxygenic photoautotrophic groups (Seuront & Leterme 2006, Chennu et al. 2013), and between centimetre (cm) and metre (m) scales for prokaryotes and viruses (Middelboe et al. 2006). However, benthic microbial biogeochemical fluxes are highly variable at micrometre (μm) to millimetre (mm) scales (Glud et al. 1999, Fenchel & Glud 2000). To put this into perspective (Fig. 7.1), if we were studying microbes at the traditional scales (cm to m) it would correspond to try determining human interactions by observing countries (in this case The Netherlands, Belgium and Northern Germany together), or at best cities (e.g. Amsterdam). It is clear that these scales are not suited to observe human interactions. I reason that the cm to m

scale for studying microbial distributions and activity is equally unsuited, and have designed the research in this thesis with that hypothesis in mind.

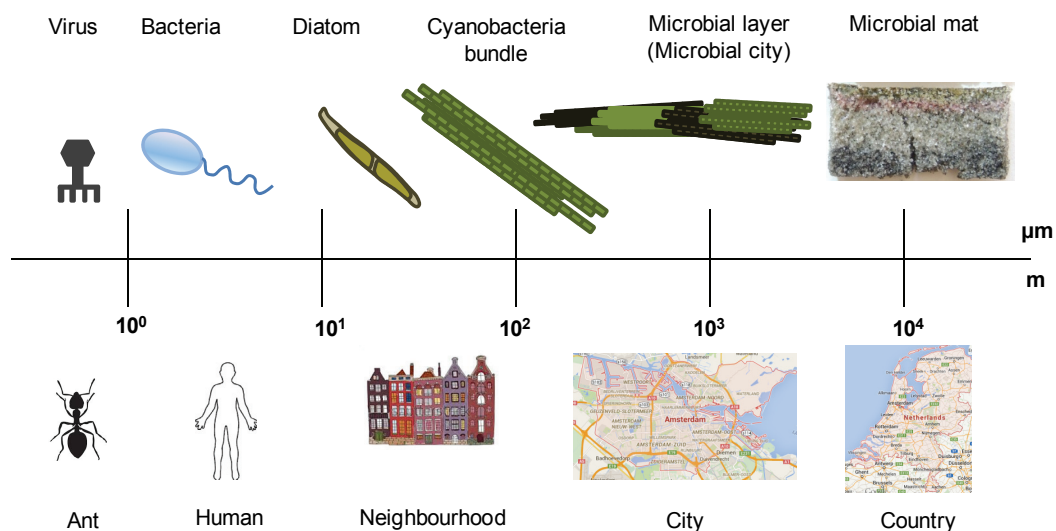


Figure 7.1. A comparison of the size ranges between microbes and human scales. Adapted from Finkel et al. (2010).

In this thesis I showed that viruses and prokaryotes were heterogeneously distributed in intertidal sediments, with abundances varying over 3-fold within mm (Chapter 2). The high variability found in the 2- and 3-dimensional spatial distribution and abundances of prokaryotes and viral, as well as oxygen concentration and uptake rates, emphasises the need for resolving the distribution of microbes on a microscale. Additional experiments studying temporal dynamics in net prokaryotic and viral production and community respiration in homogenized sediment samples showed uncoupled abundance of prokaryotes and viruses over time and in response to organic carbon addition, reflecting more the recent history of the specific environment rather than the actual activity of the microhabitat. This study implies that prokaryote-virus interactions in (intertidal) sediments occur as periodic elevations and declines of viral and prokaryotic abundances associated with continuous redistribution of microscale resource patches. The traditional study of larger scale, homogenized samples, averages out this microscale variability, which hampers our understanding of the ecology of microbial communities in the sediment. To use the analogy illustrated in Fig. 7.1, this is comparable to describing the human population distribution in The Netherlands by using only the average density (493 people km^{-2}).

However, this ignores the fact that the population is actually unevenly distributed between high density cities and low density rural areas, which has significant implications for human activities, interactions and environmental effects.

Similar microscale variability in distribution of viruses and prokaryotes was observed in the intertidal microbial mats studied (Chapter 5). Using the newly optimized method for extracting viruses (and prokaryotes) from microbial mats (Chapter 3), I found the viral and prokaryotic abundances in the mat to vary 4 and 10-fold at microscale, respectively. Photosynthetic microbial mats are very productive ecosystems and thus it was hypothesized that the viral and prokaryotic abundances would be high. Indeed I found high abundances (up to 5.4×10^{10} , with average $1.5 \pm 1.5 \times 10^{10}$ viruses g^{-1}), independent of month sampled (November 2012, April and July 2013). The high viral abundances are about an order of magnitude higher than reported thus far for other coastal and estuarine benthic ecosystems (Danovaro et al. 2008), and comparable to samples ($n = 3$) of a microbial mat in a lagoon (Pacton et al. 2014). Locally high abundances of specific prokaryotes living in the characteristically laminated photosynthetic microbial mat may have been responsible for the high viral abundances. Moreover, the distribution of viruses and prokaryotes was related to the depth of the oxygenic photoautotrophic layer, suggesting that the photosynthetic mat fuels the microbial processes in the underlying layer. The results of this study also illustrate that in order to study benthic microbial ecology we should sample at smaller scales than done so far. The microscale microbial dynamics in distribution and abundance are thus, inherent features of microbial mats and intertidal sediments, which should be better recognized.

Furthermore, studies on benthic oxygenic photoautotrophs tend to focus on the use of high performance liquid chromatography pigment analysis or the photosynthetic efficiency of the oxygenic photoautotrophs by pulse amplitude modulation (Serôdio et al. 2001, Dijkman et al. 2010). Although providing relevant information on major oxygenic photoautotrophs and activity, there is a need for a non-disruptive method that can distinguish between different benthic oxygenic photoautotrophic groups at microscale in the field and in laboratory experiments. The newly developed method (Chapter 4) allowed discrimination between the two major groups of oxygenic photoautotrophs (filamentous cyanobacteria and diatoms) in a

non-intrusive, easy and fast manner. Application of the method in the field showed that benthic cyanobacteria and diatoms were also distributed in patchy populations separated by μm to mm distances (Chapters 5 and 6).

The fungus *Emericellopsis* constituted a significant mortality factor by degrading the oxygenic photoautotrophs in the microbial mats. *Emericellopsis* sp. produced small cm -sized ring-like structures that were observed in the photosynthetic microbial mats, during different field campaigns, particularly during late summer and autumn. This has direct implications for the mat's spatial structure and species succession, as the cleared sections (due to the fungal activity) of the cyanobacteria-dominated mats were at first re-colonized by diatoms. Ultimately this is likely to influence the productivity of the microbial mats, as it has been shown that diatoms in photosynthetic microbial mats can have 5-fold higher photosynthetic maximum rates ($100 \mu\text{mol O}_2 \text{ cm}^{-3} \text{ h}^{-1}$) compared to the cyanobacteria *Microcoleus* spp. (Jørgensen et al. 1983). Furthermore, the seasonal discrepancy in the presence of the ring-like structures suggests the existence of regulatory factors that are thus far unknown. Evidently, fungal activity needs to be considered as an important constituent of the microbial mats dynamics and warrants more study.

Complex interactions between the different players in the photosynthetic microbial mats are taking place. Yet, structuring mortality agents such as fungi and viruses have been largely overlooked. The studies presented in this thesis show that viruses and fungi underlay microscale spatial distribution, succession and productivity of ecologically major players in photosynthetic microbial mats and intertidal sediments. Actually, these processes are likely to enhance the sustainability of the microbial mat on the long run. For example, when viruses and fungi kill oxygenic photoautotrophs, it opens space for re-colonization. On larger scales (spatial and temporal) this generates a more resilient multispecies environment more likely to survive disruption (Elias & Banin 2012). Still, mortality factors such fungi and viruses will affect the flow of organic matter in different manners (mortality mechanism and rate, spatial progression), with different consequences for microbial activity, distribution and stability of the microbial mat. Future studies on production, diversity, and succession in microbial mats need to plan their field sampling and experimental set-up accordingly.

7.2. Future work

Our knowledge of the importance and processes controlling the distribution of microbes in sediment and microbial mats has increased steadily over the past decades but this thesis illustrates that the overall picture is, however, still incomplete. Besides the apparent requirement for the development of methods that measure *in situ* mortality and production at the scale and conditions at which they occur to fully understand individual and combined effects of viruses and fungi in intertidal ecosystems, it is recommended to isolate key virus-host model systems as well as more fungi for detailed studies of their interactions and regulation. Performing such studies under controlled conditions will allow a better understanding of the mechanisms of lethal action from viral lysis and fungal predation of ecologically important members in natural photosynthetic microbial mats, and the consequences of these processes for mat productivity and diversity. Examining different fungi and viruses will elucidate how general their lethal activity is and whether there is a large variation in their activity. Furthermore, by combining molecular techniques with microscale sampling, insight could be obtained on the extent of mortality of specific key players in the intertidal benthic ecosystem, and particularly for the distinctly multi-layered microbial mat.

Environmental conditions in microbial mats are harsh (desiccation, UV and high light exposure, and salinity shock), therefore I recommend examining the influence of the different physico-chemical factors of interest on the activity of viruses and in particular fungi (ring-structures were observed during summer and autumn). Furthermore, it would be interesting to create an artificial photosynthetic microbial mat and test, in a controlled manner, the effects of added e.g. viruses and fungi. The imaging system developed in this thesis (Chapter 4) could be nicely used in this type of studies.

To better understand the microscale distribution and abundances of viruses and their impact in biogeochemical cycles in sedimentary habitats it is important to learn more on how viruses are transmitted. Viruses cannot move, yet they need to spread to find new hosts. One obvious way of viral dispersal in benthic habitats could be by flowing through the interstitial waters (Sutherland et al. 2004), characteristically

found in sediments and mats. Furthermore electrostatic interactions could retain or repulse viruses (Sutherland et al. 2004) to surfaces (sediment particles, host, etc), hence allowing them to attach or continue “moving”. Another possibility to “move” within the sediments would be by infecting cells that may be on the “move”, such as benthic filamentous cyanobacteria or pennate diatoms. Thus, it would be interesting to study if motile cells are more or less prone to viral infection, as compared to immobile cells. It has also been suggested (Pais-Correia et al. 2010, Thoulouze & Alcover 2011) that viruses can induce infected human T-cells to produce a biofilm layer that aggregates the newly formed viruses on top of the infected cell. Consequently viruses would be protected against external destructive factors, but also facilitating a more effective transmission after the infected cell contacts a new healthy cell. Mechanisms of movement and transmission of viruses need testing in benthic habitats. Understanding the dynamics of viral transmission in “static” systems as benthic habitats would help to understand their impact in benthic ecology.

Overall, I would like to recommend a more active exchange of ideas, concepts and hypotheses between the research field of benthic microbial mat ecology, including from now on also the mortality agents, and the more applied biofilm research (medical and industrial science). These systems show, on more than one aspect, similarities and a more free exchange of ideas, concepts and hypotheses could therefore be fruitful for both research fields.

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Summary

Marine sedimentary habitats generally have their highest microbial activity in the top few centimeters. Where light reaches the sediments, benthic oxygenic photoautotrophs grow and the organic matter released is decomposed in a series of different metabolic pathways by heterotrophic prokaryotes. If grazing pressure is low (or absent), as found more often in extreme environments such as hot springs, high salinity environments and sediments with periodic desiccation, photosynthetic microbial mats may be formed. Microbial mats are permanently vertically laminated microbial communities, with the top photosynthetic layer composed of cyanobacteria and benthic eukaryotic microalgae (such as diatoms). Much work has been done regarding the physico-chemical parameters structuring the microbial activity, but to my knowledge there are no reports on microscale (μm to mm) abundance distributions of viruses, prokaryotes and oxygenic photoautotrophs in benthic systems. It is, however, essential to collect samples at the scale relevant for microbial activity. Moreover, it is important to improve our understanding of which underlying factors, other than bottom-up (e.g. physico-chemical variables such as organic matter, light, temperature), regulate the spatial distribution of key members of benthic ecosystems.

In this thesis, I show that viruses and prokaryotes in a tidal sediment from Dunstaffnage Bay, Scotland, distribute heterogeneously both at horizontal and vertical mm scale. Furthermore, it was also shown that the dynamic viral activity over time contributed to the microscale heterogeneity observed (Chapter 2). To be able to examine microscale distributions of viruses and prokaryotes in photosynthetic microbial mats an improved method for extracting viruses and prokaryotes from these mats with their high load of organic matter was developed (Chapter 3). The method also provided better results when applied to the sediment. Furthermore, the newly developed method could be successfully combined with flow cytometry, thereby providing a fast and accurate method that can help opening the research field of viral ecology in microbial mats. Application of this method to intertidal microbial mats from the Wadden Sea island Schiermonnikoog, The Netherlands, also showed microscale heterogeneity in prokaryotic and viral abundances, the latter amongst the highest recorded ($1.5 \pm 1.5 \times 10^{10}$ viruses g^{-1}) in natural benthic habitats (Chapter 5). Viral and prokaryotic abundances were related to the depth of the oxygenic

photoautotrophic layer between the sampled periods (November 2012, April, and July 2013). Microscale observation and distinction of the two main oxygenic photoautotrophic groups (cyanobacteria and diatoms) in microbial mats was possible due to the development of a new, fast and non-intrusive, method using photoautotrophic group-specific autofluorescence imaging (Chapter 4). Applying the method in the field displayed patchy populations of cyanobacteria and diatoms at the μm to mm spatial scale. These findings will have implications on the local productivity, as cyanobacteria have different productivity rates from diatoms.

Considering that grazing pressure is typically low in microbial mats, I have focused on other factors (viruses and fungi) that may affect variability in (microscale) spatial and temporal distribution of the major groups of oxygenic photoautotrophs. The high viral abundances found in the microbial mats indicated that viruses could be significant players in the mats as potential mortality agents for prokaryotes and oxygenic photoautotrophs (Chapter 5), with particularly high abundances in the photosynthetic layer compared to the heterotrophic layers below. The fungus *Emericellopsis* sp. was found to affect the spatial distribution of oxygenic photoautotrophs by degrading benthic cyanobacteria and diatoms, during summer and autumn (Chapter 6). Consequently, small (2 - 5 cm) ring-like clearings in the photosynthetic microbial mat occurred that were re-colonised firstly by the diatoms. Fungal activity thus influenced not only oxygenic photoautotrophic species distribution and diversity (via degradation), but also succession and subsequently is expected to have an effect on the productivity of the photosynthetic microbial mat.

Overall, it was observed that microbes (prokaryotes and oxygenic photoautotrophs) in microbial mats and intertidal sediments can be distributed at μm to mm scales. Mortality agents, such as viruses and fungi, were found relevant underlying factors for the observed microscale spatial distributions. Based on these findings more research on interactions between these mortality agents (viruses and fungi) and the dominant benthic microbial groups is desired. This would improve our understanding of their role in species diversity and succession, as well as the extent to which these mortality factors affect biogeochemical cycling within the microbial mats.

Samenvatting

Marine sedimenten hebben de hoogste microbiële activiteit over het algemeen in de bovenste centimeters. Wanneer licht deze sedimenten kan bereiken worden ze vaak gekoloniseerd door bentische fototrofe organismen. Deze organismen produceren organisch materiaal dat vervolgens afgebroken wordt door bacteriën via een serie van verschillende metabolische routes. Wanneer de begrazingsdruk laag is in extreme milieus zoals o.a. warme bronnen, gebieden met erg hoge zoutconcentraties en in sediment dat geregeld uitdroogt, kunnen oxygene fototrofe microbiële matten gevormd worden. Een belangrijk kenmerk van microbiële matten is hun verticale gelaagdheid. De bovenste laag bestaat voornamelijk uit draadvormige cyanobacteriën (blauwalgen) en eukaryote micro-algen (o.a. de kiezelwieren oftewel diatomeeën). Er is al veel onderzoek gedaan naar de wijze waarop fysisch-chemische parameters de microbiële activiteit en de verticale gelaagdheid beïnvloedt. Echter, voor zover mij bekend, is er aan sediment ecosystemen nog geen onderzoek verricht naar de verdeling van virussen, bacteriën en fototrofen op millimeter (mm) schaal. Microbiële activiteit varieert op micro- en millimeter schaal en daarom denk ik dat het essentieel is om bentische ecosystemen juist op deze fijne schaal te onderzoeken. Tevens belangrijk is het vergroten van ons begrip van de onderliggende factoren die mede (naast fysisch-chemische factoren zoals voedingszouten, licht, temperatuur) verantwoordelijk zijn voor de ruimtelijke verdeling van de dominante organismen in bentische habitats.

In dit proefschrift laat ik zien dat de ruimtelijke verdeling van virussen en bacteriën in getijdsedimenten van Dunstaffnage Bay (Scotland) op mm schaal zeer heterogeen is zowel in de horizontale als de verticale richting (Hoofdstuk 2). Doordat de hoge concentratie van organisch materiaal in de microbiële matten de extractie van bacteriën en virussen bemoeilijkt (wat tot onderschatting van de aantallen leidt), was het nodig om de bestaande extractie methoden voor sedimenten te verbeteren (Hoofdstuk 3). De methode die ik ontwikkeld heb gaf niet alleen goede resultaten voor de fotosynthetische mat, maar gaf ook betere resultaten voor sediment monsters. Met de nieuwe extractie methode vond ik in intergetijdse microbiële matten op het strand van het Waddeneiland Schiermonnikoog virus aantallen ($1.5 \pm 1.5 \times 10^{10}$ virussen g^{-1}) die tot de hoogste concentraties behoorde ooit gerapporteerd voor natuurlijke bentische milieus (Hoofdstuk 5). Ook in dit ecosysteem was de

heterogeniteit in de ruimtelijke verdeling van virussen en bacteriën hoog en fijnschalig (Hoofdstuk 3). Virus en bacterie concentraties varieerden met de dieptevariatie van de fotosynthetische laag (onderzocht in november 2012 en april en juli 2013). Het was mogelijk om de ontwikkelde extractie methode in combinatie met flowcytometrie te gebruiken. Flowcytometrie is preciezer en sneller dan celtellingen met de microscoop, en kan daarom bijdragen aan de voortgang van onderzoek in het onderzoeksveld virusecologie van microbiële matten. Naast de ontwikkeling van de verbeterde extractie methode heb ik ook een methode ontwikkeld om de fijnschalige horizontale verdeling van verschillende groepen van oxygene fototrofe organismen te kunnen meten. Deze methode is gebaseerd op een verschil in absorptie van lichtkleuren van de verschillende fototrofen en hun autofluorescentie. De methode is snel en verstoort de mat niet (Hoofdstuk 4). Met deze methode heb ik laten zien dat de microbiële mat van Schiermonnikoog een fragmentarische verdeling in zowel cyanobacteriën als diatomeeën dominantie vertoonde. De verdeling cyanobacterie-diatomee bleek op micro- en millimeter schaal te variëren. Een dergelijk verschil in dominantie zal de lokale primaire produktie kunnen beïnvloeden.

Aangezien begrazing weinig voorkomt in microbiële matten heb ik in dit proefschrift van andere mortaliteitsfactoren (virussen en schimmels) onderzocht of ze invloed op de ruimtelijke verdeling van de verschillende groepen van micro-organismen in intergetijdsedimenten en microbiële matten kunnen hebben. De hoogste virus concentraties ($1.5 \pm 1.5 \times 10^{10} \text{ g}^{-1}$) werden gevonden in de bovenste 2 mm, waar de fototrofen zich bevinden. De ruimtelijke heterogeniteit en de hoge virus concentraties, lijken een indicatie dat virussen een belangrijke functie hebben in het functioneren van microbiële matten (Hoofdstuk 5).

Voornamelijk in de zomer en herfst ontstonden ringvormige structuren in de microbiële matten van Schiermonnikoog. In deze ringen (2-5 cm) waren alle bentische cyanobacteriën en diatomeeën afgebroken. Dit werd veroorzaakt door de schimmel *Emericellopsis* sp. (Hoofdstuk 6). Herkolonisatie van deze ringen vond in eerste instantie plaats door diatomeeën. Dit laat zien dat niet alleen virussen maar ook schimmels een stempel kunnen drukken op de successie, verdeling en diversiteit van fototrofe organismen in ruimte en tijd. Dit zal ook mogelijk een effect kunnen hebben op de produktiviteit van microbiële matten.

Samenvattend kan gezegd worden dat microbiële populaties (bacteriën en fototrofen) in microbiële matten en sediment een ruimtelijke verscheidenheid kunnen vertonen op μm tot mm schaal. Het lijkt erop dat virussen en schimmels hierin een belangrijke rol spelen. Dit proefschrift is een eerste aanzet tot meer onderzoek naar de interacties van schimmels en virussen met bentische fotoautotrofen. Meer inzichten in deze interacties zal tot een beter begrip leiden van hun rol met betrekking tot biologische variatie in ruimte en tijd, maar zal ook resulteren in een beter begrip van energie- en stofstromen in microbiële matten.

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I'm a PhD... Get me out of here!

Starring

Cátia Carreira	as PhD student
Corina P. D. Brussaard	as Supervisor
Mathias Middelboe	as Supervisor
Marc Staal	as Supervisor
Anna Noordeloos	as Amazing technician
Richard Doggen	as Amazing technician
Kirsten Kooijman	as Amazing technician
Robin van de Ven	as Amazing technician
Douwe	as our lab saviour
Yvonne	as the zerman
Jenny	as the English/Welsh ahah!
Sofia	as the modelling mom
Catarina Leote	as the sporty one
Catarina Silva	as the coffee break
Lisa NZ	as the crazy cool one
Lisa USA	as Born L.I.S.A.
Argiro/Sylvia	as <i>la</i> crazy greka
Martina	as the revolutionary one
Arno	as he that constructed a boat
Katrin	as the other zerman
Sapita	as always there
Christian	as the partner

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Based on the novel

"I'm a PhD...Get me out of here!"

Animal stunts

Kefla, Roef, Isabel, and Copper

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And now for something completely different.

It's been such a long time since the day I started this movie. I remember the thrill! Like a "good" movie it started "in an ordinary world" with a "flawed protagonist", then my whole scientific world was turned upside down, and I had to "take stock". I "committed to action", but the "stakes were raised". I "learned my lesson, stopped the antagonists", and "achieved my goal": to finish it. Thank you all that took part in it!

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I leave you with a slightly modified, and humorous version of a song ☺! I hope you enjoy it!

It's not time to make a change,
Just hurry up and work a lot.
You're still young, that's your fault,
There's so much you have to know.
Find a job, write more papers,
If you want you can become a professor.
Look at me, I am crazy, but I'm happy.

I was once like you are now, and I know that it's not easy,
To be calm when you've found something going on.
But hurry up, don't think a lot,
Think of everything you've got.
For you will still be here tomorrow, but your PhD may not.

How can I try to explain, when I do, he turns away again.
It's always been the same, same old story.
From the moment I could do science I was ordered to listen and work.
Now there's a way and I know that I have to go away.
I know I have to go.

It's not time to make a change,
Just hurry up, and finish the god dam PhD.
You're still young, that's your fault,
There's so much you have to go through.
Find a job, write more papers,
if you want you can become a professor.
Look at me, I am crazy, but I'm happy

All the times that I cried, keeping all the things I knew inside,
It's hard, but it's harder to ignore it.
If they were right, I'd agree, but it's them you know not me.
Now there's a way and I know that I have to go away.
I know I have to go...

Modified from Cat Stevens "Father and Son"