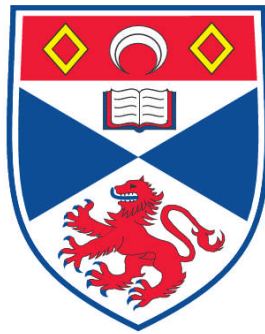


**THE IMPACT OF MICROBIAL EXTRACELLULAR POLYMERIC  
SUBSTANCES ON SEDIMENT STABILITY**

**Helen V. Lubarsky**

**A Thesis Submitted for the Degree of PhD  
at the  
University of St. Andrews**



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# **The impact of microbial extracellular polymeric substances on sediment stability**

by

**HELEN V. LUBARSKY**

Thesis submitted in accordance with the requirements of the University of St Andrews for the degree of Doctor of Philosophy



School of Biology

University of St Andrews

February 2011

The University of St Andrews 600th Anniversary

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## **Dedication**

*This thesis was written with love to my husband Gennady who has always helped me and believed that I could do it. I dedicate this thesis to the memory of my Mother, Svetlana.*

## Acknowledgements

I am heartily thankful to my primary supervisor, Prof. Dave Paterson, whose encouragement, guidance and support in a number of ways from the initial work to the final level enabled me to develop an understanding of the subject. Without his hard work in the development of the Sediment Ecology Research Group the opportunity to work here would have never arisen.

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*Helen Lubarsky*

## Abstract

The main objective of this thesis is to investigate the impact of microbial extracellular polymeric substances (EPS) on sediment stability and the related factors which influence “biogenic stabilisation” as a basis to the prediction of sediment erosion and transport.

The ability to make direct and sensitive measurements of the physical properties of the biofilm is a critical demand to further understanding of the overall biostabilisation processes. Therefore, attention has been focused on developing a new technique, Magnetic Particle Induction (MagPI) for measuring the adhesive properties of the biofilm. MagPI determines the relative adhesive properties or “stickiness” of the test surface, whether a biofilm, a sediment or other submerged material. The technique may have future applications in physical, environmental and biomedical research.

Newly developed Magnetic Particle Induction (MagPI) and traditional techniques Cohesive Strength Meter (CSM) for the determination of the adhesion/cohesion of the substratum were used to assess the biostabilisation capacity of aquatic microorganisms. Whilst these devices determine slightly different surface properties of the bed, they were found to complement each other, increasing the range of measurements that could be made and presented a strong correlation in the overlapping portion of the data.

It is recognized that microorganisms inhabiting natural sediments significantly mediate the erosive response of the bed (“ecosystem engineers”) through the secretion of naturally adhesive organic material (EPS: extracellular polymeric substances). Interactions between main biofilm consortia microalgae, cyanobacteria and bacteria in terms of their individual contribution to the EPS pool and their relative functional contribution to substratum stabilisation were investigated.

The overall stabilisation potential of the various assemblages was impressive, as compared to controls. The substratum stabilisation by estuarine microbial assemblages was due to the secreted EPS matrix, and both EPS quality (carbohydrates and proteins) and quantity (concentration) were important in determining stabilisation. Stabilisation was significantly higher for the bacterial assemblages than for axenic microalgal assemblages. The peak of engineering effect was significantly greater in the mixed assemblage as compared to the bacterial and axenic diatom culture. This work confirmed the important role of heterotrophic bacteria in “biostabilisation” and highlighted the interactions between autotrophic and heterotrophic biofilm components of the consortia.

An additional approach, to investigate the impact of toxins on biostabilisation capacity of aquatic organism was performed on cultured bacterial and natural freshwater biofilm. The data suggest a different mode of triclosan (TCS) action ranging from suppressing metabolisms to bactericidal effects depending on the TCS concentration. The inhibitory effect of triclosan on bacterial and freshwater biofilms was confirmed.

This information contributes to the conceptual understanding of the microbial sediment engineering that represents an important ecosystem function and service in aquatic habitats.

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## **Abbreviation**

**MagPI** - Magnetic Particle Induction

**CSM** - Cohesive Strength Meter

**MPB** - Microphytobenthos

**EPS** - extracellular polymeric substances

**ETDC** - erosion, transport, deposition and consolidation

**LTSEM** - Low Temperature Scanning Electron Microscopy

**BSA** - Bovine Serum Albumin

**PAM** - Pulse Amplitude Modulated

**Y(II)** - The inhibition of the photosystem

**TCA** - trichloroacetic acid

**SDS** - sodium dodecyl sulfate

**TCS** - triclosan

**HPLC** - high performance liquid chromatography

**DMSO** - dimethylsulfoxide

**PSU** - Practical Salinity Units

## **Chapter 1**

### **General Introduction**

The generic importance of the biogenic mediation of sediment erosion and transport is a matter of debate and a multidisciplinary approach is required to investigate biologically-mediated mechanisms of sediment stability (Paterson et al. 2000). Understanding the process of “biostabilisation” is essential for optimisation of the water framework directive and sediment/pollutant management strategies. The dynamic equilibrium between the erosion, transport, deposition and consolidation of aquatic sediments (ETDC-Cycle) is of key importance for the protection of coastal shorelines, especially when considering predicted environmental changes (Foerstner and Salomons 2008). This thesis describes work performed to investigate the biostabilisation potential of aquatic microbial organisms and the effect of toxins on their stabilisation capacity. This study was carried out using traditional and newly developed methods, such as the cohesive strength meter (CSM) and by magnetic particle induction (MagPI).

#### ***Sediment stability***

Natural sediments consist of a mixture of mud/sand/gravel present in varying contributions to the total bed formation. Two major classes of sediments have been described in the literature: cohesive (fine) and non-cohesive (coarse) sediments. The first type is commonly composed of silt and clay, and containing more than 10% of

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fine material by mass ( $< 63\mu\text{m}$ ). The large proportions of very small particles in this sediment are affected by the inter-particulate forces (Van der Waals forces) and by Brownian motion. Actions of these forces cause the particles to attract each other (cohesion). The bulk properties of this mixture determine an overall behaviour of the sediment (Whitehouse et al. 2000). The shear resistance of cohesive muddy sediments directly governs the susceptibility of the sediment to erosion by tidal and wave-induced currents or river flow (Tolhurst et al. 1999, Westrich and Forstner 2005). The mobility and transport of sediment depends on a variety of physical, chemical and biological processes (de Brouwer et al. 2000, Whitehouse et al. 2000, Haag and Westrich 2001). Traditionally, only physical properties of the sediment have been investigated such as bulk density, dry density, mineral density, grain-size distribution and mineralogical composition (Whitehouse et al. 2000). However a biological impact on the sediment stability has also been accepted over the past few decades. The biological impact on the sediment stability can be stabilizing (Paterson 1997), and/or destabilizing (Defew et al. 2002). Activities of macrofauna, such as bioturbation and grazing, can enhance the roughness of the sediment surface and thus, destabilize sediment. On the other hand, some activities of the organisms may influence sediment stability positively by e.g. constructing tubes (sediment traps) or coating tubes with EPS (Olafson and Thompson 1974). The most commonly recognized mechanism of biogenic stabilisation is the binding capacity of the mucilaginous extracellular polymeric substances (EPS). The EPS matrix is produced by microorganisms and some macrofauna. This process of “biostabilisation” significantly contributes to the resistance of the bed to physical forcing. Droppo (2001) has defined biostabilisation as a process whereby microbial, algal, fungal and other organism grow, along with increasing secretion of EPS, leading to an increase in bed stability. The microorganisms themselves can also establish bonding between each other and the sedimentary environment by their surface coatings. The secreted EPS can eventually envelop the sediment particles to form a three-dimensional matrix (Flemming and Wingender 2001a). The EPS matrix is held together by a range of bonding mechanisms such as electrostatic interactions, hydrogen bonds and London dispersion forces, providing the structural and functional integrity in biofilm, flocs and sludge (Flemming et al. 2000). The mechanistic relationship between EPS and sediment stability has been demonstrated by a number of authors (Yallop et al. 1994,

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Paterson 1995, Dade et al. 1996) and it has been suggested that the stability of non-cohesive sediments was greatly increased by the presence of microbial mat (Underwood and Paterson 1993, Yallop et al. 1994).

More recently, an impressive range of literature has developed concerning the biostabilisation of sediments and in particular about the mucilaginous matrix developed from EPS secretions (reviewed in Stal 2003, Underwood and Paterson 2003). However, very few publications incorporate both, biological and sedimentological variables to determine sediment stability (de Brouwer et al. 2000, Paterson et al. 2000, Haag and Westrich 2001, Underwood and Paterson 2003, Gerbersdorf et al. 2005).

Thus, the understanding of mechanisms and factors influencing cohesive sediments in riverine, estuarine and coastal habitats in terms of ecology and economy is crucial to follow up. In-depth studies of sediment erosion, transport, deposition and consolidation (ETDC-cycle) concerning sediment load and associated contaminants are central objectives for a sustainable management of waterways and coastal areas.

### ***Importance of the methods***

While investigation of the biostabilisation processes is an arguably important field of ecological research (ecosystem engineering), the measurement of adhesive potential of the biofilm is problematic and requires specialised approaches. Several devices have been introduced over the years for precise and reliable measurements of erosion resistance and erosion rates. Although these methods provide qualitative and quantitative information on the overall sediment erosion behaviour, they have limited application for studying low range shear stress and sub-critical responses. Natural depositional sediments are often dependent on mechanisms of biogenic stabilisation for their persistence against erosive forces. These mechanisms of “biostabilisation” can be varied but act together to provide an overall bed “stability” or resistance to erosion. While a number of methods are available for determining a bulk threshold for sediment stability, few techniques allow for a more sensitive assessment of the sediment surface as conditions change. One of them introduced by Prof. David Paterson (1989) the “Cohesive Strength Meter” (CSM), an *in situ* and laboratory device, working with a vertical jet flow which measures cohesive properties of the substratum. While the CSM is a well-established device to measure erosion resistance,

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it requires bed failure to occur and thus, needs a certain strength or resistance to the substratum to be developed (a threshold stress) before measurement can be made. Surface adhesion is also an important bed property reflecting the sediment system's potential to capture and retain new particles, to accumulate material or to replace eroded particles. More sensitive methods, which provide a measurement of surface adhesion of variety of surfaces, are of high demand.

### ***Ecosystem engineering***

Biofilms represent the dominant microbial life form in aquatic systems, and drive a number of important “ecological functions” such as nutrient recycling, biodegradation or pollutant retention (e.g. Battin et al. 2003). Once formed, a biofilm acts as a protective “skin” at the sediment surface and significantly affects the erosion and deposition of sediment particles (Paterson and Black 1999). While investigating different species, their growth stages, the abiotic conditions as well as emersion and immersion periods, which were mostly site-specific, are not usually considered (e.g. Riethmueller et al. 2000, Le Hir et al. 2007). Nowadays, a need has been identified to relate descriptive and structural parameters of ecological systems to their functional capabilities in order to define the “ecosystem service” of a habitat (e.g. Wimpenny et al. 2000, Paterson et al. 2008). In addition, these habitats are colonised by different types of microorganisms which play a significant role as ecosystem engineers by stabilising the sediments (Miller et al. 1996, Stal and Walsby 2000) through the production of extracellular polymeric substances (EPS) (Decho 1990, Underwood et al. 1995). Exopolymers are a ubiquitous component of marine ecosystems primarily composed of carbohydrates, proteins and lipids. They have multiple roles in aquatic systems: attachment to substrata, flotation and locomotion, feeding, protection against desiccation/UV/pollution, development of biofilms, communication (see reviews Decho 1990, Wotton 2004). For instance, in the surface layer of intertidal sediments, benthic epipellic diatoms show an endogenous migration pattern which is achieved by the secretion of highly-hydrated carbohydrate-rich exopolymers (Smith and Underwood 1998). The properties and behaviour of intertidal marine sediments can thus not be studied without taking into account these complex substances (Stal 2010), mainly because they enhance the cohesion and adhesion of sediments and

their capacity to resist to erosion, which is an important feature in these types of habitats.

***The extracellular polymeric substances (importance, producers and composition)***

The surface of submerged sediments is inhabited by meio-and macrofauna as well as microorganisms; all being able to secrete EPS (de Brouwer et al. 2000). As mentioned, the vast amount of functional groups and different bonding types within the EPS may enhance sediment stability significantly. Consequently, EPS may also enhance the mechanical stability of eroded sediment-born microbial aggregates in providing the “glue” which attaches the cells to given surface (Flemming et al. 2000). Moreover, EPS represent a sorption sites for pollutants such as heavy metals (copper, uranium, cadmium), and organic molecules; a feature used in water purification process (Flemming and Wingender 2001b). In this context, Decho (2000) speculates that the sorption of heavy metals by EPS is part of a protection strategy against toxic effects. As a result, the transfer of contaminants through food webs is significantly enhanced by EPS (Decho 2000, Flemming and Wingender 2001b, Hirst et al. 2003).

EPS also provides a nutritious food source for benthic feeders (Decho 2000) and by altering the benthic community structure via food source, sediment stability might be influenced as well. Interestingly, EPS molecules might also function as light transmitters and might support photosynthesis in deeper sediment layers (Flemming and Wingender 2001a).

The nature of the sedimentary environment helps to determine the colonization by the organisms; for instance cyanobacteria often colonize fine sand and epipelagic diatoms produce thin biofilms on mudflat surfaces (Stal 2003). In turn, organisms may change their sedimentary environment by e.g. sorting sediment particles (Wood and Armitage 1997) or secreting organic matter (Battin and Sengschmitt 1999, Perkins et al. 2004, Droppo et al. 2007) and consequently, influence future settlement of new organisms. Since different organisms will produce different EPS, the sedimentary environment is the first critical variable for organism settlement and EPS quality/quantity. The ability to secrete EPS is widespread among prokaryotic and eukaryotic organisms (Flemming et al. 2000); however the chemical composition of EPS and its binding properties are presumably very different. For instance, EPS produced from marine algae is composed primarily of polysaccharides (Staats et al.

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2000), while bacterial EPS rich in protein (Decho 1990). Diatom biofilms may contain up to 100 times more colloidal carbohydrate than cyanobacterial mats (de Winder et al. 1999). Fungal species may also play a large role in the biofilm development and structure, thus altering EPS composition (Droppo et al. 2007). Most probably the composition of the microbial produced EPS depends on the specific function and the abiotic conditions. Decho (2000) distinguishes between EPS for motility and attachment, which might explain different degrees of contribution to sediment stabilization process and described the physical state of EPS as a continuum, ranging from gels to a fully dissolved state. EPS contain large variety of chemical structures of varying proportions. Polysaccharides have often been assumed to be the most abundant component of EPS, other organic macromolecules such as proteins, nucleic acids, lipids/phospholipids or humic substances, can also predominate in the EPS (Flemming 2000). EPS present a network of molecules interconnected by chemical and physical crosslinks and defined as covalently cross linked networks (Decho 2000).

Little is known about natural EPS composition and most information originates from intertidal flats and microalgae/diatom communities. Microphytobenthos (MPB) biomass has been mostly associated with colloidal carbohydrates (Smith and Underwood 1998). Hence, it should be possible to predict the quantities of diatom EPS from chlorophyll *a* concentration (Underwood and Smith 1998). The sediment-stabilizing effect of benthic diatoms was investigated (Underwood and Paterson 2003, de Brouwer et al. 2005) and a range of factors such as species composition, complexity and physiological states of diatom mats (Decho 2000) correlated with increases in the critical erosion shear stress of the sediment, indicating that diatoms are important agents for biostabilisation process (Underwood and Paterson 1993).

Cyanobacteria is can also be found in illuminated areas and often under extreme environmental conditions. Fine sandy sediments are often characterized by the presence of microbial mats, formed by cyanobacteria (Stal 2003). The mechanism of biostabilisation from cyanobacteria is different as compared with other organism. Since the cyanobacteria absorb more light than necessary for growth, it is speculated that the cyanobacteria become embedded in a matrix of EPS through excess production that render a leathery structure and stability to the sediment (Stal 2003). EPS composition produced by cyanobacteria differs in comparison with EPS from

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diatoms, consisting mostly of tightly bound capsular carbohydrate (de Winder et al. 1999).

While the biostabilisation process is very important in terms of the prediction of sediment erosion potential and has been increasingly studied over the last decade, there are still significant gaps in our knowledge.

Despite the fact a range of meio- and microorganisms secrete EPS, most studies have focused on benthic microalgae as the main EPS producers (de Brouwer et al. 2005), and are therefore often considered as the most important contributor to biostabilisation. Thus, while microphytobenthos have been extensively researched (e.g. Paterson et al. 2000, Underwood et al. 2004), the ubiquitous heterotrophic bacterial component has been largely neglected in term of biostabilisation (Gerbersdorf et al. 2009). However in aquatic system with less light at the sediment surface, bacterial EPS production may dominate (Flemming and Wingender 2001a, Gerbersdorf et al. 2008). Microbial biofilm changes the physical and chemical microhabitat (Battin et al. 2003); bacterial activities can enhance yield stress and can be related directly to the erosion resistance (Dade et al. 1996). Also heterotrophic bacteria are critical to transformation and remineralisation of organic carbon, nitrogen and other nutrient (Decho 2000). Bacteria can produce EPS with a high protein and lipid content (Flemming and Wingender 2001a). The presence of proteins in sediment EPS may also enhance the quality of food in benthic systems, EPS in freshwater may be more available to consumers and are strongly suspected to increase transfers of contaminants through food web (Decho 2000, Flemming and Wingender 2001b, Hirst et al. 2003). Only recently the stabilisation effects by the EPS of natural bacterial assemblages (growing on inert glass beads) have been reported (Gerbersdorf et al. 2008).

The interactions of the main biofilm components such as heterotrophic bacteria, autotrophic microalgae, and cyanobacteria in terms of their individual contribution to the EPS pool and their relative functional contribution to substratum stabilisation, have rarely been studied in combination. There is also little known about the impact of meiofauna on microbial EPS production. Thus, new investigations upon the impact of EPS on sediment stability and relative contribution of EPS producers to biostabilisation are needed for the prediction of the erosion behaviour.

***The effect of toxin on microbial stabilisation capacity***

The mechanisms of biostabilisation and species interactions, including shifts in biofilms, are strongly depending on conditions in the environment. Studies of environmental risk assessment of pollutants have been the focus of much interest in the ecological field and mostly investigate the effect of toxins on aquatic organisms. They may also influence microbial biomass, EPS production and possible cause shift in community composition. However, the effect of pollution on ecological functions of aquatic organisms, such as biostabilisation is still unknown. To begin this research, triclosan (TCS) was chosen as a widely-used antibacterial and antifungal compound (McMurry et al. 1998, Villalain et al. 2001, Escalada et al. 2005) that has received much attention during recent years. It has the highest occurrence rate and maximum concentration among a group of 96 organic pollutants recently investigated (Kolpin et al. 2002, Halden and Paull 2005). Since effects of TCS on microbial biofilm may vary according to the concentration and exposure time (Tatarazako et al. 2004, Tabak et al. 2007, DeLorenzo et al. 2008, Franz et al. 2008), the range of TCS concentrations used in this study were chosen to be relevant to environmental occurrence. Despite that TCS is antibacterial agent, there is evidence that TCS also highly toxic to aquatic organisms (Orvos et al. 2002, Ishibashi et al. 2004, DeLorenzo et al. 2008), and that microalgae are the most sensitive organism to TCS (Reiss et al. 2002, Neumegen et al. 2005). While the current understanding is that TCS is acutely and chronically toxic to aquatic organisms, at the same time, the presence of toxin may elevate microbial EPS production (Fang et al. 2002, Iyer et al. 2004, Priester et al. 2006), and as result enhance bioaccumulation capacity of microbial biofilm (Schmitt et al. 1995). Based on these findings and taking into account the highly species-specific interactions between microalgae and bacteria the prediction of possible effect on biostabilisation capacity is complex and needs to be investigated by examination of biological-chemical variables (e.g. microbial biomass and EPS production) and sediment stability simultaneously.

This knowledge can provide early information about the effect of pollutants on engineering capacity of developing biofilms and significant contribute to understanding of the ecosystem functionality of “bioengineering.”

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**Objectives and hypothesises**

The aim of the work was to address gaps in our knowledge of microbial stabilisation and processes with a number of coherent aims. In Chapter 3, Magnetic Particle Induction (MagPI) (Larson et al. 2009) was developed to allow the determination of fine changes in surface properties and is suitable for investigation of young, growing biofilms. The technique is based on an original concept of using ferrous test particles and magnetism for measurements of biofilm adhesion (introduced by Prof. David M. Paterson). The method employs fluorescent microscopic magnetic particles that are added to the test surface. Thereafter an increasingly attractive force from a magnetic field (from an electro-or permanent magnet) is applied and the force at the point where the particles are recaptured by the magnet is determined as a measure of the adhesive nature of the surface. The methodology is dynamic and provides high level of precision. It can be easily controlled by fine increments of current and consequent strength of the magnetic field. For increased mobility and application in the field, the use of high power permanent magnets is possible. The methodology offers an easy and affordable way to determine the surface adhesion of a variety of surfaces rapidly and with precision. The technique may have further applications in research where the scale of determination required lies between that of atomic force microscopy (< um) and flume systems (> 10 cm).

To address this, the aim of Chapter 4 was an investigation into the interaction of aquatic organisms in terms of their coexistence and relative contribution to overall EPS pool. Coexistences of monospecific microalgae culture and their individual contribution to the EPS pool were investigated using axenic cultures of two diatoms: *Amphora coffeaeformis* and *Navicula hansenii* in combination with the cyanobacteria *Oscillatoria* species (Section 4.3.1). The individual and combined engineering capability of a natural heterotrophic bacterial assemblage with an autotrophic microalgae assemblage in terms of their innate EPS secretion was compared using benthic microbial cultures (prokaryotic, eukaryotic with natural diversity), isolated from estuarine sediments (Section 4.3.2). In addition to this, individual bacterivorous nematodes, and bacterial and microalgal cultures and in combination were used in order to determine the impact of benthic meiofauna on microbial growth and EPS production (Section 4.3.3).

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A further purpose was to test the hypothesis:

1. Higher diversity would lead to increase microbial biomass, EPS production and hence surface adhesion of the biofilm (described in Chapter 5).
2. The coexistence of bacteria and microalgae may enhance the EPS secretion, elevate microbial cell growth and the net engineering potential.
3. That bacterivorous nematodes may have a positive effect on the bacterial and microalgal growth and EPS production.

Discussions include the analysis of a shift of microbial community composition, changes of biomass and EPS production as a result of aquatic organism interaction.

Furthermore, results obtained were used to investigate relative functional contribution of microorganisms to substratum stabilisation. The aim of Chapter 5, was to assess the individual stabilisation capacity of microorganisms. Firstly, the individual stabilisation capacity of two benthic diatom species *Amphora coffeaeformis* and *Navicula hansenii* and cyanobacteria *Oscillatoria* species, separately and combined were determined using MagPI (Section 5.3.1). This data was related to biological variables and to quality and quantity of EPS obtained in Chapter 4.

Secondly, investigation of the engineering effect on a non-cohesive test bed as the surface was colonised by natural benthic assemblages (prokaryotic, eukaryotic and mixed cultures) of bacteria and microalgae. The mechanical properties of the surface were determined by newly developed magnetic particle induction technique MagPI and the CSM technique respectively to the adhesive capacity and the cohesive strength of the culture surface.

It was hypothesized that higher levels of microbial biodiversity would lead to increase stabilisation potential of the biofilm. A further purpose was to test the hypothesis that the coexistence of bacteria and microalgae might show a synergistic effect on their engineering capacity and stabilize the substratum more effectively.

The effect of triclosan (TCS) on microbial stabilisation capacity was investigated by using bacterial biofilm (Chapter 6) and natural freshwater biofilm (Chapter 7). Since bacteria are omnipresent members of aquatic biofilm and their important role on biostabilisation was confirmed in previous chapters, the aim of Chapter 6 was to study the impact of TCS on bacterial stabilisation potential. The bacterial culture was isolated from natural sediments and small glass beads were used as artificial, non-

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cohesive substratum, to support to development of bacterial biofilm. The stability of the substratum was determined using MagPI during two weeks of experimentation. The hypothesis that TCS have negative effect on bacterial population, EPS secretion and hence inhibited bacterial stabilisation potential was tested in this study. Areas discussed include biological and chemical variables (such as bacterial biomass, bacterial dividing rate and EPS quality and quantity) and sediment stability. Furthermore, obtained results were used as a basis to investigate the impact of TCS on natural freshwater microbial community described in Chapter 7.

The hypothesis that TCS have a negative effect on microbial growth and EPS production and impair stabilisation capacity of freshwater biofilm was tested and confirmed in Chapter 7. To simulate the natural scenarios of development of the biofilm in river system the natural freshwater biofilm was grown in flow-through glass channels (mini-flumes) before the start of the experiment and artificial glass beads served as the inert non-cohesive substratum. This knowledge can provide early information about the effect of pollutants on engineering capacity of developing biofilms and significant contribute to understanding of the ecosystem functionality of “bioengineering.”

## References

- Battin, T. J., L. A. Kaplan, J. D. Newbold, and C. M. E. Hansen. 2003. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* **426**:439-442.
- Battin, T. J. and D. Sengschmitt. 1999. Linking sediment biofilms, hydrodynamics, and river bed clogging: Evidence from a large river. *Microbial Ecology* **37**:185-196.
- Dade, W. B., R. L. Self, N. B. Pellerin, A. Moffet, P. A. Jumars, and A. R. M. Nowell. 1996. The effects of bacteria on the flow behavior of clay seawater suspensions. *Journal of Sedimentary Research* **66**:39-42.
- de Brouwer, J. F. C., S. Bjelic, E. de Deckere, and L. J. Stal. 2000. Interplay between biology and sedimentology in a mudflat (Biezelingse Ham, Westerschelde, The Netherlands). *Continental Shelf Research* **20**:1159-1177.
- de Brouwer, J. F. C., K. Wolfstein, G. K. Ruddy, T. E. R. Jones, and L. J. Stal. 2005. Biogenic stabilization of intertidal sediments: The importance of extracellular polymeric substances produced by benthic diatoms. *Microbial Ecology* **49**:501-512.
- de Winder, B., N. Staats, L. J. Stal, and D. M. Paterson. 1999. Carbohydrate secretion by phototrophic communities in tidal sediments. *Journal of Sea Research* **42**:131-146.
- Decho, A. W. 1990. Microbial exopolymer secretions in ocean environments-their role(s) in food webs and marine processes. *Oceanography and Marine Biology* **28**:73-153.
- Decho, A. W. 2000. Microbial biofilms in intertidal systems: an overview. *Continental Shelf Research* **20**:1257-1273.
- Defew, E. C., T. J. Tolhurst, and D. M. Paterson. 2002. Site-specific features influence sediment stability of intertidal flats. *Hydrology and Earth System Sciences* **6**:971-981.
- DeLorenzo, M. E., J. M. Keller, C. D. Arthur, M. C. Finnegan, H. E. Harper, V. L. Winder, and D. L. Zdankiewicz. 2008. Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environmental Toxicology* **23**:224-232.
- Droppo, I. G. 2001. Rethinking what constitutes suspended sediment. *Hydrological Processes* **15**:1551-1564.
- Droppo, I. G., N. Ross, M. Skafel, and S. N. Liss. 2007. Biostabilization of cohesive sediment beds in a freshwater wave-dominated environment. *Limnology and Oceanography* **52**:577-589.
- Escalada, M. G., J. L. Harwood, J. Y. Maillard, and D. Ochs. 2005. Triclosan inhibition of fatty acid synthesis and its effect on growth of *Escherichia coli* and *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **55**:879-882.
- Fang, H. H. P., L. C. Xu, and K. Y. Chan. 2002. Effects of toxic metals and chemicals on biofilm and biocorrosion. *Water Research* **36**:4709-4716.
- Flemming, H. C. 2000. Biofilms - Life on the edge of the aqueous phase. *Nachrichten Aus Der Chemie* **48**:442-+.
- Flemming, H. C. and J. Wingender. 2001a. Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects. *Water Science and Technology* **43**:1-8.
-

- Flemming, H. C. and J. Wingender. 2001b. Relevance of microbial extracellular polymeric substances (EPSs) - Part II: Technical aspects. *Water Science and Technology* **43**:9-16.
- Flemming, H. C., J. Wingender, C. Mayer, V. Korstgens, and W. Borchard. 2000. Cohesiveness in biofilm matrix polymers. Pages 87-105 in D. Allison, P. Gilbert, H. Lappin-Scott, and M. Wilson, editors. SGM symposium 59:Community structure and co-operation in biofilms, Germany.
- Foerstner, U. and W. Salomons. 2008. Trends and challenges in sediment research 2008: the role of sediments in river basin management. *Journal of Soils and Sediments* **8**:281-283.
- Franz, S., R. Altenburger, H. Heilmeier, and M. Schmitt-Jansen. 2008. What contributes to the sensitivity of microalgae to triclosan? *Aquatic Toxicology* **90**:102-108.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Gerbersdorf, S. U., T. Jancke, and B. Westrich. 2005. Physico-chemical and biological sediment properties determining erosion resistance of contaminated riverine sediments - Temporal and vertical pattern at the Lauffen reservoir/River Neckar, Germany. *Limnologica* **35**:132-144.
- Gerbersdorf, S. U., W. Manz, and D. M. Paterson. 2008. The engineering potential of natural benthic bacterial assemblages in terms of the erosion resistance of sediments. *Fems Microbiology Ecology* **66**:282-294.
- Haag, I. and B. Westrich. 2001. Correlating erosion threshold and physicochemical properties of natural cohesive sediments. *Hydraulics of Rivers Water Works and Machinery, Vol II, Theme D, Proceedings*:84-90.
- Halden, R. U. and D. H. Paull. 2005. Co-occurrence of triclocarban and triclosan in US water resources. *Environmental Science & Technology* **39**:1420-1426.
- Hirst, C. N., H. Cyr, and I. A. Jordan. 2003. Distribution of exopolymeric substances in the littoral sediments of an oligotrophic lake. *Microbial Ecology* **46**:22-32.
- Ishibashi, H., N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao, and K. Arizono. 2004. Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin. *Aquatic Toxicology* **67**:167-179.
- Iyer, A., K. Mody, and B. Jha. 2004. Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloacae*. *Marine Pollution Bulletin* **49**:974-977.
- Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, and H. T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology* **36**:1202-1211.
- Larson, F., H. Lubarsky, S. U. Gerbersdorf, and D. M. Paterson. 2009. Surface adhesion measurements in aquatic biofilms using magnetic particle induction: MagPI. *Limnology and Oceanography-Methods* **7**:490-497.
- Le Hir, P., Y. Monbet, and F. Orvain. 2007. Sediment erodability in sediment transport modelling: Can we account for biota effects? *Continental Shelf Research* **27**:1116-1142.
- McMurry, L. M., M. Oethinger, and S. B. Levy. 1998. Triclosan targets lipid synthesis. *Nature* **394**:531-532.

- Miller, D. C., R. J. Geider, and H. L. MacIntyre. 1996. Microphytobenthos: The ecological role of the "secret garden" of unvegetated, shallow-water marine habitats .2. Role in sediment stability and shallow-water food webs. *Estuaries* **19**:202-212.
- Neumegen, R. A., A. R. Fernandez-Alba, and Y. Chisti. 2005. Toxicities of triclosan, phenol, and copper sulfate in activated sludge. *Environmental Toxicology* **20**:160-164.
- Olafson, R. W. and J. A. Thompson. 1974. Isolation of heavy-metal binding-proteins from marine vertebrates. *Marine Biology* **28**:83-86.
- Orvos, D. R., D. J. Versteeg, J. Inauen, M. Capdevielle, A. Rothenstein, and V. Cunningham. 2002. Aquatic toxicity of triclosan. *Environmental Toxicology and Chemistry* **21**:1338-1349.
- Paterson, D. M. 1989. Short-term changes in the erodibility of intertidal cohesive sediments related to the migratory behaviour of epipellic diatoms. *Limnology and Oceanography* **34**:223-234.
- Paterson, D. M. 1995. Biogenic structure of early sediment fabric visualized by low temperature scanning electron microscopy. *Journal of the Geological Society* **152**:131-140.
- Paterson, D. M. 1997. Biological mediation of sediment erodibility: ecology and physical dynamics. John Wiley & Sons Ltd.
- Paterson, D. M., R. J. Aspden, P. T. Visscher, M. Consalvey, M. S. Andres, A. W. Decho, J. Stolz, and R. P. Reid. 2008. Light-Dependant biostabilisation of sediments by stromatolite assemblages. *PLOS ONE* **3**.
- Paterson, D. M. and K. S. Black. 1999. Water flow, sediment dynamics and benthic biology. *in* D. Raffaelli and D. B. Nedwell, editors. *Advances in Ecological Research*. Oxford University Press, Oxford.
- Paterson, D. M., T. J. Tolhurst, J. A. Kelly, C. Honeywill, E. de Deckere, V. Huet, S. A. Shayler, K. S. Black, J. de Brouwer, and I. Davidson. 2000. Variations in sediment properties, Skeffling mudflat, Humber Estuary, UK. *Continental Shelf Research* **20**:1373-1396.
- Perkins, R. G., D. M. Paterson, H. Sun, J. Watson, and M. A. Player. 2004. Extracellular polymeric substances: quantification and use in erosion experiments. *Continental Shelf Research* **24**:1623-1635.
- Priester, J. H., S. G. Olson, S. M. Webb, M. P. Neu, L. E. Hersman, and P. A. Holden. 2006. Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Applied and Environmental Microbiology* **72**:1988-1996.
- Reiss, R., N. Mackay, C. Habig, and J. Griffin. 2002. An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. *Environmental Toxicology and Chemistry* **21**:2483-2492.
- Riethmueller, R., M. Heineke, H. Kuehl, and R. Keuker-Ruediger. 2000. Chlorophyll a concentration as an index of sediment surface stabilisation by microphytobenthos? *Continental Shelf Research* **20**:1351-1372.
- Schmitt, J., D. Nivens, D. C. White, and H. C. Flemming. 1995. Changes of biofilm properties in response to sorbed substances - An FTIR-ATR study. *Water Science and Technology* **32**:149-155.
- Smith, D. J. and G. J. C. Underwood. 1998. Exopolymer production by intertidal epipellic diatoms. *Limnology and Oceanography* **43**:1578-1591.
-

- Staats, N., L. J. Stal, B. de Winder, and L. R. Mur. 2000. Oxygenic photosynthesis as driving process in exopolysaccharide production of benthic diatoms. *Marine Ecology-Progress Series* **193**:261-269.
- Stal, L. J. 2003. Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. *Geomicrobiology Journal* **20**:463-478.
- Stal, L. J. 2010. Microphytobenthos as a biogeomorphological force in intertidal sediment stabilization. *Ecological Engineering* **36**:236-245.
- Stal, L. J. and A. E. Walsby. 2000. Photosynthesis and nitrogen fixation in a cyanobacterial bloom in the Baltic Sea. *European Journal of Phycology* **35**:97-108.
- Tabak, M., K. Scher, E. Hartog, U. Romling, K. R. Matthews, M. L. Chikindas, and S. Yaron. 2007. Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms. *Fems Microbiology Letters* **267**:200-206.
- Tatarazako, N., H. Ishibashi, K. Teshima, K. Kishi, and K. Arizono. 2004. Effect of triclosan on various aquatic organisms. *Environmental Science* **11**:133-140.
- Tolhurst, T. J., K. S. Black, S. A. Shayler, S. Mather, I. Black, K. Baker, and D. M. Paterson. 1999. Measuring the in situ erosion shear stress of intertidal sediments with the Cohesive Strength Meter (CSM). *Estuarine Coastal and Shelf Science* **49**:281-294.
- Underwood, G. J. C., M. Boulcott, C. A. Raines, and K. Waldron. 2004. Environmental effects on exopolymer production by marine benthic diatoms: Dynamics, changes in composition, and pathways of production. *Journal of Phycology* **40**:293-304.
- Underwood, G. J. C. and D. M. Paterson. 1993. Seasonal-changes in diatom biomass, sediment stability and biogenic stabilization in the Severn Estuary. *Journal of the Marine Biological Association of the United Kingdom* **73**:871-887.
- Underwood, G. J. C. and D. M. Paterson. 2003. The importance of extracellular carbohydrate production by marine epipellic diatoms. *Advances in Botanical Research* **40**:183-240.
- Underwood, G. J. C., D. M. Paterson, and R. J. Parkes. 1995. The measurement of microbial carbohydrate exopolymers from intertidal sediments. *Limnology and Oceanography* **40**:1243-1253.
- Underwood, G. J. C. and D. J. Smith. 1998. Predicting epipellic diatom exopolymer concentrations in intertidal sediments from sediment chlorophyll a. *Microbial Ecology* **35**:116-125.
- Villalain, J., C. R. Mateo, F. J. Aranda, S. Shapiro, and V. Micol. 2001. Membranotropic effects of the antibacterial agent triclosan. *Archives of Biochemistry and Biophysics* **390**:128-136.
- Westrich, B. and U. Forstner. 2005. Sediment dynamics and pollutant mobility in rivers (SEDYMO) Assessing catchment-wide emission-immission relationships from sediment studies - BMBF Coordinated Research Project SEDYMO (2002-2006). *Journal of Soils and Sediments* **5**:197-200.
- Whitehouse, R., R. Soulsby, W. Roberts, and H. Mitchener. 2000. Dynamics of estuarine muds. Thomas Telford Ltd.
- Wimpenny, J., W. Manz, and U. Szewzyk. 2000. Heterogeneity in biofilms. *FEMS Microbiology Reviews* **24**:661-671.
- Wood, P. J. and P. D. Armitage. 1997. Biological effects of fine sediment in the lotic environment. *Environmental Management* **21**:203-217.
-

- Wotton, R. S. 2004. The ubiquity and many roles of exopolymers (EPS) in aquatic systems. *Scientia Marina* **68**:13-21.
- Yallop, M. L., B. Dewinder, D. M. Paterson, and L. J. Stal. 1994. Comparative structure, primary production and biogenic stabilization of cohesive and noncohesive marine sediments inhabited by microphytobenthos. *Estuarine Coastal and Shelf Science* **39**:565-582.

## Chapter 2

### General Methods

The methodologies described in this chapter provide the general materials, methods and techniques used throughout the thesis. The results of the experimental chapters are described with reference to the relevant sections of this chapter. The experiments conducted throughout the course of this thesis are of similar general design, which is detailed within this chapter.

#### 2.1. Study sites

The main subsurface sediments or water used for bacterial and microalgae culture were collected from the Eden Estuary, situated on the east coast of Scotland between St Andrews and the Firth of Tay (56°22'N, 2°51'W) and from the River Parthe which joins the White Elster in northwestern Leipzig, Germany (51°21'39''N 12°20'32''E).

#### 2.2. Culture preparation

##### 2.2.1. Bacterial cultures

Subsurface sediment was collected to a depth of 0-2 mm from the intertidal mudflats of the Eden estuary located in the southeast of Scotland (56°22'N, 2°51'W). One litre of 1 µm filtered seawater was mixed with the same volume of sediment and the



sediment slurry was ultrasonicated (Ultrasonic bath XB2 50-60Hz) for 10 min to enhance detachment of bacteria from the sediment grains. The sediment slurry was centrifuged twice for 10 min (6030 g, Mistral 3000E, Sanyo, rotor 43122-105) to separate sediment (pellet) and bacteria (supernatant). The supernatants were retained and centrifuged for another 10 min (17700 g, Sorval RC5B/C). This time the supernatant was discarded, while the remaining pellet containing the bacteria was resuspended and filtered through a 1.6  $\mu\text{m}$  filter (glass microfiber filter, Fisherbrand MF100). The filter size was chosen to exclude the smallest expected microalgae from the Eden estuary, such as *Nitzschia frustulum* which is typically 4-10  $\mu\text{m}$  (Aspden PhD thesis, 2005). All equipment used after this filtration step was acid-washed and possible microalgal contamination was checked regularly by epifluorescence microscopy. Standard nutrient broth (Fluka, Peptone 15 g l<sup>-1</sup>, yeast extract 3 g l<sup>-1</sup>, sodium chloride 6 g l<sup>-1</sup>, D(+) glucose 1 g l<sup>-1</sup>) was autoclaved and added (1:3) to the filtered supernatant. The bacterial stock cultures were established in 200 ml Erlenmeyer flasks under constant aeration in a dark at room temperature (15°C) and fresh nutrient broth was added once a week during a 2-week cultivation period.

### 2.2.2. Diatom cultures

Sediment surface samples (0-5 mm) were taken from the same location on the Eden estuary and were processed in a consistent manner as described for the bacterial cultures above. However, the remaining pellet was resuspended in F/2 culture media without the filtration step. To exclude bacteria, antibiotics were added (150 mg l<sup>-1</sup> streptomycin, 20 mg l<sup>-1</sup> chloramphenicol, final concentrations). To confirm the effective exclusion of bacteria the subsamples was mixed with an equal amount of SYTO Green 13 (1  $\mu\text{l}$  of stain mixed in 1 ml distilled water, Molecular Probes) and the samples were examined regularly using epifluorescence microscopy. The microalgal cultures were incubated under constant temperature (15°C) and at ambient light conditions in the laboratory for 3 weeks with fresh nutrients added regularly, once a week (Ribalet et al. 2008).

### 2.2.3. Nematode cultures

Bacterivorous nematodes (*Diplolaimelloides meyli* Timm 1961, *Diplolaimelloides oschei* Meyl, 1954) belonging to the family *Monhysteridae*, originally obtained from the

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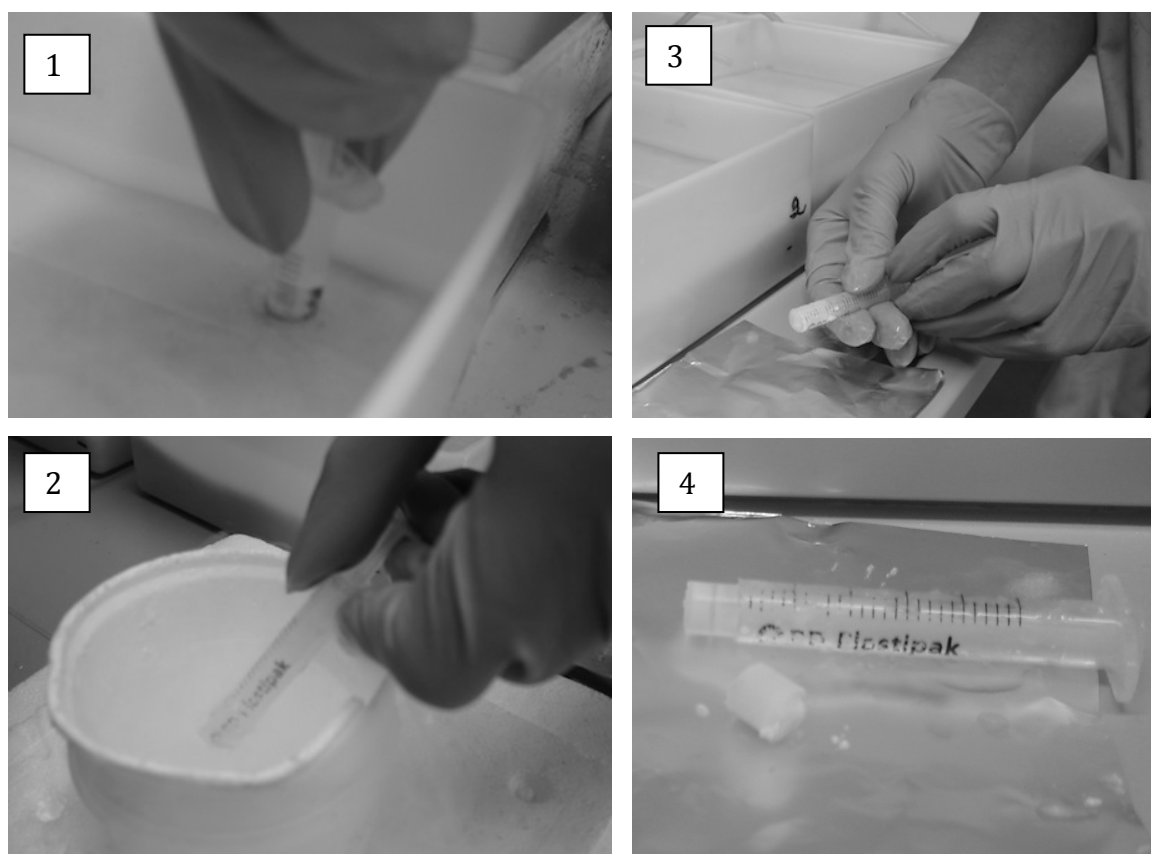
Westerschelde Estuary (SW Netherlands), were used from monospecific laboratory cultures at the Marine Biology Laboratory, Ghent University. This species was used, as they are opportunistic colonizers of various types of decaying organic matter and feed largely on bacteria. The nematodes were grown under laboratory conditions (17°C) and salinity (25) for many generations prior to the start the experiment (Hubas et al. 2010). The nematodes were grown and extracted as described by Moens and Vincx (1998). Agar media with unidentified bacteria from their natural habitat was used as food source. Prior to the experiment the nematodes were extracted from the culture plates using a density centrifugation in sucrose (40% final concentration) and carefully washed several times with artificial seawater. The nematodes were placed in non-cohesive acid washed marine sand (40-100 µm, Fisher Scientific) during the experiment.

### **2.3. Sample collection**

Surface sediment was obtained using a mini core (see section 2.3.1 for details). The sediment was fixed using methods appropriate for future analysis. For determination of bacterial cell numbers, EPS concentrations, chlorophyll *a* analysis and LTSEM the sediment samples were immediately frozen in liquid nitrogen (LN<sub>2</sub>) and stored at -80°C, to prevent microbial activity and degradation, until required for further analysis. For bacterial dividing rate, one sediment core with a depth of 10 mm was taken from each box and three cores per treatment pooled before analysis. Cores were incubated for 20 min immediately after sampling with methyl-3H thymidine with final concentration of 300 nmol l<sup>-1</sup>, methyl-3H thymidine, 50 Ci mmol l<sup>-1</sup>) according to Fuhrman and Azam (1982). The incorporation of radioactive thymidine was stopped by adding of 5 ml of 80% ethanol. The samples were stored at room temperature in a suitable container for radioactive samples until further analysis was carried out. To determine bacterial community composition the cores were fixed overnight with 3.7% formaldehyde and 70% ethanol. In order to determine microphytobenthic community composition, the cores were fixed in 4% glutaraldehyde and stored until required for further analysis.

### 2.3.1. The mini-cores

Sediment cores of 5 mm depth were taken with a cut-off syringe 10 mm diameter (Figure 2.1) by the following steps: 1-the syringe was placed into the sediment to a depth of ~5 mm, rotated 360° within the sediment and removed; 2-any sediment protruding from the syringe was removed by scraping a spatula across the face of the syringe; 3-the sediment cores for analysis of bacterial cell numbers, EPS concentrations and chlorophyll *a* concentrations were immediately frozen with liquid nitrogen after sampling; 4-the sediment was extracted from the syringe using the syringe-plunger, wrapped in labelled foil and stored at -80°C until required for further analysis.



**Figure 2.1:** *Sample extraction.*

### 2.4. EPS extraction and determination

Two millilitres of distilled water was added to the sediment samples in safety-lock Eppendorf caps. The samples were continuously rotated for 1.5 h on a horizontal mixer (Denley Spiramix 5) at room temperature (20°C). The samples were then

centrifuged (6030 g, 10 min, Mistral 3000E Sanyo, rotor 43122-105) and the supernatant containing the water-extractable (colloidal) EPS fraction was pipetted into a new Eppendorf and mixed. The supernatant was divided into 1 ml subsamples to determine EPS compounds: ~1 ml for carbohydrates concentration and ~1 ml for EPS protein concentration. Subsamples of this supernatant were analysed in triplicates for carbohydrate and proteins following the Phenol Sulphuric Assay protocol (Dubois et al. 1956) and the modified Lowry procedure (Raunkjaer et al. 1994) respectively.

#### 2.4.1. Colloidal carbohydrates analysis

For carbohydrates analysis, 200  $\mu\text{l}$  phenol (5%) and 1 ml sulphuric acid (98%) were added to 200  $\mu\text{l}$  supernatant. The samples were incubated for 35 min at 30°C and the carbohydrate concentration was measured by spectrophotometer (CECIL CE3021) at the wavelength of 488 nm (Dubois et al. 1956). The carbohydrate concentrations were calculated according to a glucose standard curve and results reported as glucose equivalents ( $\mu\text{g cm}^{-3}$  glucose equivalent).

#### 2.4.2. Glucose standard preparation

A calibration curve (Figure 2.2) of D-glucose dilutions was performed with each set of samples. Standards were made in triplicate within a range of 0, 20, 50, 100, 125, 150 and 200  $\mu\text{g ml}^{-1}$  from stock solutions of D-glucose 200  $\text{mg l}^{-1}$ .

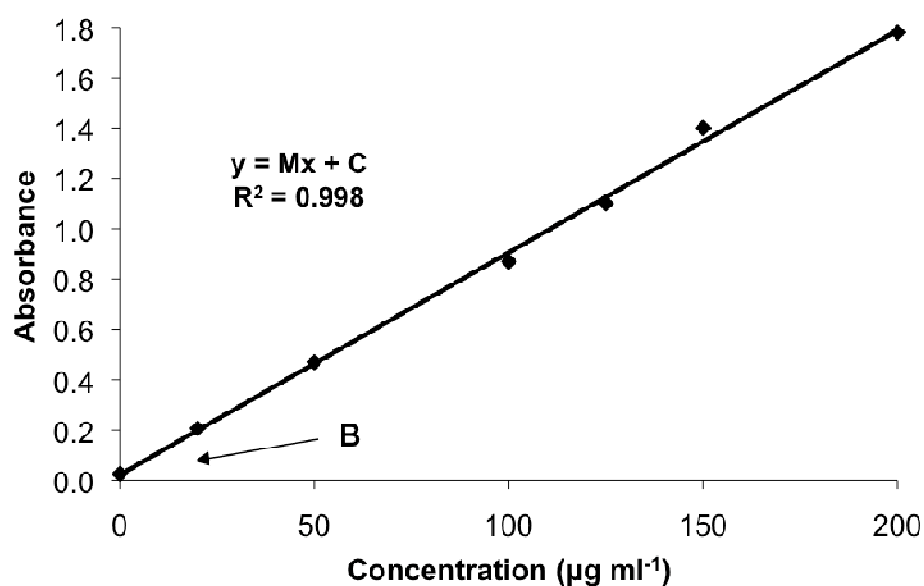


Figure 2.2: Typical glucose/carbohydrate standard curve.

Linear regressions of the relevant standard curves were used to calculate the coefficients and constants using following equation

$$\text{Colloidal Carbohydrate } [\mu\text{g cm}^{-3}] = \frac{(Abs - B) - C}{M} \times 4 \quad \text{Equation 2.1}$$

where *Abs* is the absorbance of light with wave length of 488 nm, *B* is absorbance of blank sample with zero concentration of glucose, *C* is the constant-intercept of the line and *M* is the gradient of the line. Obtained values were multiplied by a factor of 4 to correct the resulting volume of samples diluted with added water.

#### 2.4.3. Colloidal protein analysis

For protein analysis, 250  $\mu\text{l}$  supernatant was incubated for 15 min with 250  $\mu\text{l}$  of 2% sodium dodecyl sulphate (SDS) and 700  $\mu\text{l}$  of chemical reagent 4. Reagent 4 is a mixture of Reagents 1- 3 in a ratio of 100:1:1 (Reagent 1: 143 mM NaOH, 270 mM  $\text{Na}_2\text{CO}_3$ , Reagent 2: 57 mM  $\text{CuSO}_4$ , Reagent 3: 124 mM Na-tartrate), and incubated for a further 45 min at 30°C with Folin reagent (diluted with distilled water 5:6) (Raunkjaer et al. 1994, Gerbersdorf et al. 2008). The protein concentration was determined using a spectrophotometer (BUCK Scientific, CECIL CE3021, UK) at the wavelength of 750 nm. The proteins concentrations were calculated according to BSA standards curve and results are given in microgram per cubic centimetre.

#### 2.4.4. BSA standard preparation

A calibration curve of BSA standard (Bovine Serum Albumin: Sigma, cat no A 4503-10g) was produced for each set of samples. Standards were made within a range of 0, 20, 50, 125, 150, 200 and 250  $\mu\text{g ml}^{-1}$  (3 replicates of each) from stock solutions of BSA 200  $\text{mg l}^{-1}$ . Similar to calculations for colloidal carbohydrates, linear regressions of the standards were used to calculate the coefficients and constant as follow

$$\text{Colloidal Protein } [\mu\text{g cm}^{-3}] = \frac{(Abs - B) - C}{M} \times 4 \quad \text{Equation 2.2}$$

where  $A_{750}$  is the absorbance at 750 nm,  $B$  is absorbance of blank sample with zero concentration of BSA,  $C$  is the constant-intercept of the line and  $M$  is the gradient of the line. Obtained values were multiplied by factor 4 to correct the resulting volume of samples diluted with added water.

## 2.5. Pigment extraction and determination

Cores were transferred to a 15 ml Apex centrifuge tube to which 10 ml of 96% ethanol was added. The mixture was rotated for 24 h in the dark at room temperature (20 C°) by a horizontal rotator at a fixed speed of 50 rpm (Denley Spiramix 5). The samples were centrifuged for 10 min at 6030 g (Sanyo MSE, Mistral 3000E). The chlorophyll  $a$  and pheophytin concentrations in the supernatant were measured according to the BMEPC guidelines (BMEPC 1988), reading absorbance at 630, 647, 664 and 750 nm wavelength before and after acidification (Termo Biomate 5 spectrophotometer), respectively, according to Jeffrey et al. (1999). Chlorophyll  $a$  and pheophytin concentrations are given as a proxy for microphytobenthic biomass and degradation products, respectively. Chlorophyll  $a$  concentrations were calculated according to the chlorophyll  $a$  standards and results are given in microgram per cubic centimetre.

### 2.5.1. Preparation of the chlorophyll $a$ standards

Stock chlorophyll  $a$  standard solution was prepared by dissolving 1 mg of Spinach sample (Sigma-Aldrich) in 250 ml of 96% ethanol. Standards were made in triplicate within a range of 4, 2, 1, 0.5 and 0.25 mg l<sup>-1</sup> from stock solution. To inhibit pigment degradation, the standards were wrapped in tinfoil and stored at 4° C. The absorbance of a blank (96% ethanol) and each standard concentration were measured in separate 1 cm cuvettes (Termo Biomate 5 spectrophotometer), and were read at 630 nm (the correct peak maxima ( $\lambda$  max) of chlorophyll  $a$ ) and 750 nm to correct for light scattering in the sample. Chlorophyll  $a$  concentration was calculated using

$$\text{Chlorophyll } a \text{ } [\mu\text{g cm}^{-3}] = ([A_{630}] - [A_{750}] - \text{blank}) / \epsilon \quad \text{Equation 2.3}$$

where  $A_{630}$  is the absorbance at 630 nm,  $A_{750}$  is the absorbance at 750 nm,  $C$  is chlorophyll  $a$  concentration ( $\mu\text{g ml}^{-1}$ ) and  $\epsilon$  is extinction co-efficient of chlorophyll  $a$ .

## 2.6. Fluorescence measurements

Done with collaboration S. Franz, Helmholtz Centre for Environmental Research, Department Bioanalytical Ecotoxicology, Germany.

Pulse Amplitude Modulated (PAM) fluorescence is increasingly being applied in the assessment of algal photosystems (Schmitt-Jansen and Altenburger 2008). Data collected using this technique was used as a proxy of microphytobenthic biomass, and to assess the stress response of microalgae in the presence of the xenobiotic compound triclosan. Effects on photosynthetic activity were performed according to McClellan et al. (2008) using the MAXI-Imaging PAM (Fa. Walz, Effeltrich Germany) on undisturbed Petri dishes. The instrument was positioned 4 mm above the sediment surface; the position was kept constant for comparative purposes. After 5 min dark adaptation, the minimum fluorescence yield ( $F_0^5$ ) was measured and used as a surrogate of sediment surface algal biomass. Afterwards the samples were adapted to actinic light (PAR 111  $\mu\text{mol photons m}^2 \text{s}^{-1}$ ) for 2.5 min. The current fluorescence yield ( $F'$ ) and the maximum fluorescence ( $Fm'$ ) was assessed immediately after a saturating pulse of light (Schreiber et al. 1986, Honeywill et al. 2002). The measurement was repeated three times per Petri dish and an average value was calculated. These two parameters were then used to calculate the effective quantum yield that represents the photosynthetic capacity and can be used to assess the inhibition of  $Y(II)$  and therefore an indication of stress response, according to Schreiber et al. (1986) and Genty et al. (1989):

$$Y(II) = \frac{\Delta F_v}{Fm'} = \frac{Fm' - F'}{Fm'} \quad \text{Equation 2.4}$$

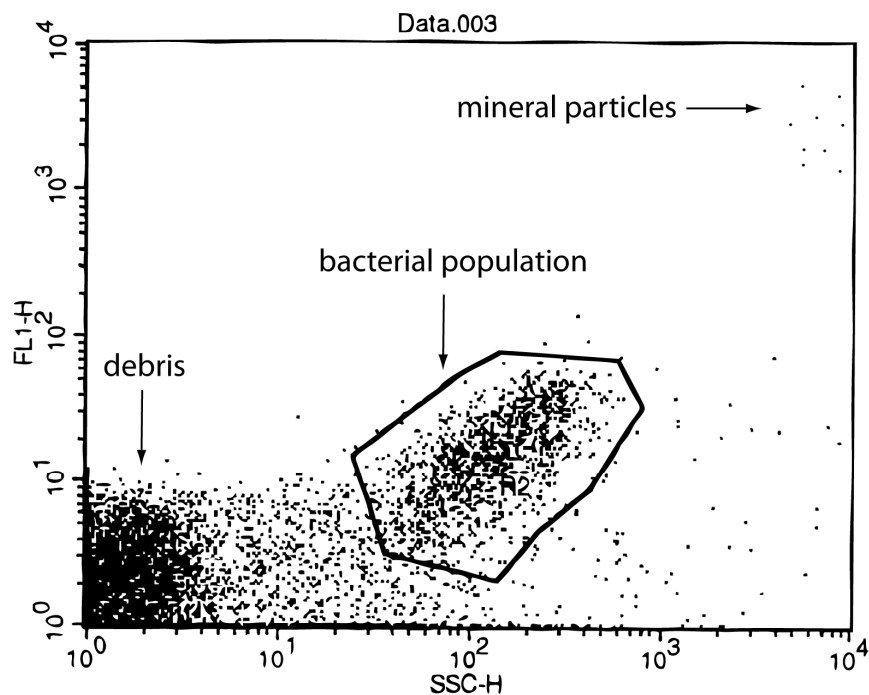
The inhibition of the photosystem is expressed as the ratio of the effective quantum yield of the treated samples  $Y(II)_{\text{treat}}$  and the effective quantum yield of each treatment's at the first day of the experiment  $Y(II)_{\text{fd}}$

$$\text{Inhibition [\%]} = \frac{100 * Y(II)_{\text{treat}}}{Y(II)_{\text{fd}}} \quad \text{Equation 2.5}$$

## 2.7. Bacterial enumeration by flow cytometry

Cores were fixed with 0.2  $\mu\text{m}$  pre-filtered glutaraldehyde solution (1% final concentration) and bacteria were stained with Syto13 (Molecular Probes, 1: 2000 v: v,

1.2  $\mu\text{mol l}^{-1}$  final concentration) for 15 min in the dark. The bacterial abundance was measured by flow cytometry (Becton Dickinson FACScan™ with a laser emitting at 488 nm). Fluorescent calibrated beads were added to some samples (PeakFlow™, 6  $\mu\text{m}$ , 515 nm, Molecular Probes) to distinguish bacterial cells from debris and mineral particles (Figure 2.3).



**Figure 2.3:** Typical example of relevant window for bacterial enumeration by flow cytometry.

The acquisition of events was thus limited to a gate encompassing only bacterial cells by plotting the side light scatter (SSC) versus green fluorescence (FL1). Data were recorded until 10,000 events were acquired or after 60 s of counting. The bacterial abundance was calculated by multiplying the acquisition rates (between 160 and 640 bacteria counted per s) by the flow rate (fixed to 60  $\mu\text{l min}^{-1}$ ).

### 2.8. Bacterial division rate

Cores were incubated for 20 min immediately after sampling with [methyl-3H] thymidine (final concentration 300  $\text{nmol l}^{-1}$ , methyl-3H thymidine, 50 Ci  $\text{mmol l}^{-1}$ ) according to Fuhrman and Azam (1982). The incorporation of radioactive thymidine was stopped by adding 5 ml of 80 % ethanol. All the samples were collected on a filter



(0.2  $\mu\text{m}$ ) after the incubation time and washed several times with 80% ethanol and 5% trichloroacetic acid (TCA) to remove excess radioactivity. The filters (containing the bacteria and the sediment particles) were mixed with 5 ml of 0.5 mol l<sup>-1</sup> of HCl and incubated at 95 °C over 16 h (Garet and Moriarty 1996) allowing the settlement of the sediment particles and the solubilisation of the stained bacteria into the supernatant. A subsample of the supernatant was taken, cooled and mixed with 3 ml of the scintillation cocktail Ultima Gold MV. The bacterial division rate (cells cm<sup>-3</sup> h<sup>-1</sup>) was calculated according to an internal standard quenching curve (Liquid scintillation analyzer "TRI-CARB 2000") while assuming that 1 mol<sup>-1</sup> incorporated thymidine is equivalent to the production of 2 x 10<sup>18</sup> bacterial cells (Lee and Fuhrman 1987, Cho and Azam 1990). The saturating concentration of <sup>3</sup>H-thymidine was chosen according to previous experiments in similar sediments. The thymidine incorporation was shown to be linear under the range of chosen concentrations (Hubas et al. 2007a, Hubas et al. 2007b). For each replicate, the radioactivity of the samples was corrected against a blank, which corresponded to the pre-fixed sediment cores submitted to the protocol described above.

## **2.9. Microbial community composition**

### **2.9.1. Bacterial assemblage/Fluorescence in situ hybridization (FISH)**

Done by Dr. W. Manz Institute for Integrated Natural Sciences, University Koblenz-Landau, Germany.

To determine bacterial community composition two sediment cores were fixed overnight with 3.7% formaldehyde and 70% ethanol to account for the different permeability of Gram negative and Gram positive bacteria, respectively (Roller et al. 1994, Manz 1999). After incubation (using a horizontal mixer, Denley Spiramix 5; Denley-Tech Ltd, Sussex, UK) and centrifugation (5 min at 16060 g<sup>-1</sup>, Biofuge pico Centrifuge, Heraeus, Rotor 7500 3325), the samples were washed twice in phosphate-buffer saline (PBS, 130 mM NaCl and 10 mM NaHPO<sub>4</sub>/ NaH<sub>2</sub>PO<sub>4</sub>, pH7.4), then the pellets were resuspended and stored in a mixture with equal parts of PBS and ice-cold absolute ethanol at -20°C (Amann et al. 1990). Prior to further analysis, sediment-associated bacteria were detached and homogenized by 5 min of sonication (Ultrasonic bath XB2 50-60Hz), thoroughly mixed for 1 min, and centrifuged at 16060 g<sup>-1</sup> (Biofuge pico Centrifuge, Heraeus, Rotor 7500 3325).

To determine total bacterial cell counts, 50  $\mu\text{l}$  aliquots of the cell suspensions were filtered through polycarbonate membranes (0.2  $\mu\text{m}$  pore size, Millipore, Eschborn, Germany) and stained with 15  $\mu\text{l}$  DAPI solution (4',6-diamidino-2-phenylindole, Sigma, Deisenhofen, Germany, 10  $\mu\text{g ml}^{-1}$ ). Applying a comprehensive set of oligonucleotide probes, intact bacterial cells have been hybridized aiming at selected parts of the 16S rRNA that are specific for bacterial groups on the domain, phylum, and subphylum level (Manz et al. 1992, Gerbersdorf et al. 2008, Gerbersdorf et al. 2009) (Table 2.1).

**Table 2.1.** Oligonucleotides used in this study (*a*-Probe nomenclature as described by Alm et al. (1996).

Target organisms	Oligonucleotide <sup>a</sup> Common name	Sequence (5'-3')	%FA	Reference
<i>Bacteria</i>	S-D-Bact-0338-a-A-18 EUB338	GCTGCCTCCCGTAGGAGT	0-50	Amann et al., (1990)
<i>Planctomycetales</i>	S-D-Bact-0338-b-A-18 EUB338 II	GCAGCCACCCGTAGGTGT	0-50	Daims et al., (1999)
<i>Verrucomicrobiales</i>	S-D-Bact-0338-c-A-18 EUB338 III	GCTGCCACCCGTAGGTGT	0-50	Daims et al., (1999)
<i>Alphaproteobacteria</i>	S-Sc-aProt-0019-a-A- ALF968	GGTAAGGTTCTGCGCGTT	35	Neef (1997)
<i>Betaproteobacteria</i>	L-Sc-bProt-1027-a-A-17 BET42a	GCCTTCCCCTTCGTTT	35	Manz et al., (1992)
<i>Gammaproteobacteria</i>	L-Sc-gProt-1027-a-A-17 GAM42a	GCCTTCCCACATCGTTT	35	Manz et al., (1992)
<i>Actinobacteria</i>	S-P-HGC-1901-a-A-18 HGC69a	TATAGTTACCACCGCCGT	25	Roller et al., (1994)
<i>Desulfobacterales,</i> <i>Desulfuromonales,</i> <i>Syntrophobacterales,</i> <i>Myxococcales, and other</i> <i>bacteria</i>	S-F-Srb-0385-b-A-18 (SRB385Db)	CGGCGTTGCTGCGTCAGG	35	Rabus et al., (1996)
<i>Cytophaga-Flavobacterium</i> <i>group of Bacteroidetes</i> <i>Flavobacteria,</i> <i>Bacteroidetes &amp;</i> <i>Sphingobacteria</i>	S-P-CyFla-0319-a-A-18 CF319a	TGGTCCGTGTCTVAGTAC	20	Manz et al., (1996)

The samples were incubated in hybridization buffer containing 0.9 M NaCl and formamide, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate (SDS), and the

oligonucleotide probe at a concentration of 20-50 ng ml<sup>-1</sup> for at least 4 h at 46°C. Under stringent conditions, the slides were washed (washing solution 20 mM Tris-HCl, pH8; 0.01% SDS) and air-dried. Total bacterial cell counts and FISH analysis was performed by epifluorescence microscopy (Zeiss Axioplan II, Carl Zeiss, Jena, Germany), fitted with Zeiss light filter set no.1 for DAPI (exciter 365 nm, dichroic mirror 395 nm, emission filter 397 nm) and for Cy3 (Exciter 535/50 nm, dichroic mirror 565 nm, emission filter 610/75/nm). For cell counts, ten areas on the slide were chosen at random to carry out cell counts (100x100 µm) were evaluated counting a minimum of 1000 cells per sample. The hybridization with a molar mixture of the probes EUB338, EUB338II, and EUB338III gave the total eubacterial counts, and the probe-specific counts were calculated on this basis as percentages.

### **2.9.2. Microphytobenthic assemblage**

Done by Dr. L.Taeuscher's Institute fuer Gewaesseroekologie Seddin, Germany

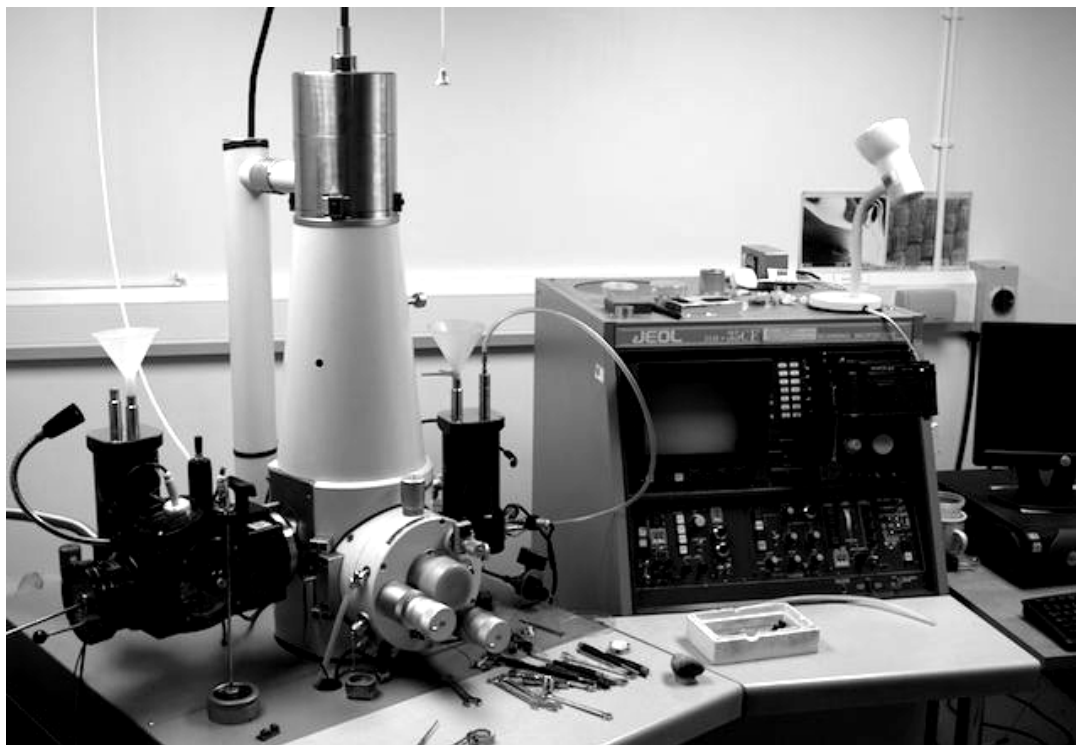
The species composition of the microalgal community was assessed from fixed samples of the diatom assemblage. The cores were fixed in 4 % glutaraldehyde and the species composition of the microalgal community was assessed within 10 subsamples per sample by light microscopy. The subsamples were further cleaned and embedded in Naphrax (refractive index  $n=1.710$ ) for precise determination of taxa. The following literature was used: (Simonsen 1962, Krammer and Lange-Bertalot 1986-1991, Pankow 1990, Lange-Bertalot 1997, Underwood et al. 1998, Witkowski et al. 2000).

### **2.10. Low Temperature Scanning Electron Microscopy**

Done with collaboration I. Davidson, Sediment Ecology Research Group, Scottish Ocean Institute, University of St Andrews.

Low Temperature Scanning Electron Microscopy (LTSEM) has often been used to visualise biofilms and microbial mats and investigate their role in the biogenic stabilisation of sediments (Perkins et al. 2006). Further details of the examination of frozen sediment/biofilm under LTSEM are discussed in Paterson (1995). Samples are frozen using liquid nitrogen to facilitate examination in an electron microscope (JEOL 35CF Scanning Electron Microscope, Japan) (Figure 2.4). This system was adapted to

perform low-temperature study (Oxford Instruments CT 1500 Cryopreparation System).



**Figure 2.4:** *Low Temperature Scanning Electron Microscope integrated with digital image recording system and Oxford cryo-SEM system*

The samples for LTSEM were collected using a cut-off syringe, as described in section 2.3.1, immediately frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until being viewed. Samples were mounted onto specifically designed cryo-stubs, whilst remaining frozen with  $\text{LN}_2$ , and transferred to the cryo-apparatus. The samples were partially freeze-dried within the electron microscope on a heated stage until enough water had sublimed into vacuum to allow a clear viewing. Images were recorded with a Digital Image Recording System (JEOL Semaphore SA20, Japan). Images were taken to give a generalised view of the sample; with higher power images taken to provide detail.

### **2.11. Application and determination of triclosan concentration**

To investigate the effect of toxin on the biostabilisation capacity of cultured bacterial biofilms (Chapter 6) and natural fresh water biofilms (Chapter 7) triclosan (TCS) which is a potent biocide that is included in a diverse range of products was employed. Two different methods of triclosan (5-chloro-2-(2,4-

dichlorophenoxy)phenol) spiking were used. Two subsections on each of these methods are described below.

#### **2.11.1. Application of triclosan-pure standard**

Triclosan stock solution was prepared by dissolving the commercially available powder (Irgasan-72779, Sigma –Aldrich) in seawater for 4 h, with the help of a magnetic stirrer. The stock solution was further diluted with seawater to gain the required concentrations and the experimental treatments were spiked via the water phase. Consequently, the actual triclosan concentrations and distribution between the water phase and the surface substratum were regularly analysed during the experiment by high performance liquid chromatography (HPLC). Overlying water was removed carefully using a 20 ml syringe, without disturbing the sediment surface, and retained for analysis. Cores were obtained using the minicore method once the overlying water had been removed. The water samples and the sediment extracts of the substrata were pre-concentrated using silica-based octadecyl bonded phase cartridges C18 6cc (Oasis HLB, Waters, Milford, MA), used to adsorb molecules of even weak hydrophobicity from aqueous solutions. Prior to use, the cartridges columns (3 ml) were activated and conditioned with 5 ml of HPLC water, acetone and finally, methanol, at a flow rate of 1 ml min<sup>-1</sup>. About 500 ml of samples volume were promptly loaded onto the cartridges at a flow rate of 5 ml min<sup>-1</sup> to avoid any degradation of the target compounds and the loss of sample integrity. After pre-concentration, the cartridges were completely dried by vacuum for about 20 min to avoid hydrolysis and kept at -20°C until analysis. Finally, the cartridges were eluted with 2 ml of methanol and directly injected onto the HPLC vials. The injection volume was set at 100 µl, and the flow rate was kept at 1 ml min<sup>-1</sup> of 80% methanol using isocratic flow. Detection of triclosan was carried out by UV-VIS detector (Waters 2489) at the wavelength of 280 nm. The triclosan peak was quantified against an absolute standard by the Empower 2 Chromatography Software (Waters). Triclosan concentrations were calculated according to the triclosan standards and results are given in microgram per litre.

##### **2.11.1.1. Triclosan standard**

Triclosan standard was purchased from Sigma-Aldrich (St. Louis, MO). Solvent used during analysis was high performance liquid chromatography grade. The standard used to quantify compounds of the highest purity available. Stock solutions (1 mg l<sup>-1</sup>)

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of individual standards were prepared by dissolving pure standards in methanol. Working solutions were obtained by further dilution of stock solution in within a range of 1-1000  $\mu\text{g l}^{-1}$ .

### 2.11.2. Application of triclosan–partitioning-based dosing

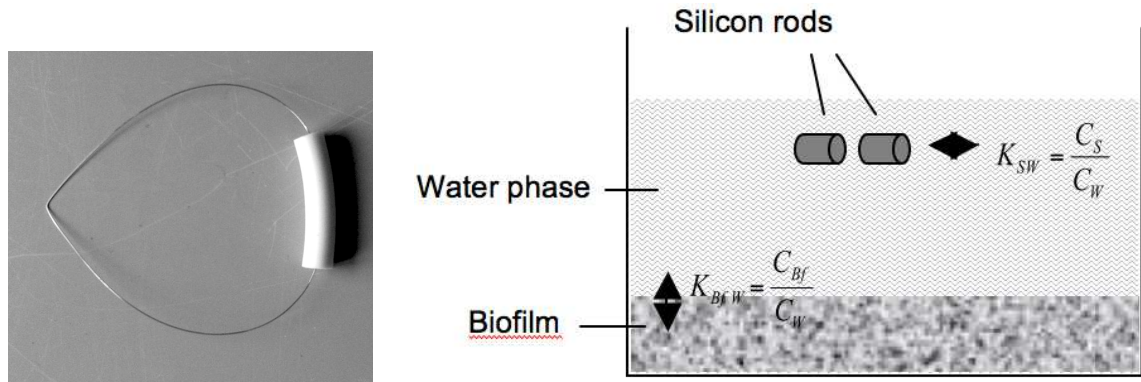
Done with collaboration S. Franz, Helmholtz Centre for Environmental Research, Department Bioanalytical Ecotoxicology, Germany.

To investigate the possible effect of triclosan on growth and biostabilisation potential of natural freshwater biofilm, triclosan commercial powder was dissolved with 1 % of dimethylsulfoxide (DMSO). In addition, to ensure the best correspondence between planned and actual concentrations, a new dosing technique based on silicone elastomer stirred bars (silicon rods) was used (Chapter 7). According to Bandow et al. (2009a), this dosing technique has a high loading capacity and a fast achievement of equilibrium in the medium establishing the equilibrium of triclosan concentration within the sediment-water-biota system. This approach maintains constant concentrations in the system by the regular compensation of decomposition losses.

Preparation of silicon rods is described in detail by Bandow et al. (2009a, 2009b). Silicon was purchased as a flexible cord with a diameter of 5 mm from Goodfellow Cambridge Ltd. (Huntingdon, U.K.). Rods were produced from this cord by cutting it into 2.52 cm lengths (Figure 2.5) to be loaded to each channel. The silicon rods were cleaned by immersing to the ultrasonic bath with a mixture of acetonitrile and methanol (ratio 80:20) three times for 15 min. The rods were dried for 8 h in an atmosphere of nitrogen at 240°C, and then loaded by soaking each silicon rod in 300  $\mu\text{l}$  of a solution of triclosan in hexane. The solvent was completely removed by gently blowing nitrogen over them for 20 min and by heating them for 2 h at 30°C. Amounts of triclosan necessary to reach intended water concentrations in the water channels (2  $\mu\text{g l}^{-1}$  to 150  $\mu\text{g l}^{-1}$ ) were estimated assuming equilibrium partitioning between the silicon rods, water and biofilm (Figure 2.5) and calculated using Equation 2.6.

The total mass of triclosan in the system at equilibrium equals:

$$m_{Load} = m_{Bf} + m_w + m_s = const. \quad \text{Equation 2.6}$$



**Figure 2.5:** A flexible silicon rod (left) and a schematic diagram of system with equilibrium (right).

where  $m_{Load}$  is total mass of triclosan loaded onto silicon rods,  $m_{Bf}$  is a mass of triclosan in the biofilm at equilibrium,  $m_W$  is a mass of triclosan in the water phase at equilibrium and  $m_S$  is a mass of triclosan in silicon rods at equilibrium.

Partitioning between the silicon rods and the water phase at equilibrium can be found as (Bandow et al. 2009b):

$$K_{SW} = \frac{C_S}{C_W} = \frac{m_S}{m_W} \frac{V_W}{V_S} \quad \text{Equation 2.7}$$

where  $C_S$  is the concentration of triclosan in the silicon rod,  $C_W$  is the concentration of triclosan in the water phase,  $V_W$  is the volume of water phase and  $V_S$  is volume of silicon rods.

The loaded amount of TCS per rod varied between 9.6  $\mu\text{g}$  and 724  $\mu\text{g}$ , then depending on the intended concentrations of TCS 14 silicon rods were prepared for each channel.

## 2.12. Sediment stability

### 2.12.1. Cohesive Strength Meter

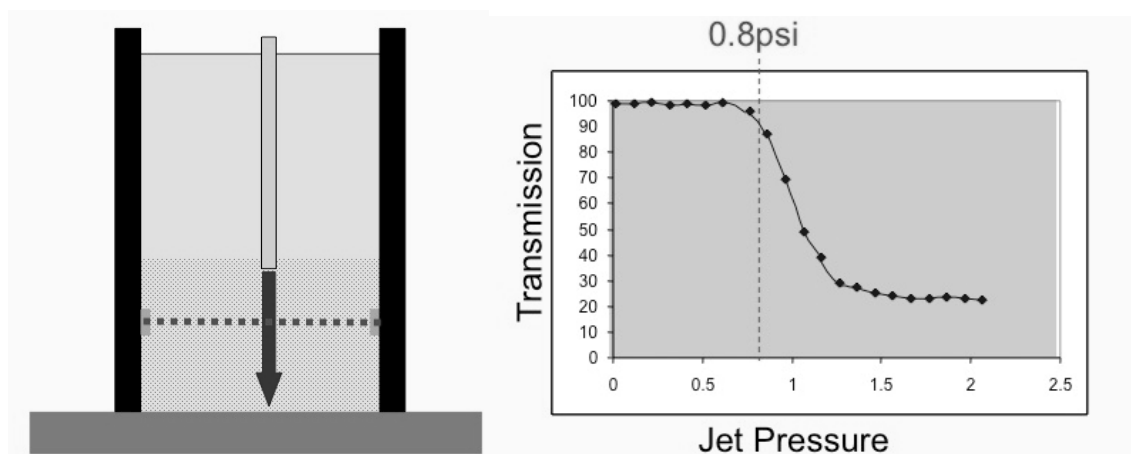
The substratum stability was determined using the Cohesive Strength Meter (CSM), a well-established technique, which allows for the rapid measurement of the erosion threshold of exposed sediments (Figure 2.6). This device was firstly described by Paterson (1989) and the sensitivity and calibration of the CSM was further improved by Tolhurst et al. (1999). The device consists of a water filled test chamber 30 mm in diameter, placed into the sediment (Figure 2.7 left).

A jet of water is expelled within the chamber towards the surface sediments. The velocity of the jet increases with each pulse until the bed fails (Vardy et al. 2007) and sediment is resuspended within the chamber.



**Figure 2.6:** *The Cohesive Strength Meter erosion device.*

The CSM system records changes in light transmission within the chamber and a 10% drop in transmission from the original undisturbed bed is taken as the indication of resuspension and erosion (Figure 2.7 right) (Tolhurst et al. 1999, Vardy et al. 2007).



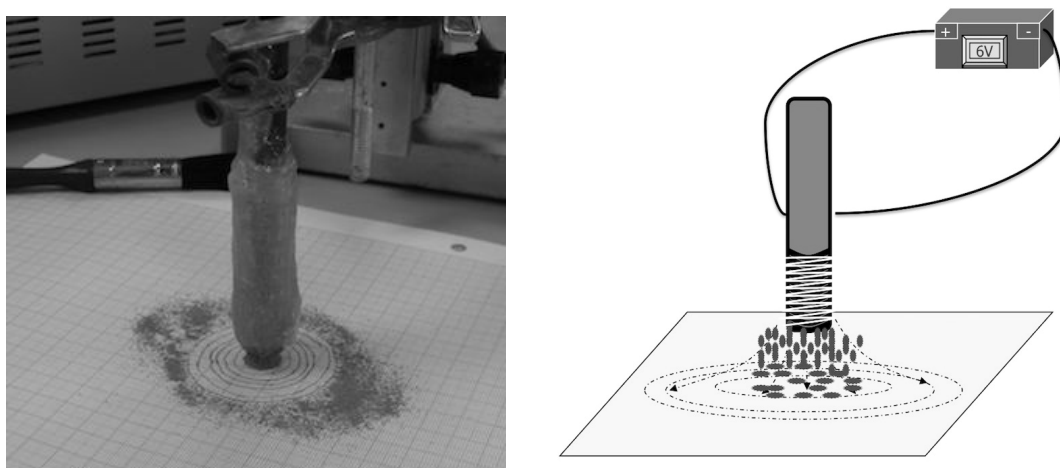
**Figure 2.7:** *The schematic diagram of CSM (left) and principle of the threshold measurements (right).*



The CSM program “Fine 1” was used as it offered the most appropriate gradual increase in pressure steps over time. The relative substratum stability was expressed as stagnation pressure at the bed surface ( $\text{N m}^{-2}$ ) causing a 10% decrease in transmission and was measured at regular intervals over the experimental period.

### 2.12.2. Magnetic Particle Induction

The adhesive property of the biofilms was studied with a new method based on the magnetic attraction of specially produced test particles. This method is suitable for recording changes of sediment surface adhesion and is described in detail within Chapter 3. Briefly, for the methodology presented here, two types of magnets were used: permanent magnets and electromagnets (Figure 2.8). In both cases a known amount of ferromagnetic fluorescent particles were distributed over the sediment surface. Then the particles were recaptured by magnetic force. The relative force required to remove the particles was used to assess the sediment adhesion.



**Figure 2.8:** *The Magnetic Particle Induction device (left) and schematic diagram of its operation (right).*

The voltage applied to electromagnet was increased gradually and the response of particles to the increasing powerful magnetic field recorded. The forces required for total removal of particles under the magnet were determined as measuring of the surface adhesion, were further calibrated by Hall probe (as described in Chapter 3) and results reported in mTesla (Larson et al. 2009). The mechanical properties of the biofilm were studied in parallel to the CSM measurements over the experimental period.

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## References

- Alm, E. W., D. B. Oerther, N. Larsen, D. A. Stahl, and L. Raskin. 1996. The oligonucleotide database. *Applied and Environmental Microbiology* **62**:3557-3559.
- Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisholm, R. Devereux, D. A. Stahl, and 1990. Combination of 16S ribosomal rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial population. *Applied and Environmental Microbiology* **56**:1919-1925.
- Aspden, R. J. PhD thesis, 2005. Studies on the biogenic mediation of sediment dynamics in coastal system. University of St. Andrews, St. Andrews.
- Bandow, N., R. Altenburger, U. Lubcke-von Varel, A. Paschke, G. Streck, and W. Brack. 2009a. Partitioning-Based Dosing: An Approach To Include Bioavailability in the Effect-Directed Analysis of Contaminated Sediment Samples. *Environmental Science & Technology* **43**:3891-3896.
- Bandow, N., R. Altenburger, G. Streck, and W. Brack. 2009b. Effect-Directed Analysis of Contaminated Sediments with Partition-Based Dosing Using Green Algae Cell Multiplication Inhibition. *Environmental Science & Technology* **43**:7343-7349.
- BMEPC. 1988. Guidelines for the Baltic monitoring programme for the third stage. Loose sheet version of the Baltic Sea environments proceedings. Baltic Marine Environment Protection Commission **27D**:16-23.
- Cho, B. C. and F. Azam. 1990. Biogeochemical significance of bacterial biomass in the oceans euphotic zone. *Marine Ecology-Progress Series* **63**:253-259.
- Daims, H., A. Bruhl, R. Amann, K. H. Schleifer, and M. Wagner. 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: development and evaluation of a more comprehensive probe. *Systematic and Applied Microbiology* **22**:434-444.
- Dubois, M., K. A. Gilles, J. K. Hemilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry* **28**:380-356.
- Fuhrman, J. A. and F. Azam. 1982. Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters-evaluation and field result. *Marine Biology* **66**:109-120.
- Garet, M. J. and D. J. W. Moriarty. 1996. Acid extraction of tritium label from bacterial DNA in clay sediment. *Journal of Microbiological Methods* **25**:1-4.
- Genty, B., J. M. Briantais, and N. R. Baker. 1989. The relationship between the quantum yield of photosynthetic electron- transport and quenching of chlorophyll fluorescence. *Biochimica Et Biophysica Acta* **990**:87-92.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Gerbersdorf, S. U., W. Manz, and D. M. Paterson. 2008. The engineering potential of natural benthic bacterial assemblages in terms of the erosion resistance of sediments. *Fems Microbiology Ecology* **66**:282-294.
- Honeywill, C., D. N. Paterson, and S. E. Hagerthey. 2002. Determination of microphytobenthic biomass using pulse-amplitude modulated minimum fluorescence. *European Journal of Phycology* **37**:485-492.

- Hubas, C., L. F. Artigas, and D. Davoult. 2007a. Role of the bacterial community in the annual benthic metabolism of two contrasted temperate intertidal sites (Roscoff Aber Bay, France). *Marine Ecology-Progress Series* **344**:39-48.
- Hubas, C., D. Lamy, L. F. Artigas, and D. Davoult. 2007b. Seasonal variability of intertidal bacterial metabolism and growth efficiency in an exposed sandy beach during low tide *Marine Biology* **151**:41-52.
- Hubas, C., C. Sachidhanandam, H. Rybarczyk, H. Lubarsky, A. Rigaux, T. Moens, and D. M. Paterson. 2010. Bacterivorous nematodes stimulate microbial growth and exopolymer production in marine sediments microcosms. *Marine Ecology Progress Series* **419**:85-94.
- Jeffrey, S. W., S. W. Wright, and M. Zapata. 1999. Recent advances in HPLC pigment analysis of phytoplankton. *Marine and Freshwater Research* **50**:879-896.
- Krammer, K. and H. Lange-Bertalot. 1986-1991. Süßwasserflora von Mitteleuropa. *in* H. Ettl, Gerloff, H., Heynig, H., Mollenhauer, D., editor. Bacillariophyceae. Gustav Fischer Verlag, Stuttgart.
- Lange-Bertalot, H. 1997. A first ecological evaluation of the diatom flora in central Europe. *Lauterbornia*:117-123.
- Larson, F., H. Lubarsky, S. U. Gerbersdorf, and D. M. Paterson. 2009. Surface adhesion measurements in aquatic biofilms using magnetic particle induction: MagPI. *Limnology and Oceanography-Methods* **7**:490-497.
- Lee, S. and J. A. Fuhrman. 1987. Relationships between biovolume and biomass of naturally derived marine bacterioplankton. *Applied and Environmental Microbiology* **53**:1298-1303.
- Manz, W. 1999. In situ analysis of microbial biofilms by rRNA-targeted oligonucleotide probing. *Methods in Enzymology* **310**:79-91.
- Manz, W., R. Amann, W. Ludwig, M. Vancanneyt, and K. H. Schleifer. 1996. Application of a suite of 16S rRNA-specific oligonucleotide probes designed to investigate bacteria of the phylum *Cytophaga- Flavobacter- Bacteriodes* in the natural environment. *Microbiology-UK* **142**:1097-1106.
- Manz, W., R. Amann, W. Ludwig, M. Wagner, and K. H. Schleifer. 1992. Phylogenetic oligodeoxynucleotide probes for the major subclasses of proteobacteria: problems and solutions. *Systematic and Applied Microbiology* **15**:593-600.
- McClellan, K., R. Altenburger, and M. Schmitt-Jansen. 2008. Pollution-induced community tolerance as a measure of species interaction in toxicity assessment. *Journal of Applied Ecology* **45**:1514-1522.
- Moens, T. and M. Vincx. 1998. On the cultivation of free-living marine and estuarine nematodes. *Helgolander Meeresuntersuchungen* **52**:115-139.
- Neef, A. 1997. Anwendung der *in situ* Einzelzell-Identifizierung von Bakterien zur Populationsanalyse in komplexen mikrobiellen Biozosen. Technische Universität München, München.
- Pankow, H. 1990. Ostsee-Algenflora. Gustav Fischer Verlag, Jena.
- Paterson, D. M. 1989. Short-term changes in the erodibility of intertidal cohesive sediments related to the migratory behaviour of epipelagic diatoms. *Limnology and Oceanography* **34**:223-234.
- Paterson, D. M. 1995. Biogenic structure of early sediment fabric visualized by low temperature scanning electron microscopy. *Journal of the Geological Society* **152**:131-140.

- Perkins, R. G., I. R. Davidson, D. M. Paterson, H. Sun, J. Watson, and M. A. Player. 2006. Low-temperature SEM imaging of polymer structure in engineered and natural sediments and the implications regarding stability. *Geoderma* **134**:48-55.
- Rabus, R., M. Fukui, H. Wilkes, and F. Widdel. 1996. Degradative capacities and 16S rRNA-targeted whole-cell hybridization of sulfate-reducing bacteria in an anaerobic enrichment culture utilizing alkylbenzenes from crude oil. *Applied and Environmental Microbiology* **62**:3605-3613.
- Raunkjaer, K., T. Hvitvedjacobsen, and P. H. Nielsen. 1994. Measurement of pools of protein, carbohydrate and lipid in domestic waste- water. *Water Research* **28**:251-262.
- Ribalet, F., L. Intertaglia, P. Lebaron, and R. Casotti. 2008. Differential effect of three polyunsaturated aldehydes on marine bacterial isolates. *Aquatic Toxicology* **86**:249-255.
- Roller, C., M. Wagner, R. Amann, W. Ludwig, and K. H. Schleifer. 1994. In situ probing of Gram-positive bacteria with high DNA G+C content using 235 ribosomal-targeted oligonucleotides. *Microbiology-Sgm* **140**:2849-2858.
- Schmitt-Jansen, M. and R. Altenburger. 2008. Community-level microalgal toxicity assessment by multiwavelength-excitation PAM fluorometry. *Aquatic Toxicology* **86**:49-58.
- Schreiber, U., U. Schliwa, and W. Bilger. 1986. Continuous recording of photochemical and nonphotochemical chlorophyll fluorescence quenching with a new type of modulation fluorometer. *Photosynthesis Research* **10**:51-62.
- Simonsen, R. 1962. Untersuchungen zur Systematik und Ökologie der Bodendiatomeen der westlichen Ostsee. *Internat. Revue ges. Hydrobiol.* **1**:1-44.
- Tolhurst, T. J., K. S. Black, S. A. Shayler, S. Mather, I. Black, K. Baker, and D. M. Paterson. 1999. Measuring the in situ erosion shear stress of intertidal sediments with the Cohesive Strength Meter (CSM). *Estuarine Coastal and Shelf Science* **49**:281-294.
- Underwood, G. J. C., J. Phillips, and K. Saunders. 1998. Distribution of estuarine benthic diatom species along salinity and nutrient gradients. *European Journal of Phycology* **33**:173-183.
- Vardy, S., J. E. Saunders, T. J. Tolhurst, P. A. Davies, and D. M. Paterson. 2007. Calibration of the high-pressure cohesive strength meter (CSM). *Continental Shelf Research* **27**:1190-1199.
- Witkowski, A., H. Lange-Bertalot, and D. Metzeltin. 2000. Diatom flora of marine coasts I. Pages 1-925 in H. Lange-Bertalot, editor. *Iconographia Diatomologica*. A.R.G. Gantner Verlag K.G.

## Chapter 3

### Methods and Tools for Sediment Stability Assessment

#### Abstract

Natural sediment stability is a product of interacting physical and biological factors, and whilst stability can be measured, few techniques allow sensitive assessment of the sediment surface as conditions change. For example, stability gradually increases as a biofilm develops or as salinity rises or it might be influenced by environmental context such as the presence of toxic compounds. In this chapter a new technique is introduced-magnetic particle induction (MagPI), based on the magnetic attraction of specially produced fluorescent ferrous particles (ParTrac, UK). The test particles were added to a surface and subjected to an incrementally increasing magnetic field produced by permanent magnets or electromagnets (section 3.5).

There was a strong correlation found between magnetic flux density (mTesla) and distance from the surface ( $r^2=0.99$ ) for permanent magnets and between magnetic flux density and the current supplied to an electromagnet ( $r^2>0.95$ ) held at a set distance from the surface. The magnetic force at which the particles are recaptured is determined as a measure of surface adhesion.

MagPI therefore determines the “stickiness” of the surface, whether a biofilm,

sediment, or other material. The average magnetic flux density required to remove test particles from diatom biofilms (15.5 mTesla) was significantly greater than from cyanobacterial biofilms (10 mTesla). Removing particles from a control bed of fine glass beads required very little force (2.2 mTesla). Surface adhesion is an important bed property reflecting the sediment system's potential to capture and retain new particles and accumulate material. MagPI offers a straightforward and economic way to determine the surface adhesion of a variety of surfaces rapidly and with precision. The technique may have applications in physical, environmental, and biomedical research.

### 3.1. Introduction

Biofilms are close to omnipresent in aquatic systems and also important across many academic disciplines including medical research (Morton et al. 1998, Jain et al. 2007, Guo et al. 2008), waste-water treatment (Liu and Fang 2003, Raszka et al. 2006), toxicant removal (Sheng et al. 2008) and biotechnology (Sutherland et al. 1998, Flemming and Wingender 2001). Considerable interest has focused on the importance of biofilms for enhancing sediment stability, largely by the microbially produced matrix of extracellular polymeric substances (EPS) that increases sediment resistance to physical force (Paterson et al. 2000, Stal 2003, Underwood and Paterson 2003, Tolhurst et al. 2008). Sediment stability is a governing factor in sediment management because sediment transport and the release of associated contaminants have important consequences for the ecological and commercial health of aquatic habitats from the watershed to the sea (Paterson et al. 2000). To assess the potential for sediments to erode under hydrodynamic forcing, several devices have been developed to determine the critical erosion threshold (Tolhurst et al. 2000) and the erosion rates of natural sediments (Amos et al. 2010). These laboratory or *in situ* devices are based on many different approaches including water flow (McNeil et al. 1996, Kern et al. 1999) or water jets impacting the surface (Paterson 1989), or the oscillation of a horizontal grid (Tsai and Lick 1986), a spinning propeller (Schuenemann and Kuehl 1991), and combined suction and flow (Gust and Mueller 1997). These methods provide relative information on the erosional behaviour of the sediment in response to hydrodynamic forcing, but all require that bed failure occurs

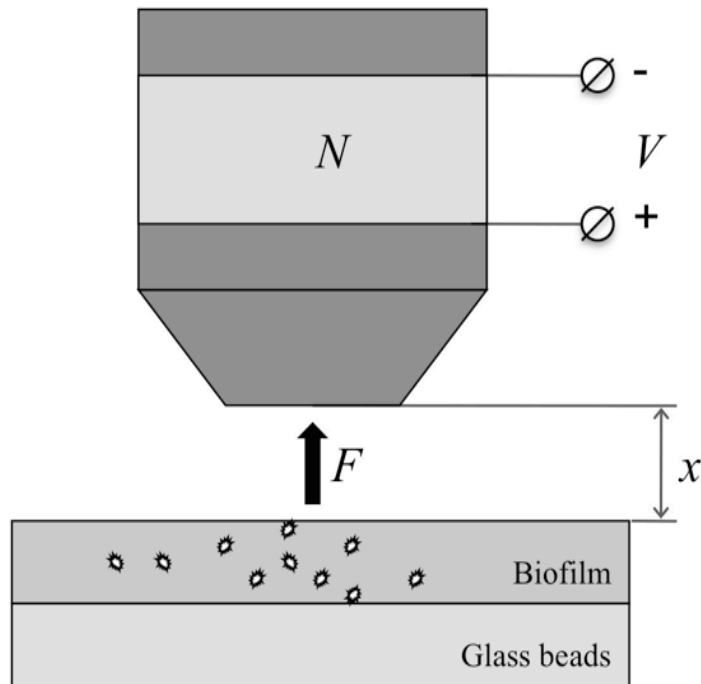
and the sensitivity of each technique is very much dependent on the conformation of the device, the area of eroding stress applied, the volume of the capture area, the sensitivity of the detector and the rate of flow (Tolhurst et al. 2000). These devices are therefore extremely useful but cannot measure any fine changes in surface properties below the point of incipient erosion. This restricts their use when it comes to determining subtle differences in surface properties and consequently more sensitive methods are needed. MagPI is not suggested as a replacement for current erosion devices but provides a tool to collect the relatively sensitive data of surface properties (adhesive capacity) influenced by cohesion and biofilm development.

The use of magnetism in bacterial biofilm research is well developed: Magnetic resonance imaging (MRI) has been used to visualise structure and detachment of biofilms (Manz et al. 2005, McLean et al. 2008), while surface bio-magnetism was used to change cell adhesion and protein secretion (Chua and Yeo 2005). Immobilisation of magnetic particles by aggregates of pathogenic bacteria has been employed to assess biofilm formation in microtitre plates (Chavant et al. 2007). The method presented here is a development of an original concept by Prof David Paterson based on the finding that the force needed to retrieve magnetic particles from a biofilm is a sensitive indicator of retentive capacity of the substratum. The degree to which the retraction of the magnetic particles from a biofilm is possible is a measure of the surface adhesion potential and a proxy for cohesion/sediment stability. It is also an ideal index for other well-known features of a biofilm such as the potential to capture pollutants, the binding of nutrients or the incorporation of deposited sediment particles (possibly an additional and cumulative stabilisation effect). The method presented here (Magnetic Particle Induction, MagPI) describes the use of both permanent magnets and electromagnets. In both cases, a defined volume of magnetic particles of a known size range was spread onto a defined area of the submerged sediment surface and the magnetic force applied to the particles until the particles were recaptured. The magnetic force was gradually increased by either reducing the distance between the magnet and the magnetic particles (permanent magnet) or by increasing the electrical current to a variable magnet statically positioned 5-10 mm above the sediment (electromagnet). The sensitivity of this method in developing microalgal (diatoms and cyanobacteria) biofilms is shown by the data presented. The

magnetic devices are cheap, easy to build and the calibration by use of Gauss Meters allows for inter-comparison of results gained in different experiments. The relative merits and use of the two types of magnets (field, laboratory) are also discussed in further detail.

### 3.2. Magnetic Particle Induction: Fundamental Principles

The magnetic field, created by magnetic materials or motion charges, at any given point can be specified by both a direction and a magnitude (or strength). Ferromagnetic objects or particles placed within the magnetic field experience a force that can lead to the movement of the object (Figure 3.1).



**Figure 3.1:** Schematic diagram of experimental setup for the magnetic particle induction device, where  $F$  is a magnetic force,  $x$  is a distance between magnet and tested surface,  $V$  is a voltage control and  $N$  is the number of turns of wire around the core.

The main part of the setup is a voltage-controlled electromagnet designed to create an electromagnetic field strong enough to displace particles captured in biofilms. The force ( $F$ ) needed to lift the ferromagnetic particles depends on the magnetic flux density and can be determined as



$$F = \frac{B^2 A}{2\mu_0} \quad \text{Equation 3.1}$$

where  $B$  is the magnetic flux density,  $A$  is the area of the pole faces and  $\mu_0$  is the permeability of free space ( $\mu_0 = 4\pi \times 10^{-7}$  [N A<sup>-2</sup>]). The magnetic flux density at the end solenoid near the centre is proportional to

$$B = \frac{\mu NI}{L} \quad \text{Equation 3.2}$$

where  $N$  is the number of turns of wire around the core,  $I$  is the current,  $L$  is the length of the magnetic circuit and  $\mu$  is permeability of the magnetic core that can be found as

$$\mu = \mu_0 \times \mu_r \quad \text{Equation 3.3}$$

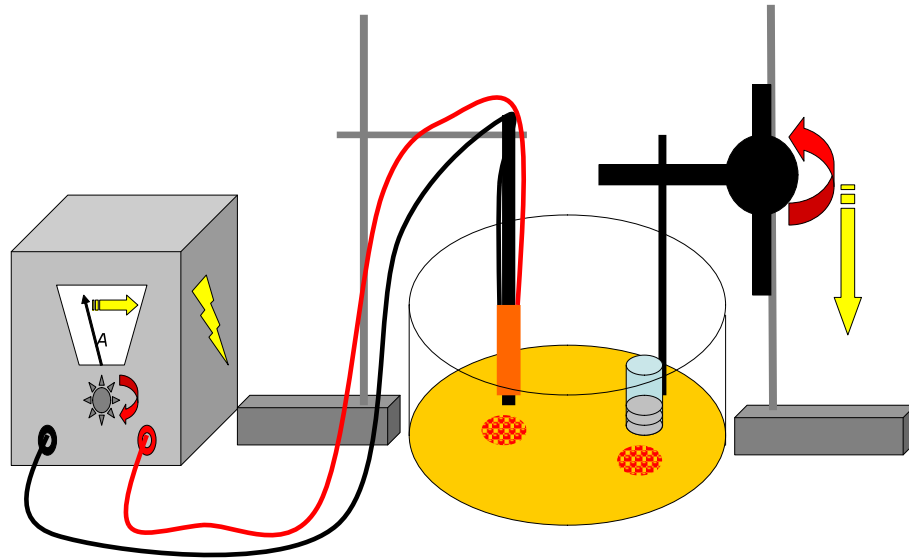
where  $\mu_r$  is the relative permeability of the material of core. In our experiment steel cores were used ( $\mu_r = 8.75 \times 10^4$  [NA<sup>-2</sup>]). Thus, the maximum force can be determine from equation

$$F = \frac{\mu^2 N^2 I^2 A}{2\mu_0 L^2} \quad \text{Equation 3.4}$$

The force ( $F$ ) applied to the particle can be controlled by varying the voltage ( $V$ ) or the distance ( $x$ ). Design of electromagnet depends on the material and the diameter of a core ( $D$ ), length of magnetic circuit and number of turns of wire around the core, while all other factors are held constant.

### 3.3. Experimental set-up

Two types of magnets were used: permanent magnets and electromagnets (Figure 3.2).



**Figure 3.2:** Schematics of experimental setup. Two variants of MagPI are shown. The electromagnet on the left and the permanent magnet on the right.

### 3.3.1. Permanent neodymium magnets

After extensive testing of a variety of permanent magnets, neodymium alloy ( $\text{NdFe}_3\text{B}$ ) disc magnets were chosen for their superior magnetic strength. Neodymium (Nd) is the most magnetic element found on earth (Lebech et al. 1975, Coey 1995). The Nd-magnets used were discs of the size 20×5 mm (E-magnets, UK), and were used either individually or as a stack of five, depending on strength requirements. Adding any more than five discs gave no further increase in magnetic strength, since the extra discs were too far away to influence the active magnetic field reaching the test surface. The force from the permanent magnets acting upon the surface was regulated using distance to the surface, controlled by an adjustable vernier-scaled manipulator (Figure 3.2).

### 3.3.2. Electromagnets

Electromagnets were used, combined with a precision power supply to allow fine control of voltage and current supply, (Rapid 5000 variable power supply) (Figure 3.2). A wide range of commercially available electromagnets was tested, but none showed the required functionality. The most common problems being that magnets were too big, obscuring the surface below, or that they were too weak to be able to retract the added particles from the different test surfaces. Bespoke electromagnets

were therefore constructed from metal rods of ferrous alloy that were coiled with insulated copper thread. To widen the overall range of the magnetic field, two sizes of magnets were produced: “the strong” and “the weak”, the properties of these electromagnets are presented in Table 3.1.

**Table 1** *Properties of magnets used in experiments*

	<b><math>D</math>, m</b>	<b><math>d_1</math>, mm</b>	<b><math>N</math></b>	<b><math>L</math>, m</b>
<b>Strong</b>	0.01	0.375	500	0.1
<b>Weak</b>	0.004	0.13	1540	0.1

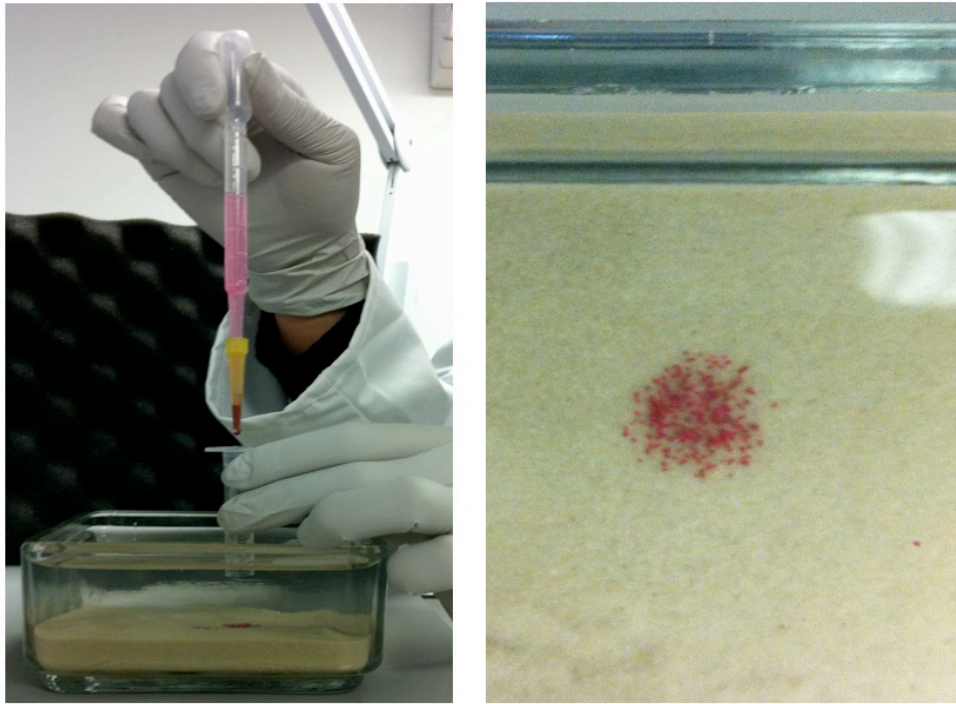
Where  $D$  is diameter of a core,  $d_1$  is diameter of a wire,  $N$  is number of turns of wire around the core and  $L$  is length of magnetic circuit.

The coil was spread over a length of 100 mm on both magnets. The full coil resistance of the larger electromagnet was 35  $\Omega$ , and it was limited to a power input range of 0-12 V (0-3.4 A). The smaller magnet had a coil resistance of 24  $\Omega$  and was limited to a power input range of 0-20 V (0-1.8 A). Exceeding these limits burnt the coils, since at this level of supply an increased current is dissipated as heat due to resistance.

### 3.3.3. Ferrous particles

Particles were produced from an amalgam of ferrous material to provide a magnetic response, mixed with fluorescent pigment to increase their visibility. An inert transparent binding agent combines the material into a solid, which is then ground to produce a particle spectrum (ParTrac, UK). The test particles were then sieved into different size classes. The size range selected for the trials was 180-250  $\mu\text{m}$ , similar to fine/medium beach sand. The particles have to be applied to the test surface in a consistent manner to allow repeatable measurements. To achieve a relatively even, single layer of particles on the test surface took practice but was achieved with experience. The test particles were suspended in water, and the mixture was drawn into a plastic pipette (Figure 3.3). The suspended particles were allowed to settle toward the tip of the pipette before being ejected as a single drop in the media above the surface. A cutoff 2 ml syringe, submerged into the water and held at short distance

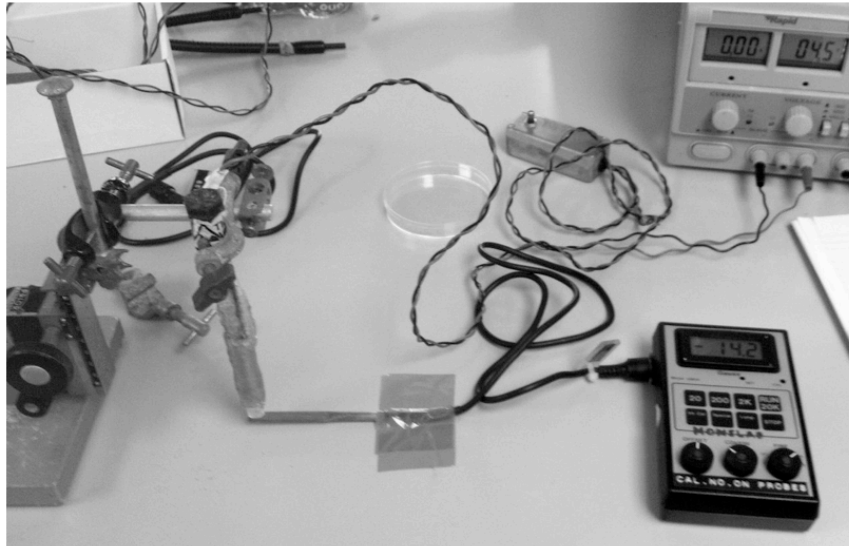
above the test surface, was served as a guide to confine the particles to the selected test area.



**Figure 3.3:** *The suspension particles procedure.*

### **3.4. Calibrations**

To calibrate the device magnets were placed over a Hall sensor connected to a Gauss Meter (Unilab, Blackburn, England) (Figure 3.4). The permanent magnets were lowered toward the probe in small incremental steps (1 mm), as applied for a test measurement. The magnetic field flux in mTesla was recorded for each step. For the electromagnets, the voltage and current were increased in small increments (0.2 V/0.05 A) and the mTesla for each increase was recorded. The Hall sensor calibrations were performed in air as well as submerged in water using a waterproof sensor. Calibrations were performed both before and after an experiment. During the experiment, the resistance of electromagnets was regularly checked. A decrease in coil resistance would be evidence of a fault and thereby a loss in magnetic field strength.



**Figure 3.4:** *The calibration process using Hall probe.*

### 3.5. Measuring procedure

The magnetic measurements were initiated immediately after the application of the particles. The magnet was lowered into position a set distance above the test surface (Figure 3.5). The distance to the test surface is critical; to insure correct placement, a small guide rod (glass or plastic, not metal) was attached to the end of magnet to set the distance to the surface consistently (usually 10 mm). The magnet was lowered until the tip of the guide just contacted the test surface. This could be checked by use of a magnifying glass. The magnetic field was increased in small incremental steps.



**Figure 3.5:** *Measuring procedure.*

Four stages of particle response were noted. The first stage: (I) is when the particles show initial orientation (alignment) along the magnetic field; (II) the first particles are retracted to the magnet; (III) large groups of particles are attracted to the magnet; and finally (IV) total clearance of the surface from particles under the magnet. The least subjective points are the first and last ones. If the replicate is intended for repeated measurements, after total clearance, all particles accidentally left outside the area affected by the magnet should be cleaned from the surface with a permanent magnet to prevent compromising subsequent measurements.

### 3.6. Precision and statistics

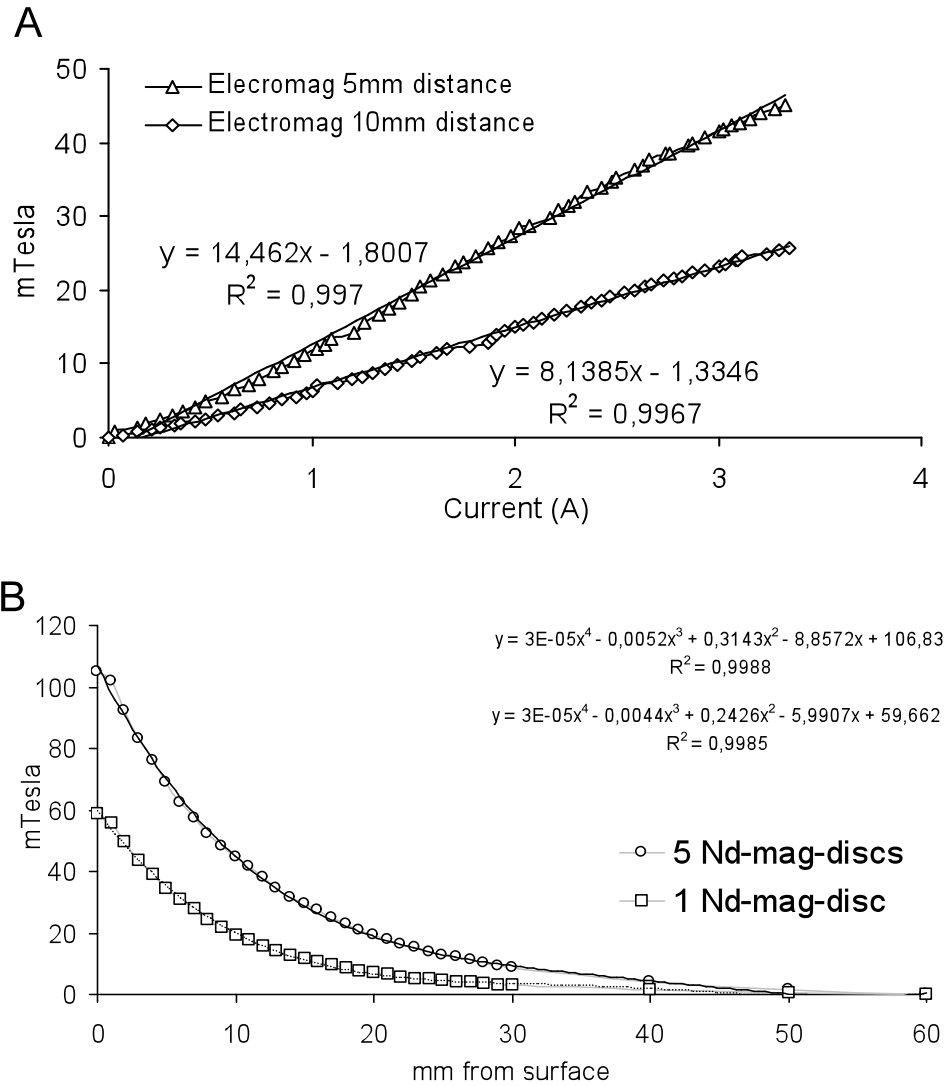
The precision of the method was tested through repeated calibrations ( $n=25$ ). Based on 95% confidence intervals and average precision of 0.1% for the electromagnet measurements was determined ( $\pm 0.22\%$  in the low current range,  $\pm 0.35\%$ , in the mid-current range and  $\pm 0.45\%$  in the high current range). The use of a different electromagnets and/or other power source requires a separate precision test to be conducted, but as long as a suitably sensitive power supply is used, a similar range could be expected. Data were assessed for normality and homogeneity of variance, and a one-way ANOVA was applied (significance level  $\alpha=0.05$ ) and post-hoc test (Tukey) to determine differences in surface adhesion between varying surface and biofilm compositions.

## 3.7. Results

### 3.7.1. Calibration

Electromagnets provide strong linear relationships ( $r^2=0.996-0.997$ ) between current ( $I$ ) and the magnetic flux density (mTesla) (Figure 3.6 A), whereas the permanent magnets showed an exponential relationship (Figure 3.6 B). In contrast to the electromagnets, the permanent magnets have to be moved toward the surface during the measurement to increase  $F$ . Consequently, the area of the magnetic field that interacts with the surface increases with decreasing distance, and this corresponds to a nonlinear increase of field strength (Figure 3.6 B). The line of best fit for the

calibration of the permanent magnet strength versus distance required a sixth-order polynomial as opposed to the linear function used for the electromagnet calibration.

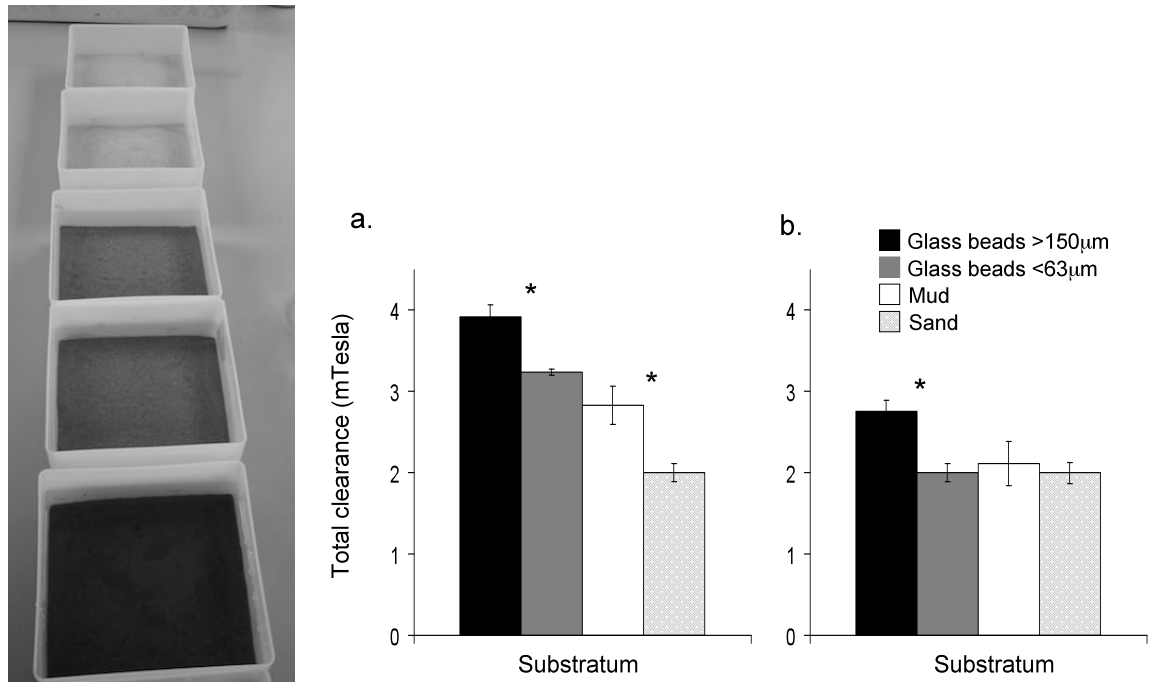


**Figure 3.6:** Examples of calibration curves for the electromagnetic (A) and permanent magnet (B) devices.

### 3.7.2. Abiotic particulate surfaces

Different substrata were tested during the pilot studies. Firstly clean glass beads of two sizes (<63  $\mu\text{m}$  and >150  $\mu\text{m}$  Ballotini beads), sand and mud (both heat-treated to remove organic material). The surfaces were submerged in seawater and freshwater to compare abiotic particle responses and account for ionic interactions. The two size groups (180-250  $\mu\text{m}$  and 425-500  $\mu\text{m}$ ) of magnetic particles were used throughout

the tests. This provided background knowledge of the adhesion properties of common abiotic substrata of variable particle size.



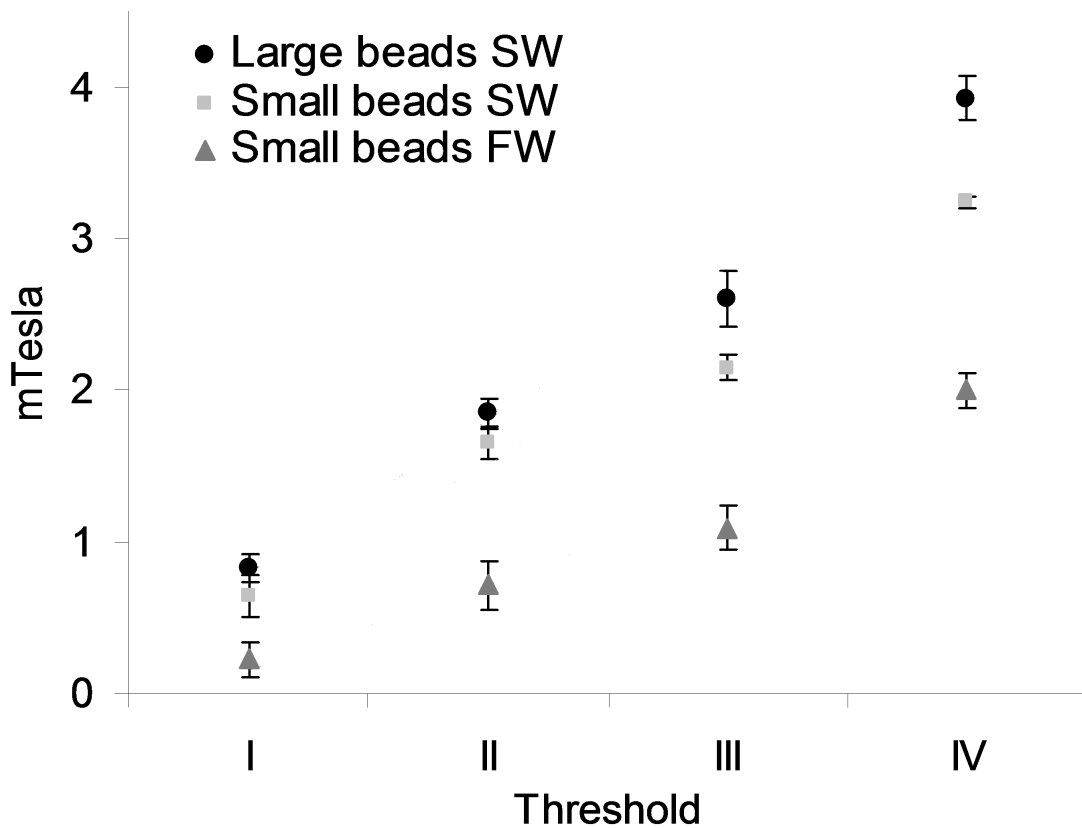
**Figure 3.7** Examples of tested substratum: glass beads, sand and mud (left). Test of abiotic particulate beds of different materials (right) in seawater (a) and in freshwater (b) to attract test particles (180-250 µm) by MagPI (n=6, ±SE). \* Significant difference between adjacent groups by ANOVA,  $\alpha=0.05$ , and Tukey test.

The force required to recapture the test particles (size 180-250 µm) from different surfaces varied between seawater and freshwater conditions (Figure 3.7). Under saline conditions, it was more difficult to capture test particles from the bed composed of larger glass beads than from the smaller glass beads, followed by mud, and then the cleaned sand. Under freshwater conditions, the magnetic force needed to retrieve the test particles was similar for all surfaces except the larger glass beads, which showed a significantly higher retentive capacity (Figure 3.7). For the sand, similar forces were needed to retrieve particles in seawater and freshwater, but relatively greater force had to be applied in seawater to recapture particles from the other substrata (Figure 3.7). However, increasing binding capacity was also noted in freshwater from the large glass bead substratum.



### 3.7.3. Threshold conditions

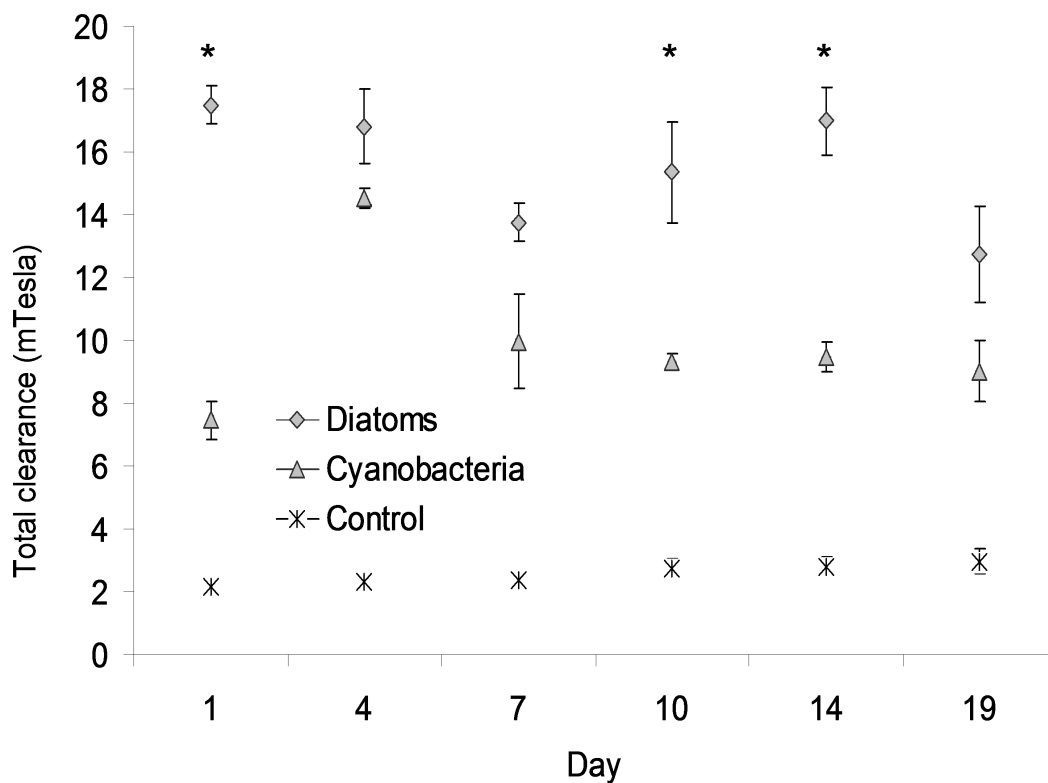
Total clearance (IV) was the preferred measure for threshold condition (Figure 3.8), because this is the least subjective measure and the data gained by different researchers are almost identical. This threshold shows significant differences between treatments that neither were nor always observed using three other thresholds. Under laboratory conditions more sophisticated observation using microscopy of the particles is possible and the first and second threshold measurements can be used as an alternative and complementary value if needed.



**Figure 3.8** The thresholds used in the magnetic measurements: (I) particle orientation to magnetic field; (II) first particles captured by the magnet; (III) larger groups of particles attracted; (IV) total clearance of particles under the magnet. Three treatments are given as examples: small glass beads submerged in seawater (SW) and freshwater (FW) and large glass beads in SW using test particles of size range 180-250  $\mu\text{m}$  ( $n=6$ ,  $\pm\text{SE}$ ).

### 3.7.4. Adhesion due to surface biofilms (Biotic experiment example)

The influence of biotic adhesion was examined using glass beads with diameter of  $<63 \mu\text{m}$  (Ballotini) covered with filtered ( $1 \mu\text{m}$ ) and autoclaved seawater. Biofilms were cultured with benthic cyanobacteria (dominated by *Oscillatoria* spp.) and benthic diatoms (dominated by *Nitzschia* spp.). Both cultures were grown on clean glass beads in a temperature-controlled room ( $15^{\circ}\text{C}$ ) under a 13/11 h light-dark cycle ( $\sim 250 \mu\text{mol m}^{-2} \text{s}^{-1}$ ). Similar glass beads covered with seawater without microorganisms were used as a control group. For the treatments and controls, plastic weighing trays ( $55 \times 55 \times 23$  in mm) were filled with 5 mm layer of the glass beads and filled with seawater. The experimental period lasted 19 days to follow changes in the surface properties of developing biofilm cultures. The “weak” electromagnet described above was employed for these tests. The biotic test experiments revealed that the biofilms developed by benthic diatoms under these conditions had a more adhesive surface than the cyanobacterial biofilms (Figure 3.9).



**Figure 3.9** Biotic example with cultured biofilms grown with diatoms and cyanobacteria. The threshold reported is the strength of the magnetic field needed to provide total clearance of particles ( $n=6$ ,  $\pm$  SE). \* Significant difference between experimental groups by ANOVA,  $\alpha=0.05$ , and Tukey test.

The magnetic flux density in control treatments did not show any variations over experimental period and was below 2 mTesla. The important aspect of this experiment was that the MagPI method was able to detect even quite small differences in surface adhesion with high precision.

### 3.8. Discussion

The equipment required for the method described here is simple and affordable (Figures 3.2 and 3.10 A). Production of suitable electromagnets, however, does demand some technical skill to achieve the acquired magnetic strength.

In the laboratory, electromagnets were preferred over permanent magnets because of the accuracy of their calibration and ease of deployment. Depending on the design and power source, electromagnets offer the possibility to increase the magnetic force in small steps, thus offering a high resolution within the applied magnetic strength range. A fixed distance marker (non-metallic) fitted at the tip of the electromagnet helps to ensure positional accuracy between measurements. However, permanent magnets had a higher strength to compare with electromagnets and can be used for investigation of matured biofilm and are also easier to deploy in the field (e. g., tidal flats) because of the logistical ease for field use and the lack of requirement of an external power source. The permanent magnet still produces an accurate and stable force at each set of distance, although the precise manipulation of the distance between the magnet and the test surface is critical. The vernier scale ( $\pm 100 \mu\text{m}$ ) was then used to move the magnet in small incremental steps, and the results were recorded. The test surface must be set parallel to the magnet face.

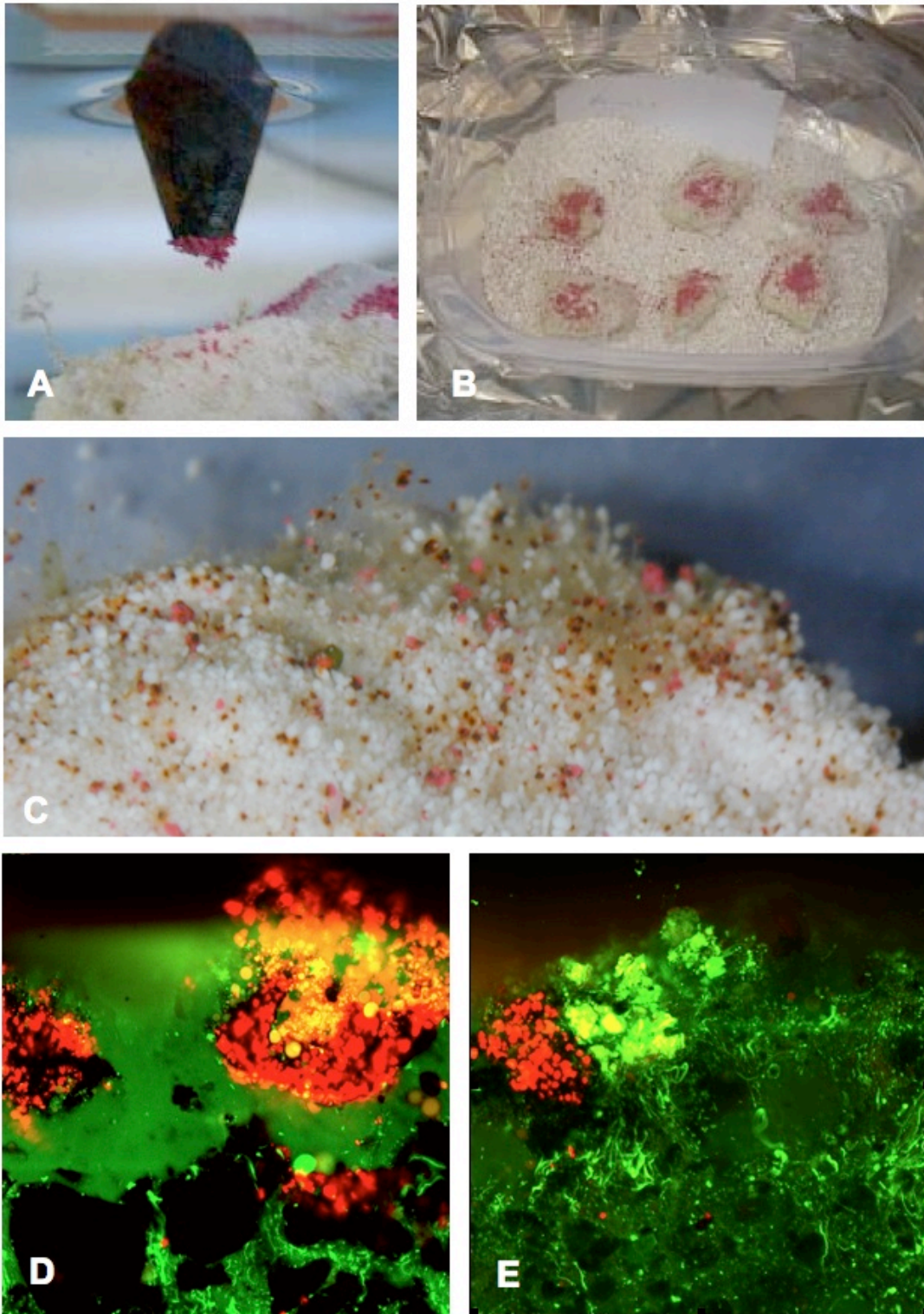
In MagPI experiments magnetic forces needed to be applied to remove particles were also depended on the substratum and size of glass beads. This may be explained by the fact that the magnetic particles become physically trapped between the larger glass beads. Thus, choice of the size of the particles is also very important decision. It is sensible to select a size range of the particles that does not deviate too much from the test sediment, preferably being slightly larger to prevent trapping in surface pore space (Figure 3.10C). Although this type of trapping may not be an issue on surface where biofilm has developed, the test particle size is also important for the easy of observation on the surface. It is also sensible to use a narrow size range of test

particles, to enhance the uniformity of the particle interactions with the surfaces since the force required recapturing the test particles from different surfaces varied between seawater and freshwater conditions. This is probably due to the ionic nature of seawater increasing the potential for electrostatic and other physicochemical attractions between particles (e.g., mud with silt and clay content known for their surface charge variation). This could imply that the ionic milieu facilitates the cohesion of the surface as measured by MagPI. However, both the smaller and larger glass beads showed enhanced surface cohesion in seawater as opposed to freshwater, which suggests both mechanisms may be responsible for the binding capacity of the larger glass beads.

Another variable is the incubation time, or period that particles are left on the test surface before performing the measurement. Because this depends on the characteristics of the investigated surface as well as on the objectives of a particular study, it should be decided by the operator on the basis of the question to be addressed in each experiment.

The simplest way to ensure a repeatable measure of the test surface is to retract particles directly after their addition, and the most appropriate value of the surfaces “stickiness” for our purposes can be gained directly after adding the particles. When particles are left for a longer time, they will be partly or fully incorporated into the biofilm, and the measured variable becomes a combination of the adhesion of the surface and the capacity to entrap particles by biofilm development (Figure 3.10E).

The influence of biotic adhesion using glass beads and axenic diatom culture was firstly examined in present experiment. Results suggest that the biofilms developed by benthic diatoms had a more adhesive surface than the cyanobacterial biofilms. One plausible explanation for this was that the experimental irradiance was relatively high, and cyanobacteria, in this case dominated by *Oscillatoria* spp., tend to prefer lower light levels, thus forcing them deeper into the sediment matrix and reducing surface EPS production. Diatoms, in contrast, are better adapted to higher irradiances. This experiment was further developed and described in details in Chapter 5.



**Figure 3.10:** *MagPI placed above the sediment surface. Test particles can be seen adhering to the magnet (A). Samples prepared for measurement (B). Surface shows fluorescent particles and diatoms (C). Confocal microscopy of fluorescent beads incorporated into the biofilm (D and E). The green coloration represents organic material and the red fluorescence represents the test particles. Images courtesy of Prof. D. M Paterson. Confocal images supplied by Dr. A. Decho.*

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The MagPI methodology introduces an easy way to measure the variable surface adhesion. This provides very useful data for depositional systems such as intertidal flats, shallow submerged sediment systems and stromatolites as obvious examples (Figure 3.10 B). MagPI is also easily applicable for monitoring and detection of changes over time in shallow water biofilm based systems. Analysis of different systems with accompanying data on the composition and density of biofilm organisms will provide useful comparative insights. However the method cannot easily be used if a wet biofilm is not submerged, such as during emersion periods. The measurements have to be performed underwater by the help of a water-filled chamber, otherwise the magnetic particles interact with the surface tension of the water-film and these forces confound the measurement of adhesion.

#### **Declaration**

The results presented in this chapter have formed all or part of the following publication:

- Larson, F., Lubarsky, H., Gerbersdorf, S. U., and Paterson, D. M. (2009). Surface adhesion measurements in aquatic biofilms using magnetic particle induction: MagPI. *Limnology and Oceanography-Methods* 7, 490-497.

## References

- Amos, C. L., G. Umgiesser, C. Ferrarin, C. E. L. Thompson, R. J. S. Whitehouse, T. F. Sutherland, and A. Bergamasco. 2010. The erosion rates of cohesive sediments in Venice lagoon, Italy. *Continental Shelf Research* **30**:859-870.
- Chavant, P., B. Gaillard-Martinié, R. Talon, M. Hebraud, and T. Bernardi. 2007. A new device for rapid evaluation of biofilm formation potential by bacteria. *Journal of Microbiological Methods* **68**:605-612.
- Chua, L. Y. and S. H. Yeo. 2005. Surface bio-magnetism on bacterial cells adhesion and surface proteins secretion. *Colloids and Surfaces B-Biointerfaces* **40**:45-49.
- Coey, J. M. D. 1995. Rare-earth magnets. *Endeavour* **19**:146-151.
- Flemming, H. C. and J. Wingender. 2001. Relevance of microbial extracellular polymeric substances (EPSs) - Part II: Technical aspects. *Water Science and Technology* **43**:9-16.
- Guo, L. H., H. L. Wang, X. D. Liu, and J. Duan. 2008. Identification of protein differences between two clinical isolates of *Streptococcus mutans* by proteomic analysis. *Oral Microbiology and Immunology* **23**:105-111.
- Gust, G. and V. Mueller. 1997. Interfacial hydrodynamics and entrainment functions of currently used erosion devices. Pages 149-175 in N. Burt, R. Parker, and J. Watts, editors. *Cohesive Sediments*. Wiley, Chichester.
- Jain, A., Y. Gupta, R. Agrawal, P. Khare, and S. K. Jain. 2007. Biofilms - A microbial life perspective: A critical review. *Critical Reviews in Therapeutic Drug Carrier Systems* **24**:393-443.
- Kern, U., V. Shuerlein, M. Holzwarth, I. Haag, and B. Westrich. 1999. Ein stromungskanal zur ermittlung der tiefenabhängigen erosionsstabilität von gewässersedimenten: das SETEG system. *Wasserwirtschaft* **89**:72-77.
- Lebech, B., K. A. McEwen, and P. A. Lindgard. 1975. Magnetism in praseodymium-nedymium single-crystal alloys. *Journal of Physics C-Solid State Physics* **8**:1684-1696.
- Liu, H. and H. H. P. Fang. 2003. Hydrogen production from wastewater by acidogenic granular sludge. *Water Science and Technology* **47**:153-158.
- Manz, B., F. Volke, D. Goll, and H. Horn. 2005. Investigation of biofilm structure, flow patterns and detachment with magnetic resonance imaging. *Water Science and Technology* **52**:1-6.
- McLean, J. S., O. N. Ona, and P. D. Majors. 2008. Correlated biofilm imaging, transport and metabolism measurements via combined nuclear magnetic resonance and confocal microscopy. *Isme Journal* **2**:121-131.
- McNeil, J., C. Taylor, and W. Lick. 1996. Measurements of erosion of undisturbed bottom sediments with depth. *Journal of Hydraulic Engineering-Asce* **122**:316-324.
- Morton, L. H. G., D. L. A. Greenway, C. C. Gaylarde, and S. B. Surman. 1998. Consideration of some implications of the resistance of biofilms to biocides. *International Biodeterioration & Biodegradation* **41**:247-259.
- Paterson, D. M. 1989. Short-term changes in the erodibility of intertidal cohesive sediments related to the migratory behavior of epipelagic diatoms. *Limnology And Oceanography* **34**:223-234.
- Paterson, D. M., T. J. Tolhurst, J. A. Kelly, C. Honeywill, E. de Deckere, V. Huet, S. A. Shayler, K. S. Black, J. de Brouwer, and I. Davidson. 2000. Variations in

- sediment properties, Skeffling mudflat, Humber Estuary, UK. *Continental Shelf Research* **20**:1373-1396.
- Raszka, A., M. Chorvatova, and J. Wanner. 2006. The role and significance of extracellular polymers in activated sludge. Part I: Literature review. *Acta Hydrochimica Et Hydrobiologica* **34**:411-424.
- Schuenemann, M. and H. Kuehl. 1991. Experimental investigations of the erosional behaviour of naturally formed mud from the Elbe estuary and adjacent Wadden sea, Germany. Pages 314-330 *in* Nearshore and Estuarine Cohesive Sediment Transport Workshop.
- Sheng, G. P., M. L. Zhang, and H. Q. Yu. 2008. Characterization of adsorption properties of extracellular polymeric substances (EPS) extracted from sludge. *Colloids and Surfaces B-Biointerfaces* **62**:83-90.
- Stal, L. J. 2003. Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. *Geomicrobiology Journal* **20**:463-478.
- Sutherland, T. F., J. Grant, and C. L. Amos. 1998. The Effect of Carbohydrate Production by the Diatom *Nitzschia curvilineata* on the Erodibility of Sediment. *Limnology and Oceanography* **43**:65-72.
- Tolhurst, T. J., K. S. Black, D. M. Paterson, H. J. Mitchener, G. R. Termaat, and S. A. Shayler. 2000. A comparison and measurement standardisation of four in situ devices for determining the erosion shear stress of intertidal sediments. *Continental Shelf Research* **20**:1397-1418.
- Tolhurst, T. J., M. Conalvey, and D. M. Paterson. 2008. Changes in cohesive sediment properties associated with the growth of a diatom biofilm. *Hydrobiologia* **596**:225-239.
- Tsai, C. H. and W. Lick. 1986. A portable device for measuring sediment resuspension. *Journal of Great Lakes Research* **12**:314-321.
- Underwood, G. J. C. and D. M. Paterson. 2003. The importance of extracellular carbohydrate production by marine epipelagic diatoms. Pages 183-240 *Advances In Botanical Research*, Vol 40.



## Chapter 4

### **Interactions between diatoms, cyanobacteria and nematodes in terms of their production of extracellular polymeric substances**

#### **Abstract**

Many organisms grow in close association in surficial sediments. Bacteria, protists (algae, fungi and slime moulds), meiofauna and macrofauna live in close proximity and all produce some form of extracellular polymeric substances (EPS). However the interaction between these groups in terms of polymer production is not yet known, and may be important in terms of the carbon dynamics of the system and as a mechanism of sediment stabilisation. In this chapter, the interactions between two groups of cohabiting organisms (diatoms and bacteria) is examined individually and in association and then in the presence of selected meiofaunal representative (nematodes).

Benthic microalgae, cyanobacteria and bacterial assemblages are recognised as the main producers of extracellular polymeric substances (EPS) in intertidal sediment deposits. Yet little is known about the individual engineering capability of the main biofilm consortia (autotrophic microalgae, cyanobacteria and heterotrophic bacteria), in terms of their contribution to the EPS pool. In the first experiment interaction of

axenic microalgae cultures in terms of their contribution to the EPS pool was examined. Single or combined axenic cultures of two diatoms: *Amphora coffeaeformis* and *Navicula hansenii* in combination with the cyanobacteria *Oscillatoria* species were used in this experiment. The aim of this experiment was to find out whether the combination of two or three species in a biofilm would lead to additive or synergistic effects on the microbial growth and EPS production. The results suggest that coexistence of three of the species had a positive effect on microbial growth and mixed cultures exhibit a greater EPS concentration value than single cultures.

Since neither bacteria nor microalgae exist independently in nature, the aim of the second experiment was to examine the interactions between autotrophic and heterotrophic biofilm consortia in terms of microbial growth, community composition and EPS secretion. Cultures of natural benthic bacteria, microalgae and mixtures of the two, growing on artificial sediments were used in this study. The data suggest that bacterial cultures produce lower EPS carbohydrate than diatom cultures, however they both produced similar levels of EPS protein. In the mixed assemblage, while there was no clear evidence for synergistic or additive effects in terms of the microbial community composition or growth; a synergism in EPS carbohydrate production was found. There was no such effect for EPS protein production.

An additional trophic level was added for the next study. Two cultures including (i.e. *Diplolaimelloides meyli* and *Diplolaimelloides oschei*) were produced in addition to the bacterial and microalgal cultures and grown both separately and simultaneously on non-cohesive acid washed marine sand. The main aim of this study was thus to estimate the effect of nematodes on the EPS production in marine sediment. The study showed a positive impact of bacterivorous nematodes on microbial density and enhancement of the EPS production.

The data from this chapter will be further addressed in Chapter 5, when investigating the stabilisation potential of individual and mixed assemblages of aquatic organisms. This information contributes to the conceptual understanding of the microbial sediment engineering that represents an important ecosystem function and service in aquatic habitats.

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#### 4.1. Introduction

In estuarine systems, wherever light can penetrate with sufficient radiance to support photosynthesis, biofilms mainly consist of unicellular eukaryotic benthic microalgae (microphytobenthos) and cyanobacteria, that grow within the upper several millimetres of illuminated sediments (MacIntyre et al. 1996). However, heterotrophic benthic bacteria are also important members of this community, since neither microalgae nor bacteria exist independently in natural sediment (Gerbersdorf et al. 2009). Such communities produce pronounced biofilms, which may be transient or become fully developed into microbial mats (de Winder et al. 1999). Over the last years, there has been increasing awareness of the importance of biogenic mediation of sediment systems, namely by the mucilaginous matrix of extracellular polymeric substances (EPS) produced by bacteria, microalgae and macrofauna. Organisms produce EPS for different reasons such as feeding, communication and protection of microbial cells from desiccation or toxicants, thus enhancing cell survival and success (Yallop et al. 1994, Decho 2000, Wotton 2004).

EPS consists of a relatively undefined complex of a heterogeneous mixture of mainly polysaccharides, proteins, nucleic acids, lipids and humic acids (Flemming and Wingender 2001) and plays an important and multifunctional role in biofilm ecology (Decho 2000). However due to bacterial degradation, consumption by deposit-feeding invertebrates or removal by overlying water, EPS may be lost from sediments. To quantify these pathways, investigations on the structural and physical properties of EPS are needed to understand its role in the environment (reviewed in Underwood and Paterson 2003).

In intertidal muddy sediments, the biomass of phototrophic microorganisms is supported by the easy availability of nutrients and light (Underwood and Smith 1998). These assemblages are the major primary producers in many aquatic habitats and it has been estimated that diatoms are responsible for approximately 40% of the total global primary production in marine systems (Medlin 2002, Underwood and Paterson 2003). Due to their ability to release and fix organic and inorganic nutrients, microphytobenthos support higher, grazing trophic levels and perform many

important functions in food webs and nutrient dynamics (Miller et al. 1996). Microphytobenthos (MPB) are involved in a number of ecological and evolutionary processes, such as gene flow or bioaccumulation, and may also have a role in water conservation and bio-weathering (Wynn-Williams et al. 1997), however very little information is available at the molecular level about their biology (Falciatore and Bowler 2002), as well as the factors that may control the distribution and ecological response of cyanobacteria and diatoms (Watermann et al. 1999). Investigating the influence different light levels (Defew et al. 2004), temperature (Admiraal 1977, Watermann et al. 1999) or nutrient stress (Admiraal et al. 1982, Villbrandt et al. 1990) on microbial growth, suggest that effects of abiotic factors are complex and coupled, and thus it can be difficult to distinguish these effects (Defew et al. 2004). These factors also may have effect on co-occurrence of species would result in high intraspecific competitions of these populations and a greater capability of survival under stress of one species than another (Admiraal et al. 1984). Admiraal and Peletier (1980) found some species of diatoms were more tolerant to the presence of toxins, and Joseph and Jacob (2010) found that both *Oscillatoria* and *Navicula* were pollution tolerant genera, therefore potentially controlling the relative domination of these populations. This may also be a density dependent effect, such that the dominant species maintain high population densities thereby suppressing the growth of invading species and decreasing the diversity of the population. Thus, in addition to several cases of coexistence of species, there is also a strong tendency towards segregation of some species (Dejong and Admiraal 1984). The diversity response of species to these selective forces may lead to a stable co-occurrence of species or to a gradual shift in abundance (Admiraal et al. 1984). Thus engineering consequences of the co-occurrence of species in terms of their ecological functionality is great of interest. Three species were used in this study (experiment 1): *Navicula hansenii*, *Amphora coffeaeformis* and *Oscillatoria* species as single or combined cultures. *Navicula* species are a dominant genera (44%) in the Eden Estuary, Scotland (Defew et al. 2004). *Amphora coffeaeformis* is a common benthic marine pennate diatom (Round 1990) which has many advantages as a biological model: they are easy to maintain in culture, have a short generation time (< 1 day Round 1990), are single-celled and eukaryotic and give a large population of cloned individuals (Davies et al. 1998). The reason for including benthic cyanobacteria was to investigate the

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difference in the EPS production and surface adhesion produced by bacterial autotroph. A further purpose was to test the hypothesis that higher diversity would lead to increase microbial biomass, EPS production and hence surface adhesion of the biofilm (described in Chapter 5).

While a range of meio- and microorganisms secrete EPS, most studies focused on benthic microalgae as the main EPS producers with carbohydrates as their main product (reviewed in Stal and Walsby 2000, Stal 2003, Underwood and Paterson 2003). Heterotrophic bacteria have been mainly regarded as decomposers of the organic matrix (Goto et al. 2001) and as acting in response to microalgal exudates (Haynes et al. 2007, Bruckner et al. 2008). However, bacteria are able to produce copious amounts of EPS as known from biomedical, biotechnology or industrial fields (Raszka et al. 2006, Jain et al. 2007, Vu et al. 2009).

To-date, there is a common agreement that the co-existence of bacteria and microalgae might be of mutual advantages mainly in terms of nutrient recycling (Goto et al. 2001, Klug 2005). Some microalgal species even seem to depend on the association with certain bacteria groups ("satellite bacteria", Schaefer et al. 2002), and in some pelagic diatoms, the presence of certain bacteria is crucial for their growth and EPS release (Grossart and Simon 2007). Bruckner et al. (2008) suggest that the monomer composition of microalgal EPS carbohydrates varied along with the presence of different bacterial groups. On the other hand, some microalgae species suppress bacteria with polyunsaturated aldehydes that have strong bactericidal effects (Wichard et al. 2005, Ribalet et al. 2008); while bacteria can effectively control microalgal growth and EPS secretion through the release of specific algicidal compounds (Fukami et al. 1997, Kang et al. 2005, Mu et al. 2007, Jung et al. 2008). There is evidence that these bacteria-microalgae interactions are highly species-specific and help to shape the composition of the biofilm assemblages (Boivin et al. 2007), with possible implications for their EPS secretion and ecological function. Presumably, the various bacteria-microalgae interactions are strongly driven by abiotic and biotic conditions from both within and outside of the biofilm. For instance, external nutrient addition can cause shifts within the natural microbial assemblage to influence EPS concentration, EPS composition and sediment stability (Gerbersdorf et al. 2009). Still, the mechanisms and species interactions inducing these shifts in

biofilms are far from understood and nutrients are not the only condition that varies in the environment.

It was hypothesized that the coexistence of bacteria and microalgae might show synergistic effects on EPS secretion, cell growth and the net engineering potential. For this purpose, benthic microbial cultures were isolated from estuarine sediments and were grown, separately (prokaryotic and eukaryotic) and combined (natural diversity), on non-cohesive glass beads over a period of 25 days (experiment 2). The data presented on this chapter, compared the individual and combined capability of natural heterotrophic bacterial assemblages (B), axenic autotrophic microalgal/diatom assemblages (D) and mixed assemblages of both (BD) in terms of microbial growth and EPS secretion. The microbial growth (bacterial cell numbers, bacterial dividing rate, and microalgal biomass) and EPS secretion (concentrations and composition of carbohydrates and proteins) were monitored and further addressed to the adhesive capacity as well as the cohesive forces, both proxies for sediment stability (described in Chapter 5).

It is likely that the vast majority of these polymers are produced by micro-organisms but the impact of benthic meiofauna on this microbial production has seldom been studied. Benthic animal populations also effect on the biogeochemical and physical characteristics of their environment. For instance, they enhance the oxygen input into the sediment and denitrification by bioturbation (Hansen and Kristensen 1997, de Deckere et al. 2001). Feeding activity, either by grazing directly on the microbial EPS producers (Riera et al. 1996, Hagerthey et al. 2002) or by using the exopolymers themselves as a food source (Decho 1990, Smith and Underwood 1998) may cause loss of EPS from system. On the other hand meiofauna and macrofauna organisms can also secrete important amounts of exopolymers and significantly contribute to overall EPS pool. The selected nematodes, *Diplolaimelloides meyli* (Timm 1961) and *Diplolaimelloides oschei* (Meyl 1954) are two congeneric species which typically occupy several types of decomposing macrophyte detritus in estuarine intertidal areas (dos Santos et al. 2008), where they feed largely but not exclusively on bacteria (Moens and Vincx 1997). These and other monhysterid nematodes have been shown to affect bacterial activity and detritus decomposition rates (De Mesel et al. 2006). Even at relatively low densities, they can also significantly impact bacterial

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community composition in a very species-specific way. This may result from differential food preferences (De Mesel et al. 2004) and/or from more indirect interactions (Moens et al. 2005). Thus investigations into how they interact with each other (e.g. synergism-parasitism, predator-prey interactions) and the effect of this interaction on EPS production need to be performed. In order to estimate their respective influence on microbial growth and EPS production, bacterivorous nematodes were included in bacteria-microalgae interaction system (experiment 3). For this propose, bacteria, microalgae and nematodes were both grown separately and simultaneously in laboratory microcosm. Nematodes were chosen because they generally dominate soft-sediment meiofauna communities. It was hypothesized that bacterivorous nematodes would impact the bacterial and microalgae growth and EPS production. These results may provide future support for investigation of biostabilisation processes in presents of meiofauna.

## **4.2. Experimental set-up**

### **4.2.1. Investigation of growth and EPS production of axenic microalgae culture (Experiment 1)**

To investigate the effect of coexistence and stabilisation potential of the microalgae, axenic cultures of *Navicula hansenii*, *Ampfora coffeaformis* and *Oscillatoria* species were obtained from monospecific laboratory cultures at the SAMS CCAP, Dunstaffnage Marine Laboratory, UK. A layer of 0.5 cm of <63 µm glass beads in total was placed in disposable plastic trays (7L×7W×2.5H in cm) and 50 ml of autoclaved seawater were added in each box. Five trays containing glass beads and seawater served as controls. The controls (C) were additional treated with a mixture of antibiotics (150 mg l<sup>-1</sup> streptomycin and 20 mg l<sup>-1</sup> chloramphenicol, final concentrations) and antibiotics were replenished regularly. The following treatments were established in five replicates each: A, N, O, AN, AO, NO and ANO. Treatments names refer to the first letter(s) of the corresponding culture(s) inoculated: A for *Amphora coffeaformis*, N for *Navicula hansenii* and O for *Oscillatoria* species. About 10 ml of the single cultures or their combinations were added to each tray of the corresponding treatments. Antibiotics were not added as it appeared toxic to microalgae culture. The possible bacterial contaminations were monitored regularly by epifluorescence microscopy. Bacterial contamination was not found to be a problem in this case. All treatments

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were illuminated at 220–250  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  under a light/dark cycle of 10/14 h and kept at constant temperature (15°C) over a period of 2 weeks.

**Sampling strategy (Experiment 1).** Sampling took place every 3 days during the experiment using cut-off syringe 10 mm diameter (see 2.3.1). In all the trays, two cores were sampled to measure chlorophyll *a* concentration (described 2.5), and EPS concentrations (protein and carbohydrates) (described 2.4).

#### **4.2.2. Investigation of growth and EPS production of individual and mixed assemblages of natural bacteria and microalgae (Experiment 2)**

A 3 cm layer of  $<63 \mu\text{m}$  glass beads was placed in Rotilab deep-freeze boxes (208Lx208Wx94H in mm). A layer of buoyant plastic was placed onto the surface of the sediment to protect the bed during the addition of the medium (autoclaved sea water) (Gerbersdorf et al. 2008). Two litres of autoclaved seawater were carefully added to each box. Bacteria and diatom cultures were isolated from natural sediment (as described 2.2.1 and 2.2.2) and served as inoculums to grow biofilms on non-cohesive artificial substratum (Ballotini balls, glass beads). The following treatments were established in six replicates each: controls (C), bacterial cultures (B), diatom cultures (D), as well as mixed assemblages of bacteria and diatom cultures (BD). The controls containing only glass beads and seawater were regularly treated (once a week) with a mixture of antibiotics (150  $\text{mg l}^{-1}$  streptomycin and 20  $\text{mg l}^{-1}$  chloramphenicol, final concentrations) to prevent bacterial colonisation. The other boxes were initially inoculated from the stock cultures with 15 ml each for bacterial and diatom cultures, and 30 ml (15/15 ml, B/D) for the mixed cultures. All treatments were gently aerated and kept at constant temperature (15°C) over a period of 4 weeks. The diatoms and the bacteria+diatom cultures were illuminated at 220-250  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$  from a neon tube with a light/dark cycle of 10/14 h (Figure 4.1).





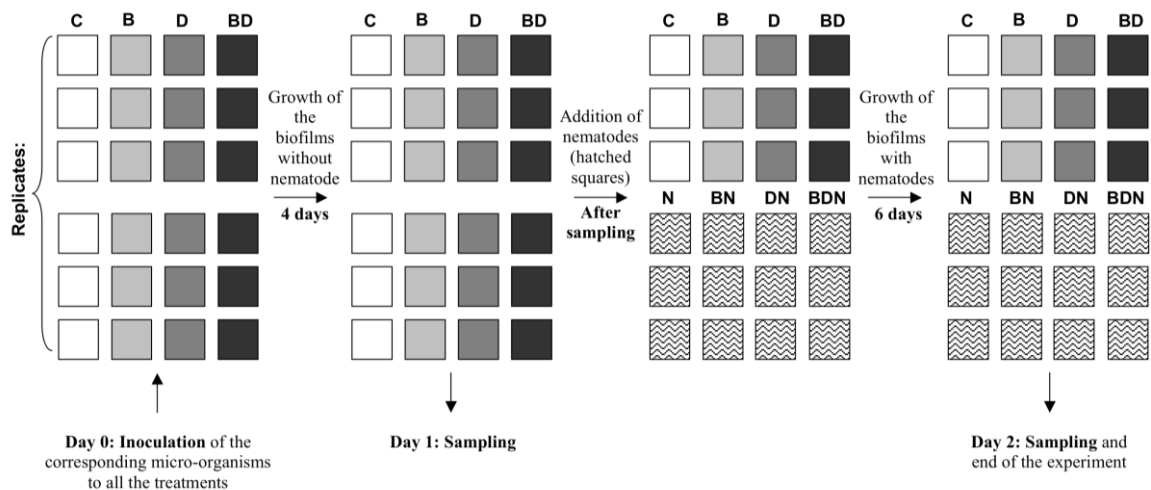
**Figure 4.1:** *Experimental setup.*

**Sampling strategy (Experiment 2).** Sampling took place every 3 days during the experiment. For each treatment, 3 boxes out of 6 replicates were sampled in turn at each measurement. From each box, 4 sediment cores of 5 mm depth were taken by syringe 10 mm diameter (see 2.3.1) to determine bacterial cell numbers (described in 2.7), bacterial assemblage (2 cores for 2 fixation protocols) (described in 2.9.1), and extracellular polymeric substances (EPS) (described in 2.4). For the diatoms treatments (D) and the mixed assemblage (BD), 2 additional cores were taken to determine chlorophyll *a* (described in 2.5), and the microphytobenthic species composition (described in 2.9.2). To determine bacterial dividing rate, 1 additional sediment core (depth 10 mm) was taken from the box and the 3 cores per treatment pooled before analysis (described in 2.8), while all other sediment cores were processed individually. For LTSEM 1 additional core of 10 mm depth was taken and immediately frozen with liquid nitrogen and stored at  $-80^{\circ}\text{C}$  until the sediment could be viewed (see 2.10).

#### **4.2.3. Investigation into the effect of nematodes on microbial growth and exopolymer production in marine sediments microcosms (Experiment 3)**

For this experiment microbial (diatom and bacteria) and nematode cultures were obtained as described in (2.2.1, 2.2.2 and 2.2.3). Microbes (bacteria and diatoms) and nematodes were grown both separately and simultaneously on non-cohesive acid

washed marine sand (40-100  $\mu\text{m}$ , Fisher Scientific). A control group (C) and seven different treatments (B, D, BD, N, BN, DN, and BDN) were tested, each in three replicate microcosms incubated under the same conditions (a total of 24 boxes, 12x12 x6 cm, Figure 4.2). Treatment names refer to the first letter(s) of the corresponding culture(s) inoculated: B for bacteria, D for diatoms and N for nematodes. For all the boxes, a layer of sediment (3 cm deep) was moistened with 200 ml of autoclaved seawater. About 20 ml of the bacterial and diatom cultures were added to each box of the corresponding treatments. All the nematodes extracted from the cultures were resuspended in artificial seawater and distributed equally in the corresponding boxes. The nematode density in the corresponding treatments was about 4 nematodes  $\text{cm}^{-2}$  which was low compared to natural sediments (Heip et al. 1985).



**Figure 4.2:** Schematic diagram of experimental setup and sampling strategy.

For treatments C and N, an antibiotic cocktail (streptomycin and chloramphenicol in final concentrations of 150  $\text{mg l}^{-1}$  and 20  $\text{mg l}^{-1}$ , respectively) was added to limit bacterial proliferation (Lee 1993). Treatments D and DN were supplemented with 150  $\text{mg l}^{-1}$  streptomycin only (non lethal for Bacillariophyceae, Berland and Maestrini 1969) to avoid bacterial proliferation. Chloramphenicol was not added here as it appeared toxic to benthic diatoms. All the treatments were oxygenated, placed at room temperature (18°C-20°C) and submitted to a daily 10 h photoperiod throughout

the experiment (at a saturating light of about 200  $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$ , PAR 400-700 nm).

**Sampling strategy (Experiment 3).** The microorganisms (bacteria and diatoms) were added at the beginning of the experiment (Figure 4.2, day 0) to allow biofilm growth. Sediment cores were sampled 4 days later (Figure 4.2, Sampling Day 1) using a cut-off syringe 1  $\text{cm}^2$  and 0.2 cm depth (as described 2.3.1). In all the boxes, four cores were sampled to measure, respectively, bacterial cell number (described 2.7), chlorophyll *a* concentration (described 2.5) and EPS concentrations (proteins and carbohydrates) (described 2.4). The sampling was immediately followed by the addition of nematodes in the corresponding treatments. The experiment was maintained during 6 more days (10 days after day 0) after which sediment cores were sampled again as described above (Figure 4.2, Sampling Day 2). All the sediment cores were immediately frozen with liquid nitrogen after sampling and stored at  $-20^\circ\text{C}$  until analysis.

#### 4.2.4. Statistics

The data violated assumptions of normality and homogeneity of variance (visual assessment of the frequency histogram and normal plot, Kolmogorov-Smirnov and Barlett tests), thus differences between treatments were assessed using a non-parametric Kruskal-Wallis ( $\chi^2$ ) test (KW), followed by the non-parametric Student-Newman-Keuls (SNK) test to correct for multiple comparisons. Additionally, the Mann-Whitney test was used to compare pairs of treatments.

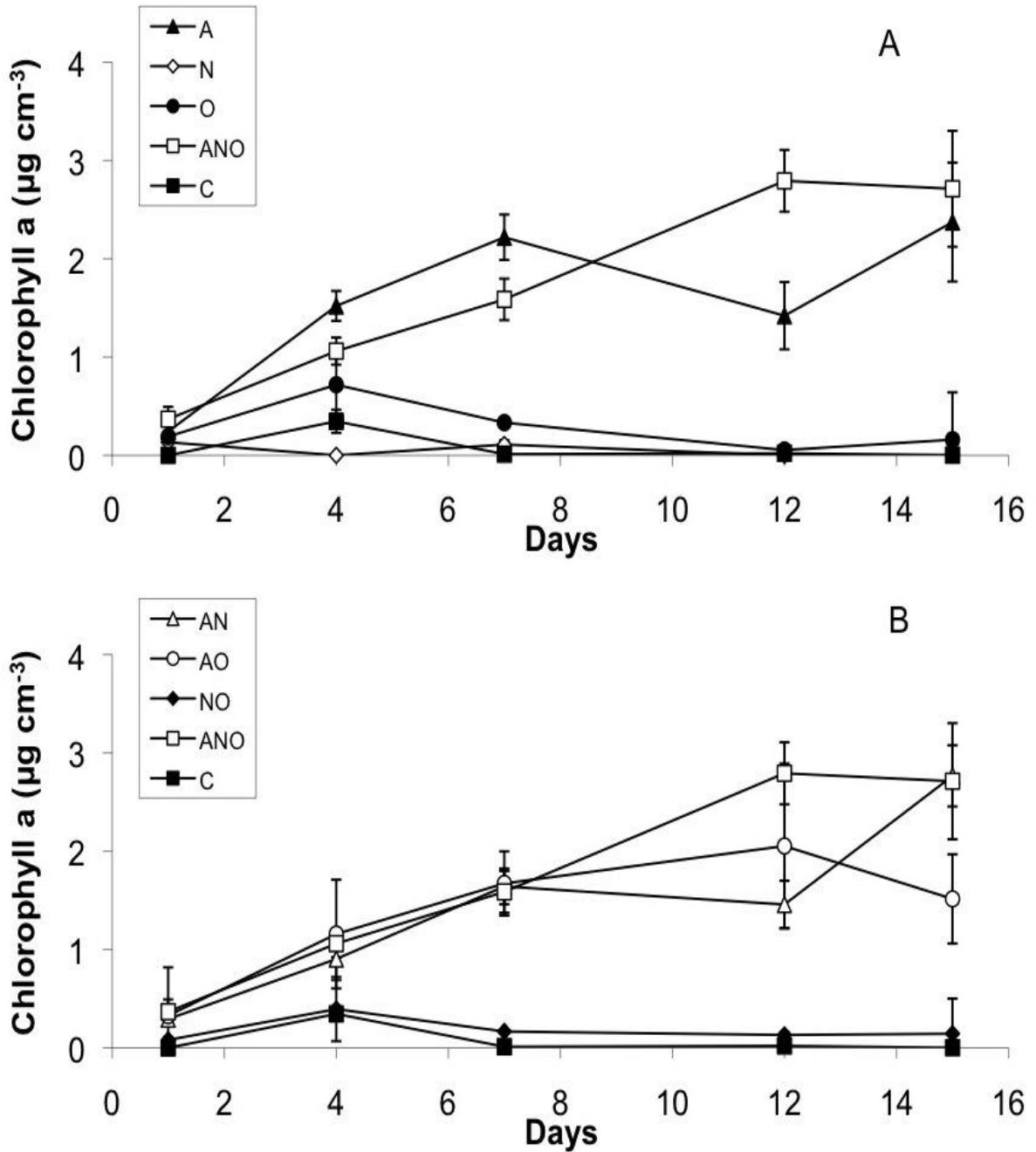
### 4.3. Results

#### 4.3.1. Experiment 1: Investigation into the interaction of axenic microalgae culture with respect to microbial growth and EPS production using *Navicula hansenii*, *Amphora coffeaeformis* and *Oscillatoria species*.

##### **Microbial biomass**

Chlorophyll *a* concentrations in treatment N, O and NO (Figure 4.3 A) increased during the first week of the experiment and decreased rapidly afterwards. In all other treatments chlorophyll *a* concentrations continuously increased until the end of the experiment, except in treatment AO (Figure 4.3 B). At the end of the experiment AO was significantly lower in Chl *a* value than ANO and AN (MW test,  $U=2$ ,  $p<0.05$ ).

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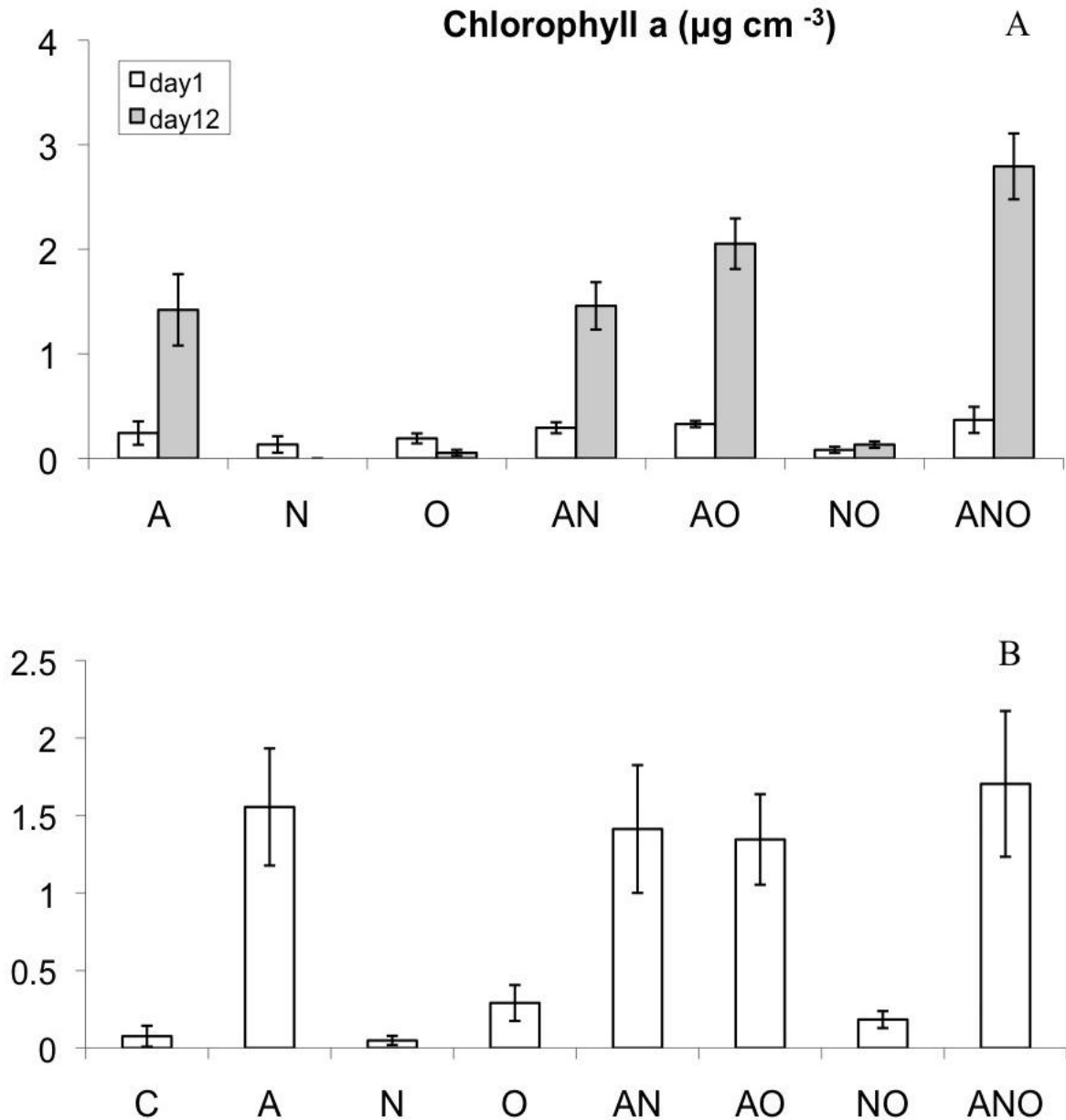


**Figure 4.3:** Mean values ( $n=5$  per treatment  $\pm SE$ ) of measurements over the course of the experiment. (A) The different treatments were single culture: ▲ - *Amphora*; ◇ - *Navicula*; ● - *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*. (B) Pairs of mixed cultures: △ - *Amphora* + *Navicula*, ○ - *Amphora* + *Oscillatoria*; ◆ - *Navicula* + *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*; ■ - Control.

The most pronounced increases were observed for treatments A, AN, AO, ANO (Figure 4.4 A, Table 4.1). There were significant differences in the medians of the chlorophyll *a* concentrations (KW,  $\chi^2=34.7$ ,  $df=6$ ,  $p<0.001$ ). For instance on day 12 (given as example) treatments ANO and AO were significantly higher than N, O and NO (MW test,  $U=0$ ,  $p<0.01$ ) and ANO was significantly higher than AN (MW test,  $U=2$ ,  $p<0.05$ ). The single culture A and AN were significantly higher than N, O and NO (MW test,  $U=0$ ,  $p<0.01$ ). The highest microbial biomasses were observed in mixed treatments AN, AO and ANO and the single culture A where chlorophyll *a* concentration was up to 9 times higher than the other treatments N, O and NO (Figure 4.4 B, Table 4.1).

**Table 4.1.** Quotient/ factors for Chlorophyll *a*, EPS carbohydrates, EPS proteins between the first day of sampling (day 1) and day 12 where most of the variables showed their maximum value as well as differences between mixed assemblages “ANO” and the given treatments (A, N, O, AN, AO, NO, ANO).

Factor	Treatment	<i>Chlorophyll a</i>	<i>Carbohydrates</i>	<i>Proteins</i>
Between minimum and maximum values reached	A	9.8	1.8	2.5
	N	0	1.1	1.7
	O	1.7	1.3	2.8
	AN	9.4	2.6	3.2
	AO	6.2	2.2	3.7
	NO	4.8	1.6	3.8
	ANO	7.6	2.7	2.6
Between ANO and single and combined treatments	A	1.1	1.2	1.3
	N	34.8	2.6	1.7
	O	5.8	2.1	1.0
	AN	1.2	1	1.0
	AO	1.3	1	0.7
	NO	9.2	2.2	0.9



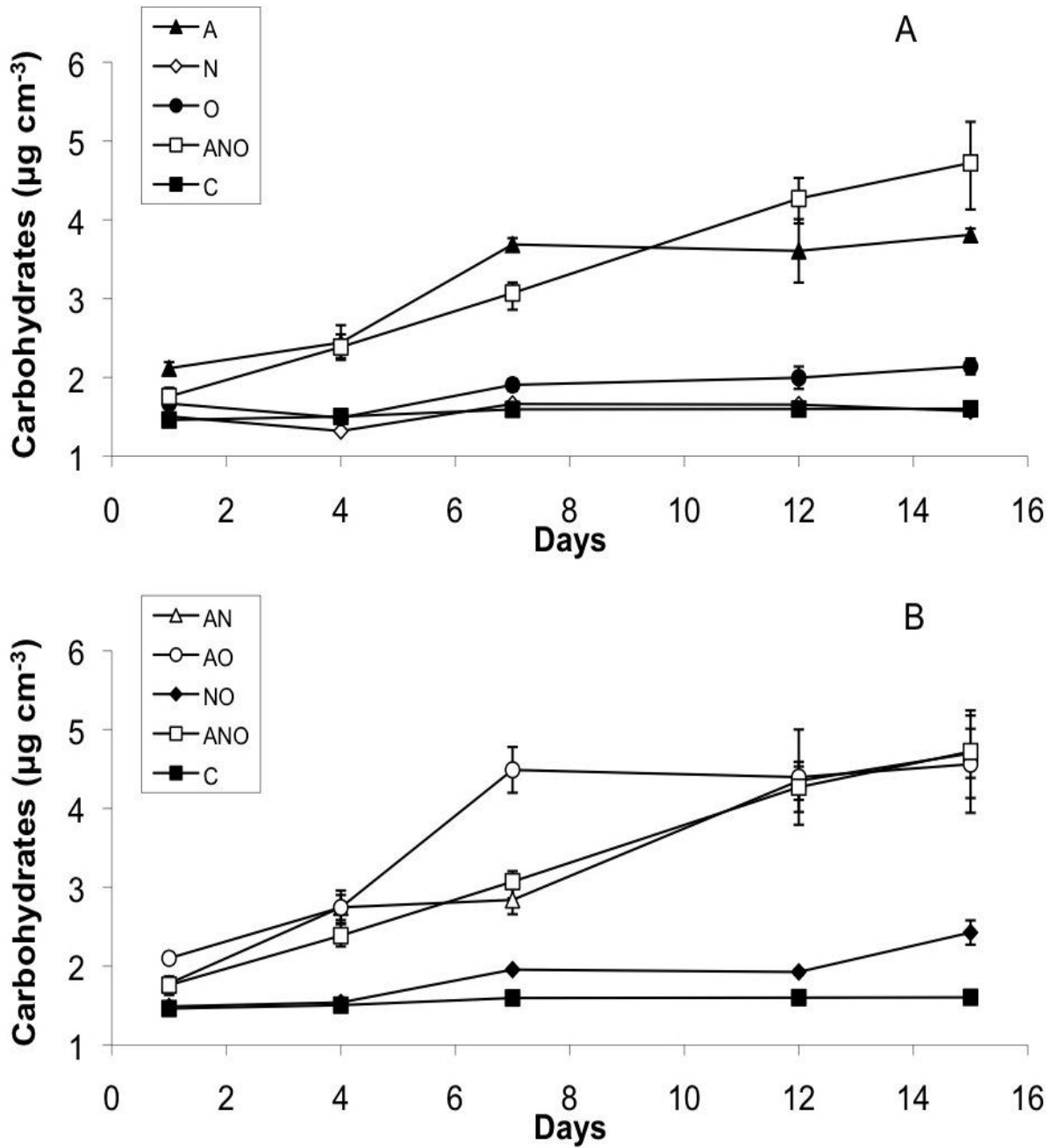
**Figure 4.4:** The differences in chlorophyll a concentrations: (A) between the first sampling day and day 12 where most of the variables showed their maximum value. (B) Cumulative chlorophyll a concentrations ( $n=25$ ) during 2 weeks of experiment. The treatment name (Diatom species) was given according to the first letter of the corresponding culture (s) inoculated: A for Amphora, N for Navicula, O for Oscillatoria and their mixture AN for Amphora and Navicula, AO for Amphora and Oscillatoria, NO for Navicula and Oscillatoria and ANO for Amphora, Navicula and Oscillatoria.

**Changes in EPS components**

The colloidal carbohydrate concentrations increased in all treatments over time (except the control) and reached a maximum on day 15 (Figure 4.5 A and B). However this increase was more pronounced in treatments ANO, AO and AN rather with single treatment A (Figure 4.6 A and B Table 4.1). Statistical testing per day revealed that on day 12, treatments ANO, AN and AO were significantly higher than treatments N, O and NO (KW test,  $\chi^2=33.4$ ,  $df=6$ ,  $p<0.001$ , MW test,  $U=0$ ,  $p<0.001$ ). The carbohydrates in the control were below detection limits.

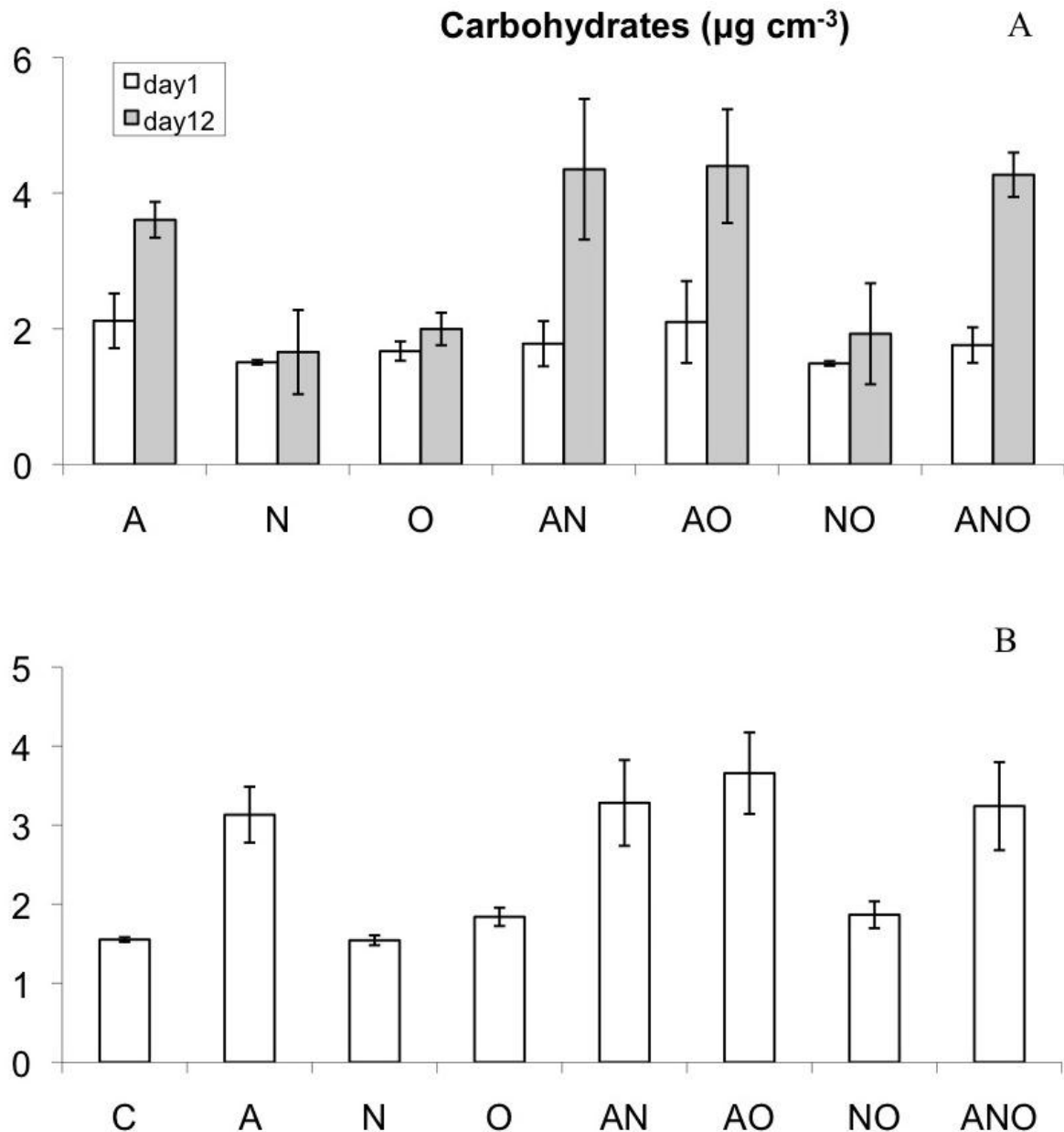
Water-extractable (colloidal) protein concentrations increased in all treatments, (except in the control) up to day 4, followed by a rapid decrease to day 7 and then continuous increase until the end of experiment day 15 (Figure 4.7 A and B).

Protein concentration for all treatments reached a maximum on the last day of the experiment with a more pronounced increase (up to 3.8 times) for treatments AO and NO (Figure 4.8 A, Table 4.1), however cumulative protein concentration was higher in treatments O, AO, NO and ANO followed by AN, A and N (Figure 4.8 B). On the last day of the experiment treatment AO was significantly higher than all other treatments (KW test,  $\chi^2=32.2$ ,  $df=7$ ,  $p<0.001$ , MW test,  $U=0$ ,  $p<0.001$  for all treatments), treatment NO was significantly higher than A and N (MW test,  $U=1$ ,  $p<0.05$ ), treatments AN and ANO was significant higher than A and N (MW test,  $U=1$ ,  $p<0.05$ ) and treatment O was significant higher than N (MW test,  $U=0$ ,  $p<0.01$ ).

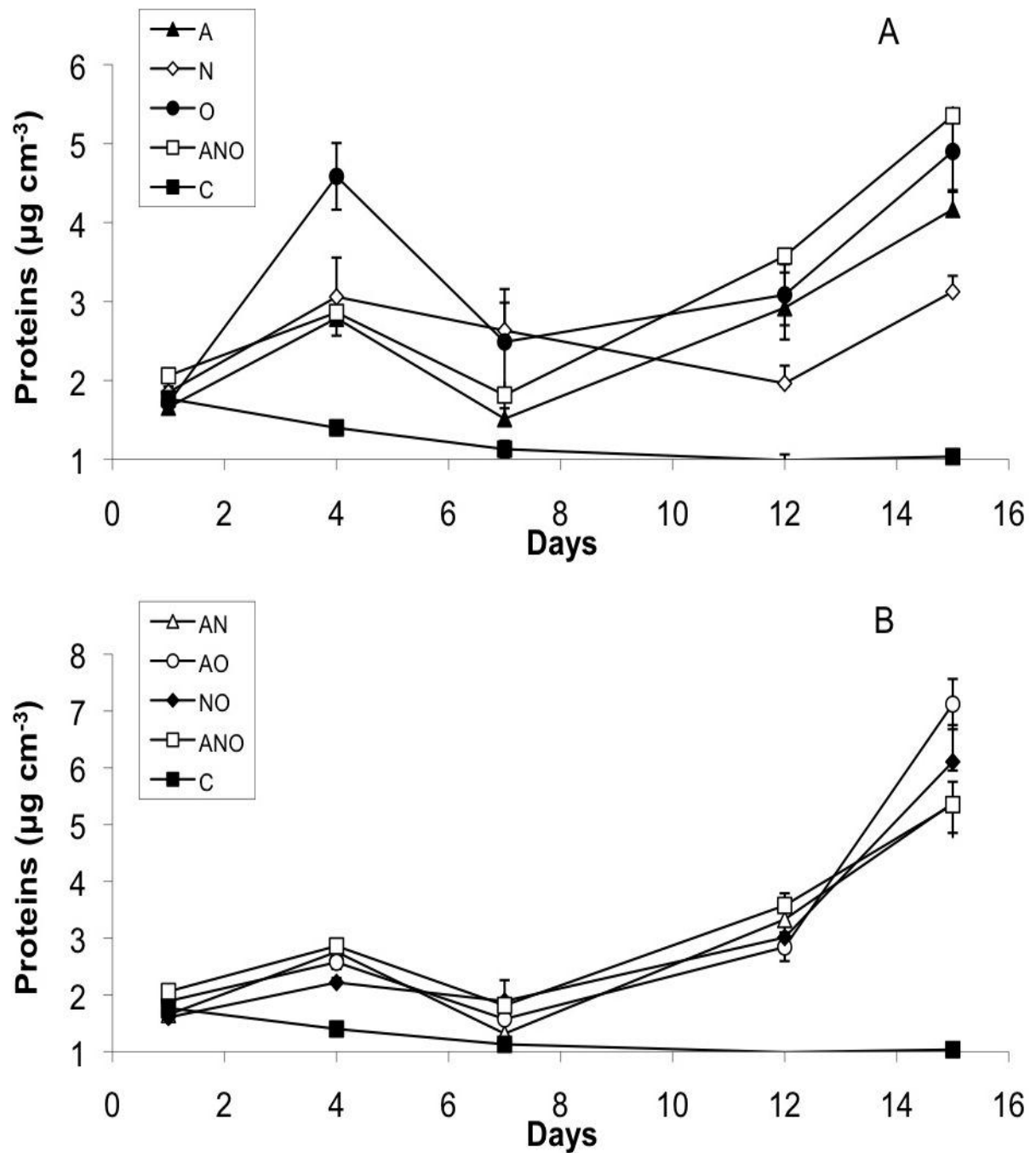


**Figure 4.5:** Mean values ( $n=5$  per treatment) with SE of colloidal carbohydrate measurements over the course of the experiment. (A) The different treatments were single culture: ▲ - *Amphora*; ◇ - *Navicula*; ● - *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*. (B) Pairs of mixed cultures: △ - *Amphora* + *Navicula*, ○ - *Amphora* + *Oscillatoria*; ◆ - *Navicula* + *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*; ■ - Control.

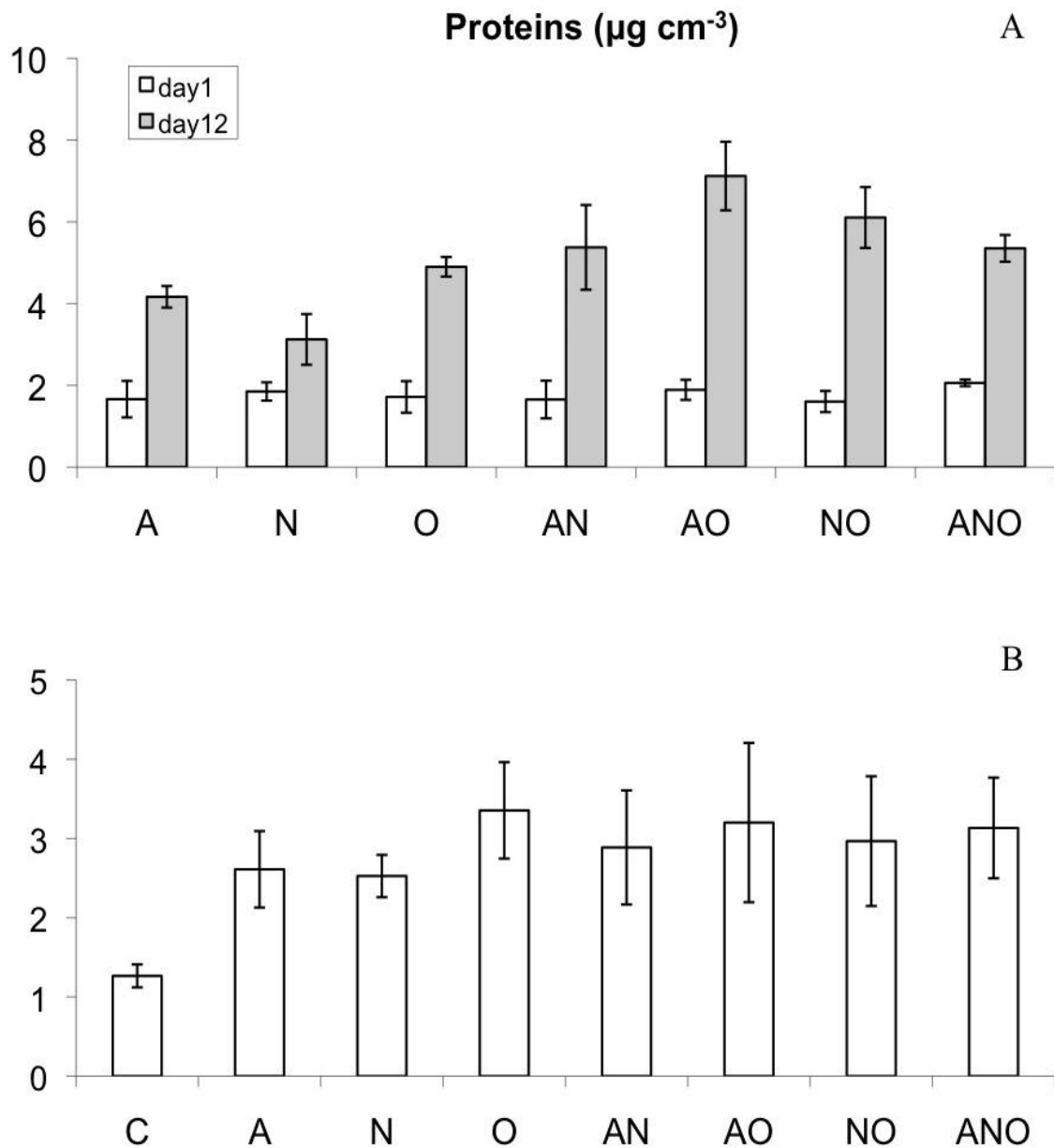




**Figure 4.6:** The differences in colloidal carbohydrates concentrations: (A) between the first sampling day and day 12 where most of the variables showed their maximum value. (B) Cumulative carbohydrates concentrations ( $n=25$ ) during 2 weeks of experiment. The treatment name (Diatom species) was given according to the first letter of the corresponding culture (s) inoculated: A for Amphora, N for Navicula, O for Oscillatoria and their mixture AN for Amphora and Navicula, AO for Amphora and Oscillatoria, NO for Navicula and Oscillatoria and ANO for Amphora, Navicula and Oscillatoria.



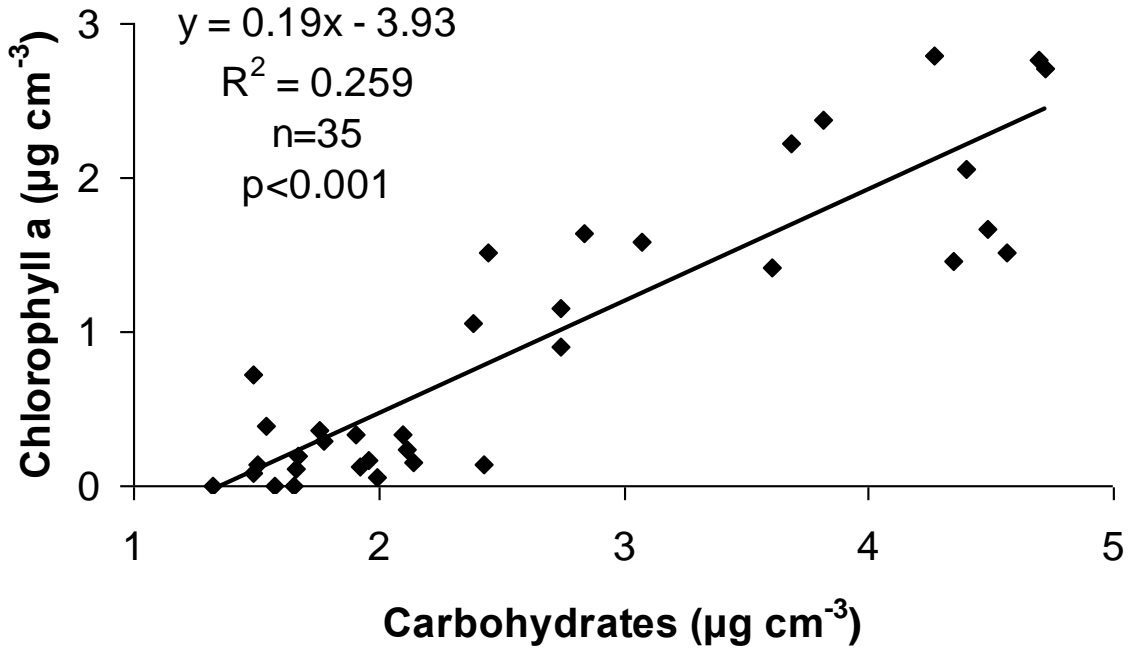
**Figure 4.7:** Mean values ( $n=5$  per treatment) with SE of colloidal protein measurements over the course of the experiment. (A) The different treatments were single culture: ▲ - *Amphora*; ◇ - *Navicula*; ● - *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*. (B) Pairs of mixed cultures: △ - *Amphora* + *Navicula*, ○ - *Amphora* + *Oscillatoria*; ◆ - *Navicula* + *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*; ■ - Control.



**Figure 4.8:** The differences in colloidal protein concentrations: (A) between the first sampling day and day 12 where most of the variables showed their maximum value. (B) Cumulative carbohydrates concentrations ( $n=25$ ) during 2 weeks of experiment. The treatment name (Diatom species) was given according to the first letter of the corresponding culture (s) inoculated: A for Amphora, N for Navicula, O for Oscillatoria and their mixture AN for Amphora and Navicula, AO for Amphora and Oscillatoria, NO for Navicula and Oscillatoria and ANO for Amphora, Navicula and Oscillatoria.

**Relationship between biological variables**

A positive correlation was determined between colloidal carbohydrates and colloidal proteins (Pearson correlation coefficient,  $r=0.410$ ,  $n=35$ ,  $p<0.01$ ). A positive linear relationship was determined between colloidal carbohydrates and microalgal biomass, as indicated by Chl *a* concentrations (Figure 4.9), however no significant relationship was found between colloidal proteins and chlorophyll *a*.



**Figure 4.9:** Relationship between colloidal carbohydrates and chlorophyll *a*.

#### 4.3.2. Experiment 2: Interaction of microorganisms (heterotrophic bacteria and autotrophic microalgae)

##### **Microphytobentos composition**

In the mixed assemblage (bacteria+diatoms, BD), diatoms of the genera *Achnanthes*, *Caloneis*, *Navicula* and *Nitzschia* were present on the substratum at the beginning of the experiment (day1). While the large species *Achnanthes longipes* and *Caloneis amphisbaena* seemed to dominate the samples, the majority of species were represented by the genus *Navicula* (*N. cinta*, *N. digitoradiata*, *N. flantica*, *N. gregaria*, *N. crytocephala*, *N. perminuta/diserta*, *N. phyllepta*, *N. salinarum*) and *Nitzschia* (*N. epithemioides*, *N. frustulum*, *N. hungarica*, *N. sigma*). Over time, smaller species such as *Navicula* became dominant together with *Nitzschia* and *Cymbella* species. After 4 weeks, only small

*Navicula* species remained. In the diatom assemblage (D), treated with antibiotics to inhibit bacterial colonization, the species composition was quite similar to the mixed assemblage with *Achnanthes*, *Cylindrotheca*, *Cymbella*, *Navicula* and *Nitzschia* species but smaller *Navicula* species dominated in this culture from the beginning. *Achnanthes*, *Cymbella*, and *Nitzschia* species were characteristic for this treatment for about 3 weeks. By the end of the experiment, only small *Navicula* species remained.

Most of the diatom species typically occur in poly- and hypertrophic environments, except for some species of *Achnanthes* and *Cymbella*, which rather require mesotrophic conditions. Although the benthic diatom community was isolated from natural sediments, species richness seemed less diverse as compared to the natural habitats.

### **Bacterial assemblages**

The proportion of the active cells as determined by EUB mix was higher in the beginning for the pure bacterial assemblage (B, 58%) as compared to the mixed assemblage (BD, 38%); however at the end of the experiment the proportion of active cells was similar for both treatments (54% B and 55% BD) indicating that most of the bacterial community was metabolically active at the sampling time. In the control measurements (C) as well as in the diatom assemblage (D), hybridizing with oligonucleotide probes was below levels of detection.

The application of domain, phylum, and subphylum specific oligonucleotide probes revealed that the samples were predominated by gram-negative Proteobacteria, while gram-positive Actinobacteria were determined with less than 1% (Table 4.2). In the mixed assemblage, the Alphaproteobacteria accounted for 18%, the Betaproteobacteria for 35%, the Gammaproteobacteria for 15%, the Delta-subclass for 5% and the Cytophaga Flexibacter Subphylums for 15%. Over time, a noticeable shift could be determined within the assemblage: while the Alphaproteobacteria increased to 20%, the Betaproteobacteria decreased to 18%, and Sulphate deoxidizer/Delta-subclass decreased below detection limit (Table 4.2).

The Actinobacteria accounted for less than 1% and were thus negligible. The pure bacterial assemblage showed similar proportions of the subphylums (Alphaproteobacteria 10%, Betaproteobacteria 30%, Gammaproteobacteria 10%, Cytophaga/Flexibacter 13%), but the Delta-subclass could not be detected.

**Table 4.2.** Percentage of the specific bacterial groups (marked by the oligonucleotide probes named on the left) of the total eubacterial counts; given for the treatments bacteria and diatoms (BD), bacteria (B) and diatoms (D) for the beginning (1) and the end (2) of the experiment.

	BD, 1 FA (%)	BD, 2 FA (%)	B, 1 FA (%)	B, 2 FA (%)
ALF968	18	20	10	12
BET42a	35	18	30	25
GAM42a	15	15	10	25
HGC69a	<1	-	-	<1
SRB385Db	5	-	-	<1
CF319a	15	15	13	18

Over time, Alphaproteobacteria increased (to 12%) and the Betaproteobacteria decreased, but to a much lesser extend (to 25%) as compared to the mixed assemblage. Noticeably different to the “BD” treatment was also the increase in Gammaproteobacteria (to 25%) and Cytophaga/Flexibacter (to 18%) over time. As for the mixed assemblage, the gram-positive Actinobacteria were present at low relatively abundance of <1% (Table 4.2).

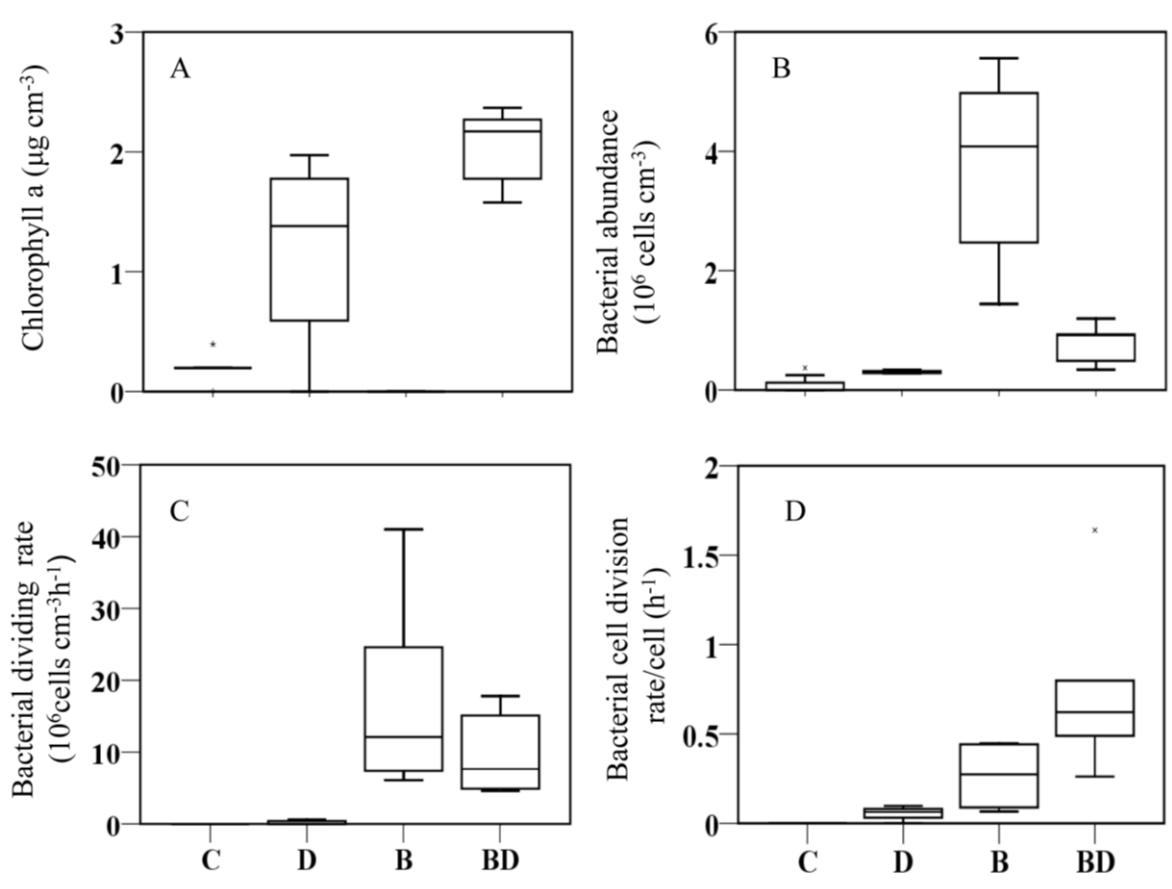
#### **Microbial biomass, cell number and growth rate**

The chlorophyll *a* and pheophytin concentrations were significantly different between the treatments for most of the sampling days (Kruskal-Wallis ( $\chi^2$ ) test (KW),  $p < 0.05$ ). Chl *a* concentrations in the mixed treatment BD ranged between 1.5 and 2.17  $\mu\text{g cm}^{-3}$  and were significantly higher than the D treatment (Figure 4.10 A) with values ranging between 1.38 and 1.97  $\mu\text{g cm}^{-3}$  (for example, day 14: KW,  $\chi^2=6.77$   $df=2$ ,  $p < 0.05$ , with post-hoc Student-Newman-Keuls (SNK) test).

Like the microbial biomass, the bacterial cell numbers determined by flow cytometry significantly differed between the treatments on most of the days (KW,  $p < 0.05$ ). The bacterial cell numbers in the treatment B and BD varied between  $1.44 \times 10^7$  and

$5.56 \times 10^7$  cells  $\text{cm}^{-3}$  as well as  $0.34 \times 10^6$  and  $1.19 \times 10^7$  cells  $\text{cm}^{-3}$ , respectively (Figure 4.10 B). Thus, the bacterial cell numbers were significantly higher in the pure bacterial culture (for example, day 14: KW,  $\chi^2=3.8$ ,  $\text{df}=3$ ,  $p<0.05$ , with post-hoc SNK test).

Based on the calculated [*methyl*- $^3\text{H}$ ] thymidine incorporation, there was no significant difference for bacterial division rate between the bacterial and mixed assemblages (Figure 4.10 C). Like the bacterial cell numbers, the bacterial division rates were negligible in the controls and in the axenic diatom assemblage.



**Figure 4.10:** Box plots of the different treatments: mixed assemblages (BD), diatoms (D), bacteria (B) and control (C). (A): chlorophyll a ( $n=21$ ), (B) bacterial cell numbers ( $n=24$ ), (C) bacterial division rates ( $n=18$ ), (D) bacterial specific rates ( $n=18$ ).

The specific rate of bacterial division per cell per hour can be calculated by dividing the division rate of the bacterial community (cells  $\text{cm}^{-3} \text{h}^{-1}$ ) by the bacterial cell numbers (cells  $\text{cm}^{-3}$ ). The specific rate of bacterial division was significantly higher for

BD as compared to B (Figure 4.10 D); especially on day 3 where the specific rate of bacterial division in the BD assemblage was 18.2 times higher than in B (KW,  $\chi^2=6.2$  df=2,  $p<0.05$ , with post-hoc SNK tests).

There was no significant correlation between the bacterial cell division rates and bacterial cell numbers in the bacterial treatment or in the mixed assemblage. Despite ongoing growth of microalgae and bacteria, no significant relationships between chlorophyll *a* as a proxy for microalgal biomass and the bacterial cell numbers or bacterial division rates could be determined within the mixed assemblage.

**Changes in EPS components**

Over time, the water-extractable (colloidal) carbohydrate concentrations increased in all treatments to a maximum on day 14 (Figure 4.11 A, Table 4.3), but the increase was most pronounced for the mixed assemblage.

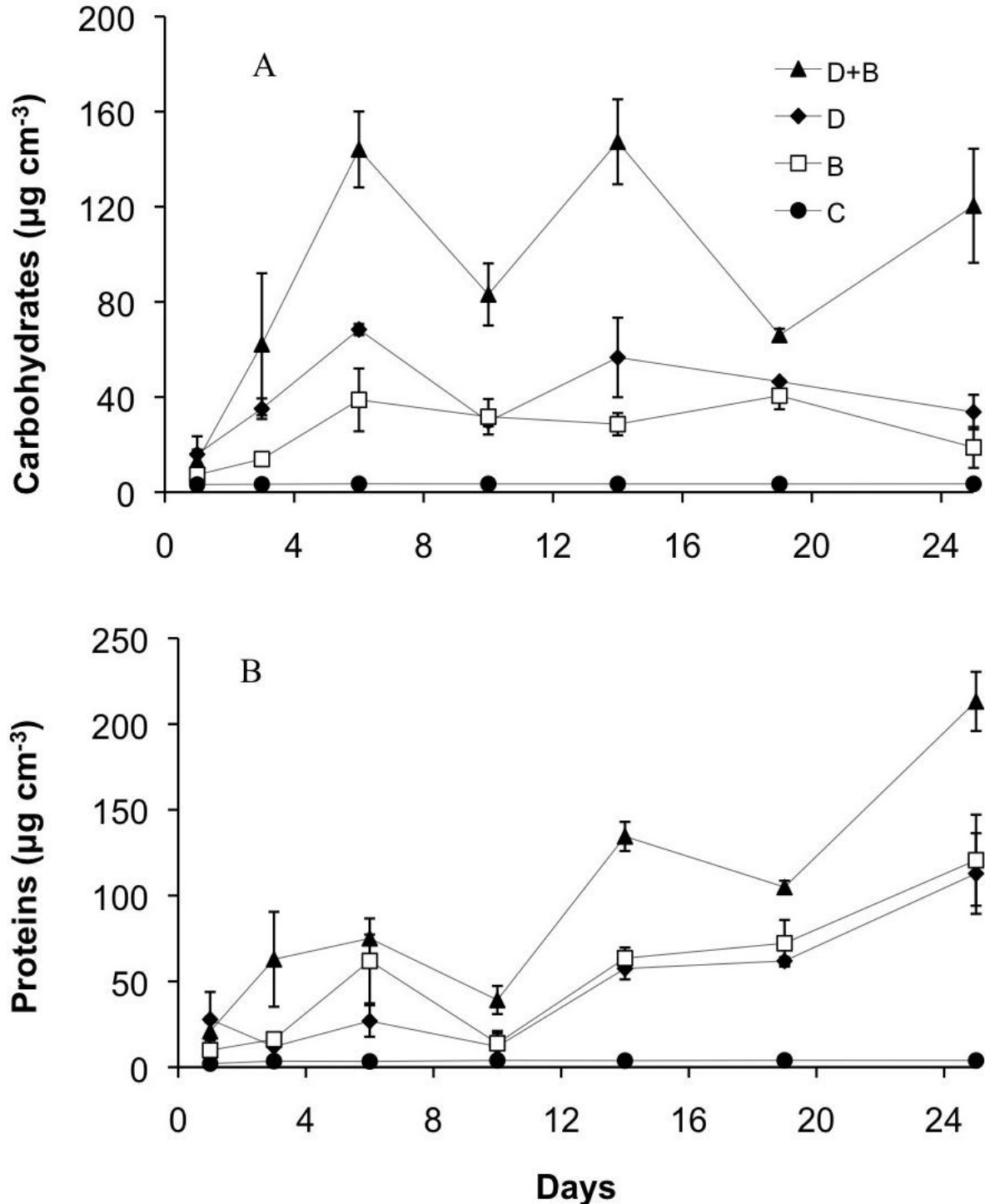
**Table 4.3.** Differences between the first day of sampling (day 1) and day 14 where most of the variables showed their maximum value as well as differences between the given treatments (mixed: BD, Bacteria B, Diatom D); both times expressed as quotient/factors for colloidal carbohydrates, colloidal proteins.

Factors		<i>Carbohydrates</i>	<i>Proteins</i>
<b>between day 1-14</b>	<b>B</b>	5.5	6.4
	<b>D</b>	3.6	2.1
	<b>BD</b>	11	6.4
<b>between treatments</b>	<b>BD/B</b>	5.1	1.7
	<b>BD/D</b>	2.6	1.9
	<b>B/D</b>	0.714	-

The carbohydrate concentrations varied between 13-147.3  $\mu\text{g cm}^{-3}$ , 7.3-40.5  $\mu\text{g cm}^{-3}$  and 15.9-56.6  $\mu\text{g cm}^{-3}$  for BD, B and D, respectively (Figure 4.11 A) with significantly different means in the treatments for all sampling dates except at the beginning of the experiment (KW,  $p<0.05$ ). The carbohydrate concentrations were significantly higher in BD as compared to D and B (for example, day 14: KW,  $\chi^2=9.66$ , df=3,  $p<0.05$ ,



followed by post-hoc SNK test) (Figure 4.11 A, Table 4.3). The treatments B and D were not significantly different from each other. The controls showed negligible amounts of colloidal carbohydrates.

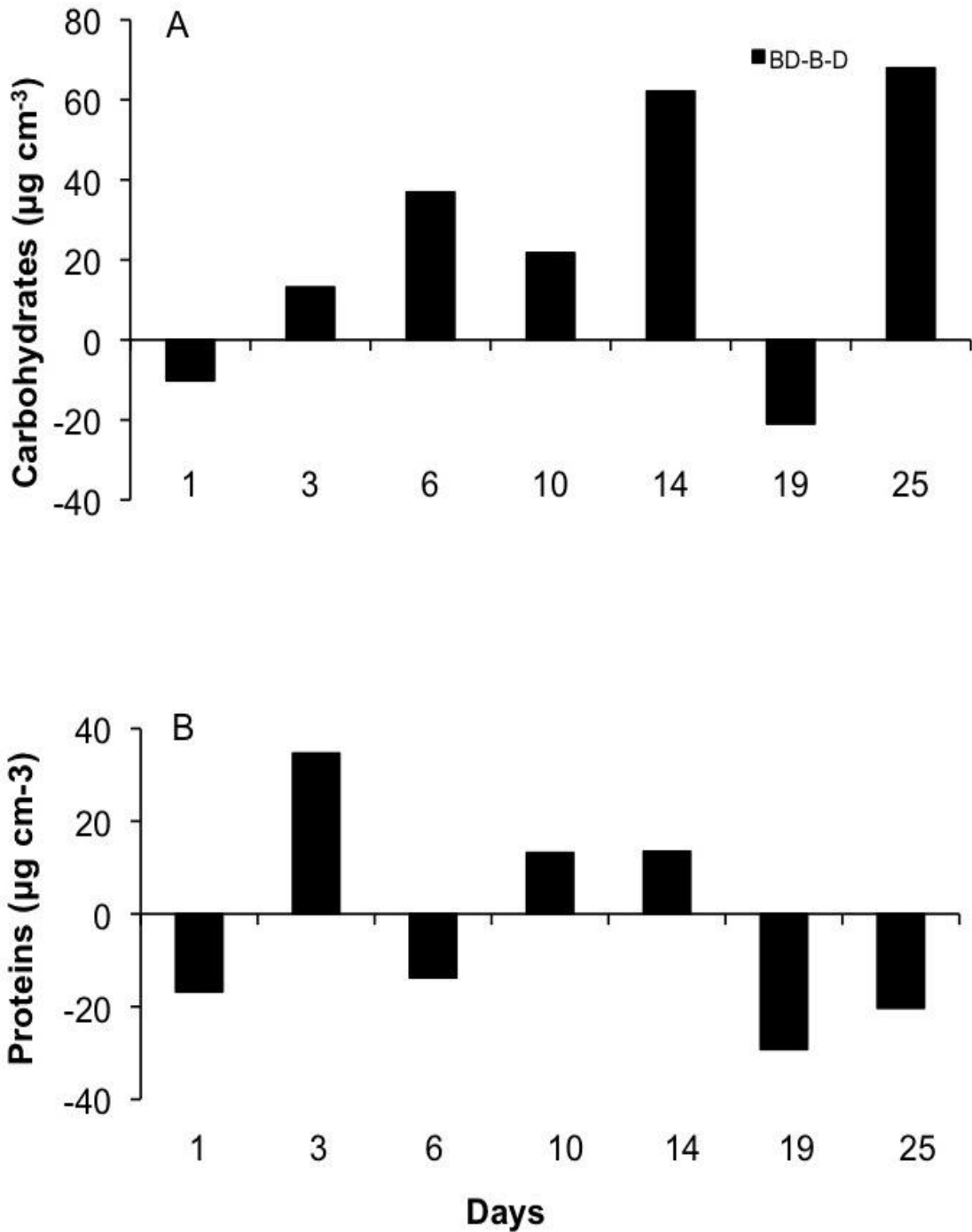


**Figure 4.11:** Mean values of colloidal carbohydrates (A) and colloidal protein (B). Mean values ( $n = 3$  per treatment, based on  $n = 3$  replicates per box  $\pm$  SE) in the treatments bacteria and diatoms (BD,  $\blacktriangle$ ), diatoms (D,  $\blacklozenge$ ), bacteria (B,  $\square$ ) and controls (C,  $\bullet$ ).

The pattern of the water-extractable (colloidal) protein concentrations over time was similar to that of the carbohydrates, with an increase towards on day 14 in all treatments (Figure 4.11 B, Table 4.3). The protein concentrations for the treatments BD, B and D varied between 20.9-213.1  $\mu\text{g cm}^{-3}$ , 9.8-120.6  $\mu\text{g cm}^{-3}$  and 27.8-112.8  $\mu\text{g cm}^{-3}$ , respectively (Figure 4.11 B) with significantly different means in the treatments for most of the sampling dates (KW,  $p < 0.05$ ). The protein concentrations in the treatment BD were significantly higher than in the treatments B and D (for example, day 14: KW,  $\chi^2 = 9.67$ ,  $df = 3$ ,  $p < 0.05$ , followed by post-hoc SNK test). The treatments B and D were not significantly different from each other. The colloidal proteins in the controls were below detection limits.

To explore possible additive, inhibitory or synergistic effects between the effects of single and combined assemblages, the amount of EPS produced in each single assemblage (B and D) was assessed relative to the amount of EPS produced in the mixed assemblage ([BD]-[B+D], Figure 4.12 A and B).

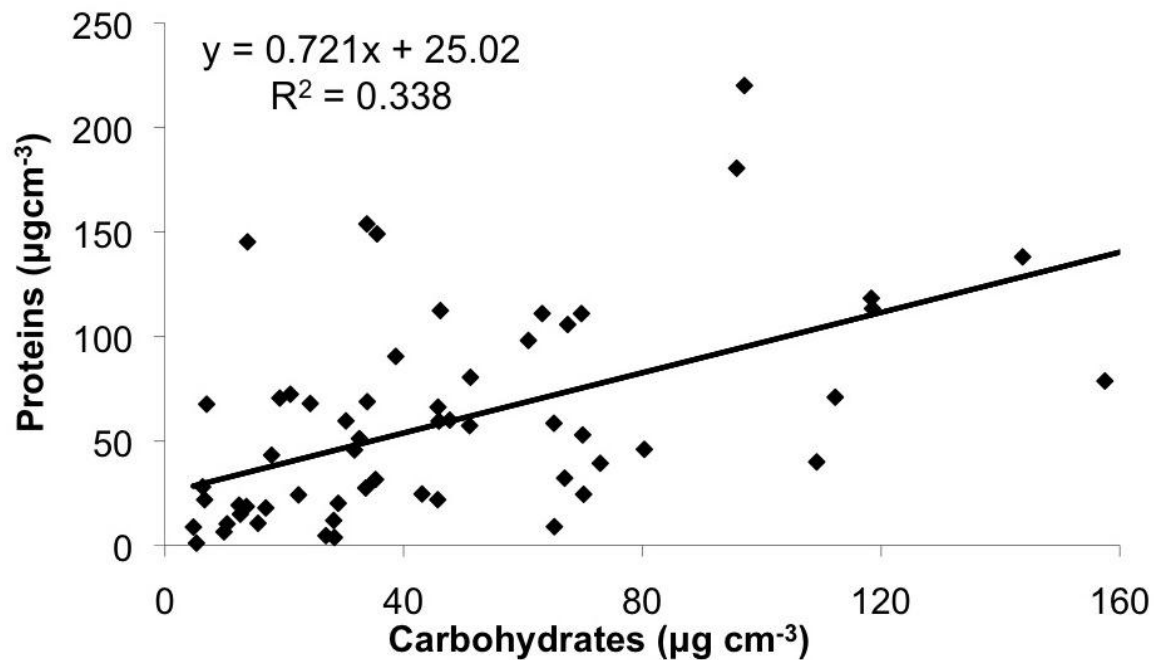
Where this relationship is close to zero, production by B and D is additive with respect to BD, the more negative the relationship then the lower the relative production of BD against the combined values of B and D (inhibitory effect) suggesting that either EPS production in BD is reduced or that cycling is more rapid in the combined culture. A strongly positive value for the relationship ( $BD \gg B+D$ ) would suggest synergy in the mixed culture. There were 2 sampling dates on which the relationship was inhibitory for [EPS] and on all other occasions the value was strongly positive suggesting a synergistic effect (Figure 4.12 A). The results in terms of protein production were more equivocal with a balance in response across the sampling dates (Figure 4.12 B).



**Figure 4.12:** The relative assessment between treatments. The EPS concentration of the mixed cultures (BD) relative to the contribution of the single cultures (B and D) such that the value “BD-B-D” is reported for carbohydrates (A) and proteins (B). Where the production of carbohydrate or protein from mixed cultures (BD) exceeds that of the added single cultures (B and D) the value is positive (synergistic effect) and vice versa (inhibitory effect). If the added values of the single cultures exactly equal the mixed cultures then there is an additive effect.

**Relation between biological variables**

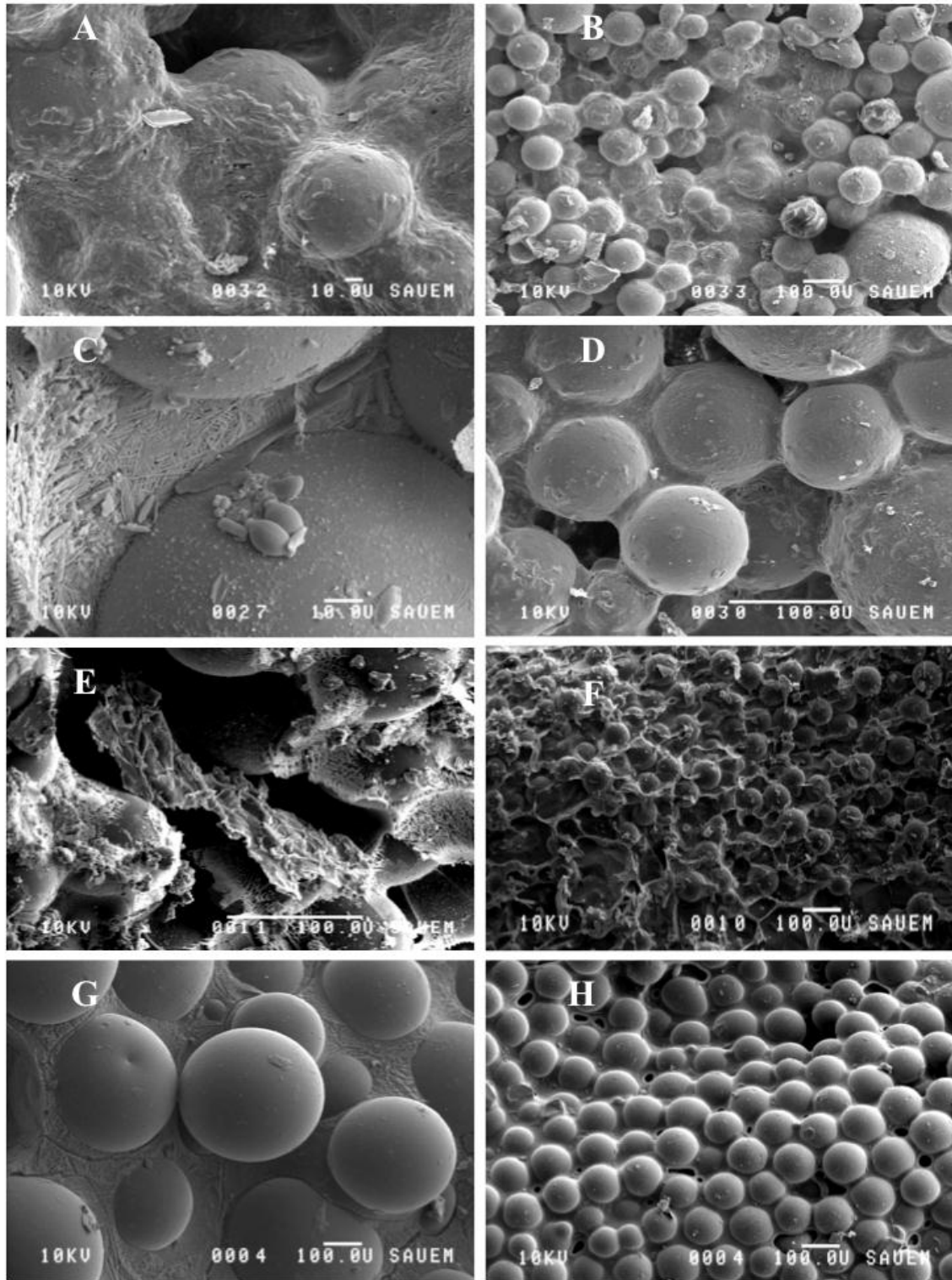
A strong positive correlation was determined between colloidal carbohydrates and colloidal proteins (Figure 4.13). The colloidal carbohydrates and proteins showed a significant positive relation to microalgal biomass, as indicated by Chl *a* concentrations ( $r=0.385$ ,  $n=56$ ,  $p<0.001$  and  $r=0.310$ ,  $n=57$ ,  $p<0.01$ , respectively) as well as to the bacterial cell numbers ( $r=0.649$ ,  $n=18$ ,  $p<0.01$  and  $r=0.518$ ,  $n=18$ ,  $p<0.01$ , respectively).



**Figure 4.13:** Scatter plot to show the relationship between colloidal carbohydrates ( $\mu\text{g cm}^{-3}$ ) and colloidal proteins ( $\mu\text{g cm}^{-3}$ ).

**Low Temperature Scanning Microscopy**

Microbial colonization resulted in the development of a biofilm, which significantly stabilized the test substratum. As the chosen substratum was composed of non-cohesive glass beads, the binding force must have been entirely due to microbial attachment and the secretion of a polymeric matrix (Figure 4.14). LTSEM highlighted that in treatments with microorganisms, the EPS matrix is visible, heavily covering the glass beads and permeating the intermediate pore space, on the other hand, frozen water (ice) on the surface produces a solid matrix around the glass beads on the controls (Figure 4.14).

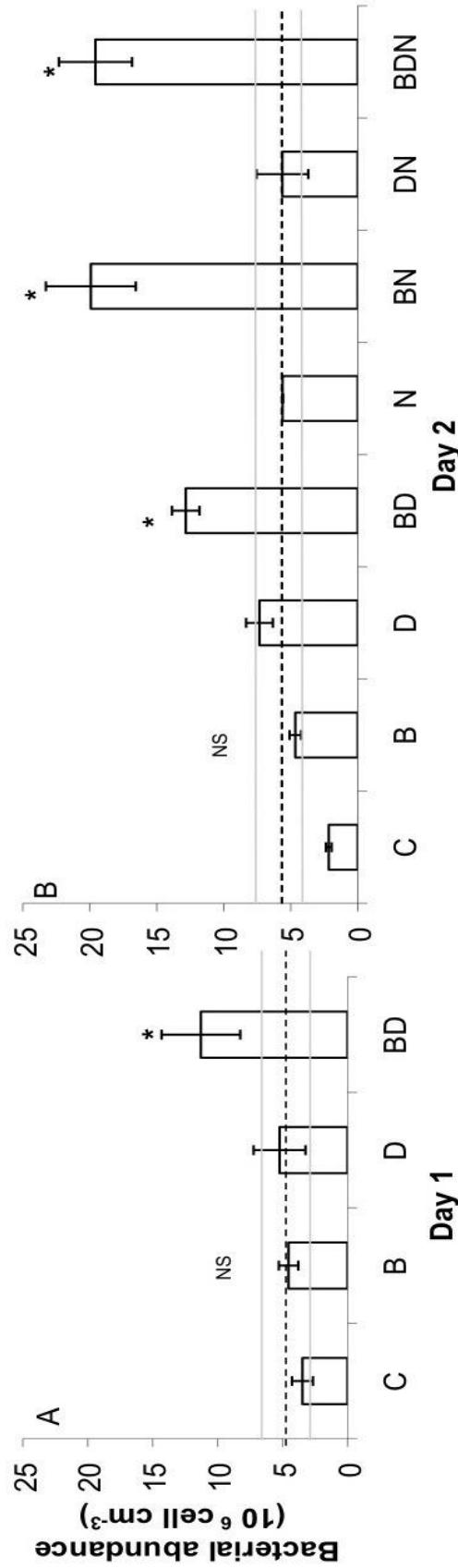


**Figure 4.14:** Low-temperature scanning electron microscope images using different magnifications. A-B. The mixed assemblages bacteria + diatom. C-D. The diatom treatment. E-F. The bacteria treatment. G-H. The control substratum.

### 4.3.3. Experiment 3: The effect of nematodes on microbial growth and EPS production

#### ***Microbial biomass***

For bacterial cell number, a baseline was calculated by averaging the results of the controls and all the treatments which were not originally inoculated with bacteria and which were supplemented with antibiotics (see the dashed lines in Figure 4.15 A, B mean  $\pm 95\%$  interval of confidence). For the first sampling date (Figure 4.15 A), bacterial abundance in treatment B was not significantly different from the baseline (Mann-Whitney test,  $U=28.00$ ,  $p>0.05$ ). The difference was significant for treatment BD (Mann-Whitney test,  $U=5.00$ ,  $p<0.01$ ). For the second sampling date (Figure 4.15 B), bacterial abundance was between 2 and  $24 \times 10^6$  cells  $\text{cm}^{-2}$  depending on the treatment considered which is slightly lower than natural bacterial abundances of intertidal mud and sand flats i.e.  $10^9$  cells  $\text{cm}^{-2}$  (Epstein et al. 1997, Goni-Urriza et al. 1999, Bottcher et al. 2000, Danovaro et al. 2001). Bacterial abundance was relatively stable between the first and last sampling dates in treatments C, B, D and BD and was higher in presence of nematodes. As for sampling Day 1, bacterial abundance in treatment B was not significantly different from the baseline (Mann-Whitney test,  $U = 22.00$ ,  $p>0.05$ ). At the sampling Day 2 the difference was significant for treatment BD, BN and BDN (Mann-Whitney test,  $p<0.01$  for all the treatments).



**Figure 4.15:** Bacterial cell number in superficial sediments for the first (A) and second (B) sampling dates (mean  $\pm$  SE). The dashed lines correspond to the average ( $\pm$  95% interval of confidence represented by the grey area) of all the treatments which were not originally inoculated with bacteria (e.g. the dashed line in fig. B was calculated with treatments C, D, N and DN). The differences between the dashed line and the remaining treatments was tested (NS: not significant, \*: significant difference).

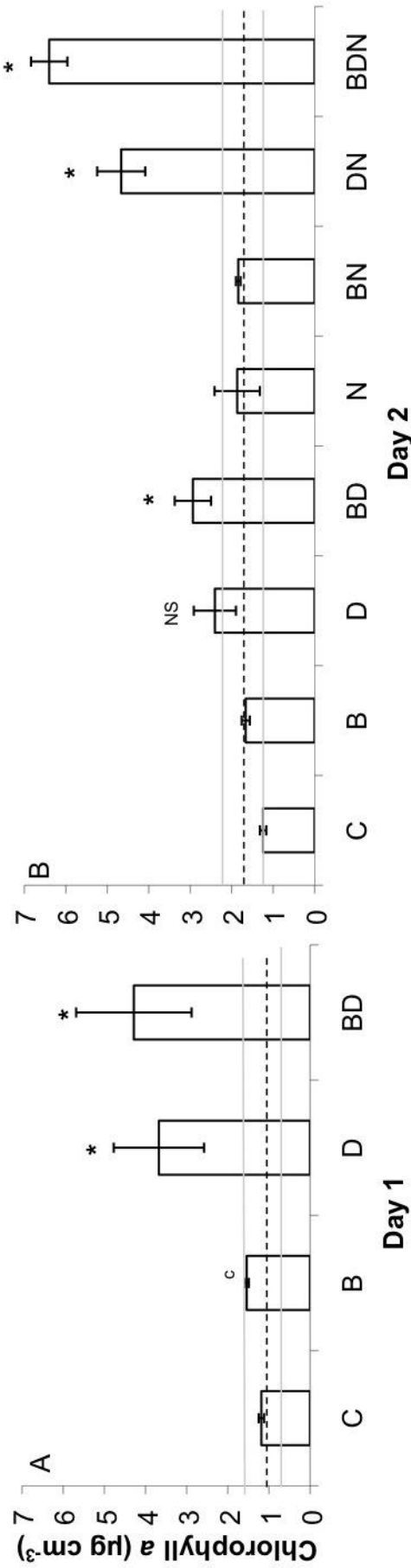
For chlorophyll *a* (Chl *a*), a baseline was calculated as described above from the treatments which were not originally inoculated with diatoms (Figure 4.16 A and B). For the first sampling date (Figure 4.16 A), chlorophyll *a* concentration in treatments D and BD were significantly different from the baseline (Mann-Whitney test,  $p < 0.001$  and  $p < 0.01$  respectively). For the second sampling date (Figure 4.16 B), chlorophyll *a* concentration ranged from 1 to 7  $\mu\text{g cm}^{-3}$  depending on the treatment considered which is also lower than chlorophyll *a* concentration of natural intertidal mud- and sandflats (Dejonge and Colijn 1994, Barranguet et al. 1997, Paterson and Hagerthey 2001). Chl *a* concentration increased slightly from the first to last sampling dates in treatments D and BD and was slightly higher in presence of nematodes. As for sampling Day 2, chlorophyll *a* concentration in treatment D was not significantly different from the baseline (Mann-Whitney test,  $U=5.50$ ,  $p > 0.05$ ). The difference was significant for treatment BD, DN and BDN (Mann-Whitney test,  $p < 0.01$  for all the treatments).

#### ***Changes in EPS components***

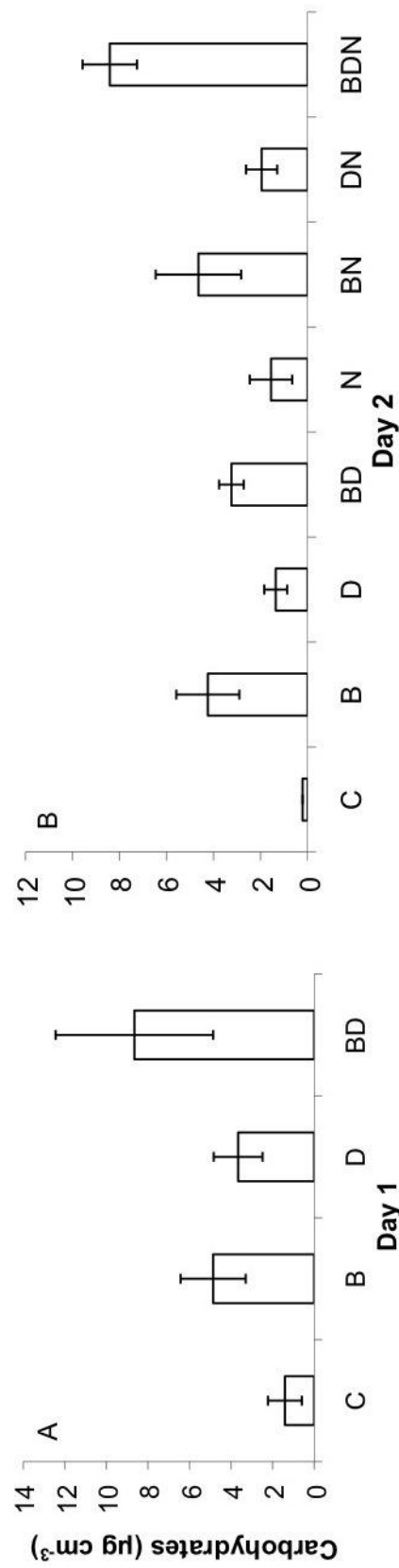
For the first sampling date (Figure 4.17 A), colloidal carbohydrate concentration of all the treatments were significantly different from the control and the treatment BD displayed the highest average concentration (Kruskal-Wallis,  $H=13.18$ ,  $df=3$ ,  $p < 0.01$ , followed by a non-parametric SNK test). For the second sampling date (Figure 4.17 B), the colloidal carbohydrates concentrations were significantly different from the control and treatment BDN displayed the highest carbohydrates concentration (Kruskal-Wallis,  $H=12.71$ ,  $df=6$ ,  $p < 0.05$ , followed by a non-parametric SNK).

For the first sampling date (Figure 4.18 A), treatment BD displayed the highest average colloidal protein concentration (Kruskal-Wallis,  $H=10.17$ ,  $df=3$ ,  $p < 0.05$ , followed by a non-parametric SNK). For the second sampling date (Figure 4.18 B), the colloidal protein concentrations were significantly different from the control and treatment BDN again displayed the highest protein concentration (Kruskal-Wallis,  $H=18.51$ ,  $df=7$ ,  $p < 0.01$ , followed by a non-parametric SNK).

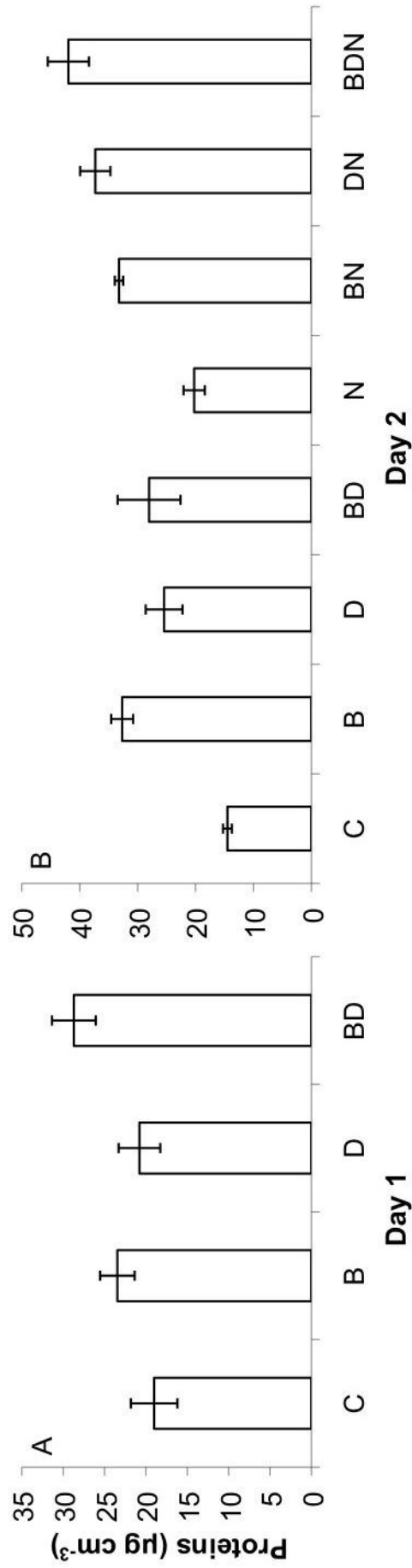




**Figure 4.16:** Chlorophyll a concentration in superficial sediments for the first (A) and second (B) sampling dates (mean  $\pm$  SE). The dashed lines correspond to the average ( $\pm$  95% interval of confidence represented by the grey area) of all the treatments which were not originally inoculated with diatoms (e.g. the dashed line in fig. B was calculated with treatments C, B, N and BN). The differences between the dashed line and the remaining treatments was tested (NS: not significant, \*: significant difference).



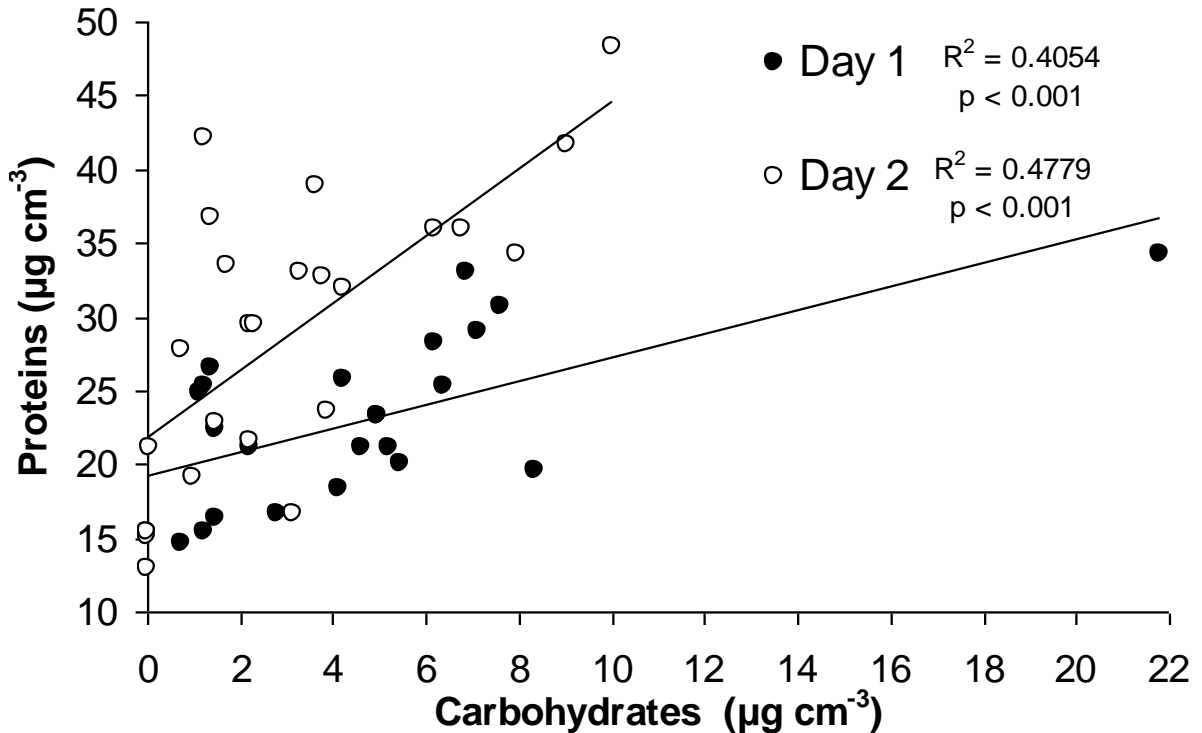
**Figure 4.17:** Mean value ( $n=3$ ) of water - extractable (colloidal) carbohydrates concentrations in superficial sediments for the first (A) and second (B) sampling dates (mean  $\pm$  SE).



**Figure 4.18:** Mean value ( $n=3$ ) of water - extractable (colloidal) proteins concentrations in superficial sediments for the first (A) and second (B) sampling dates (mean  $\pm$  SE).

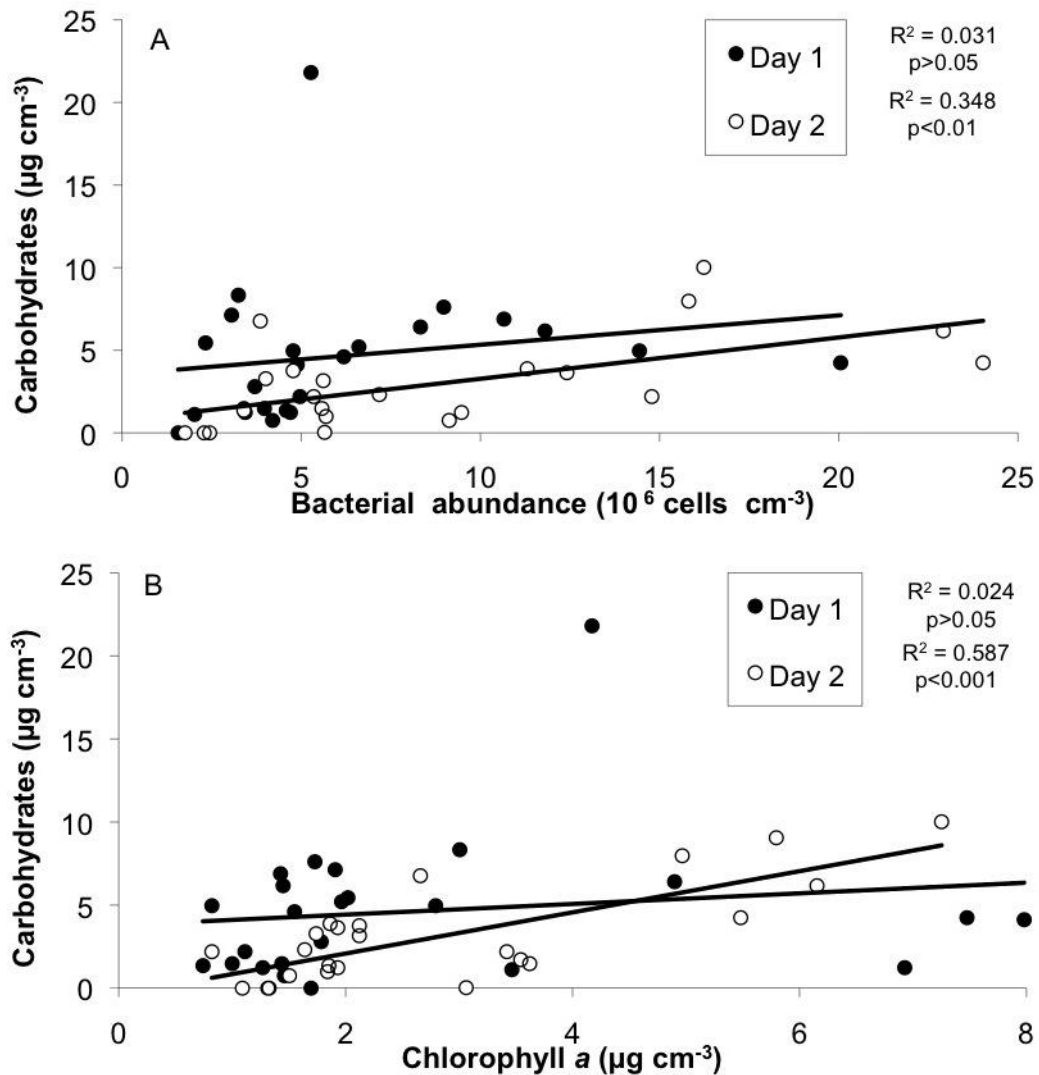
**Relationship between microbial biomass and EPS compounds**

For the first and the second sampling dates, colloidal carbohydrates and proteins were always significantly correlated (Pearson correlation coefficient  $r=0.64$  and  $0.69$ ,  $p<0.001$ , Figure 4.19).



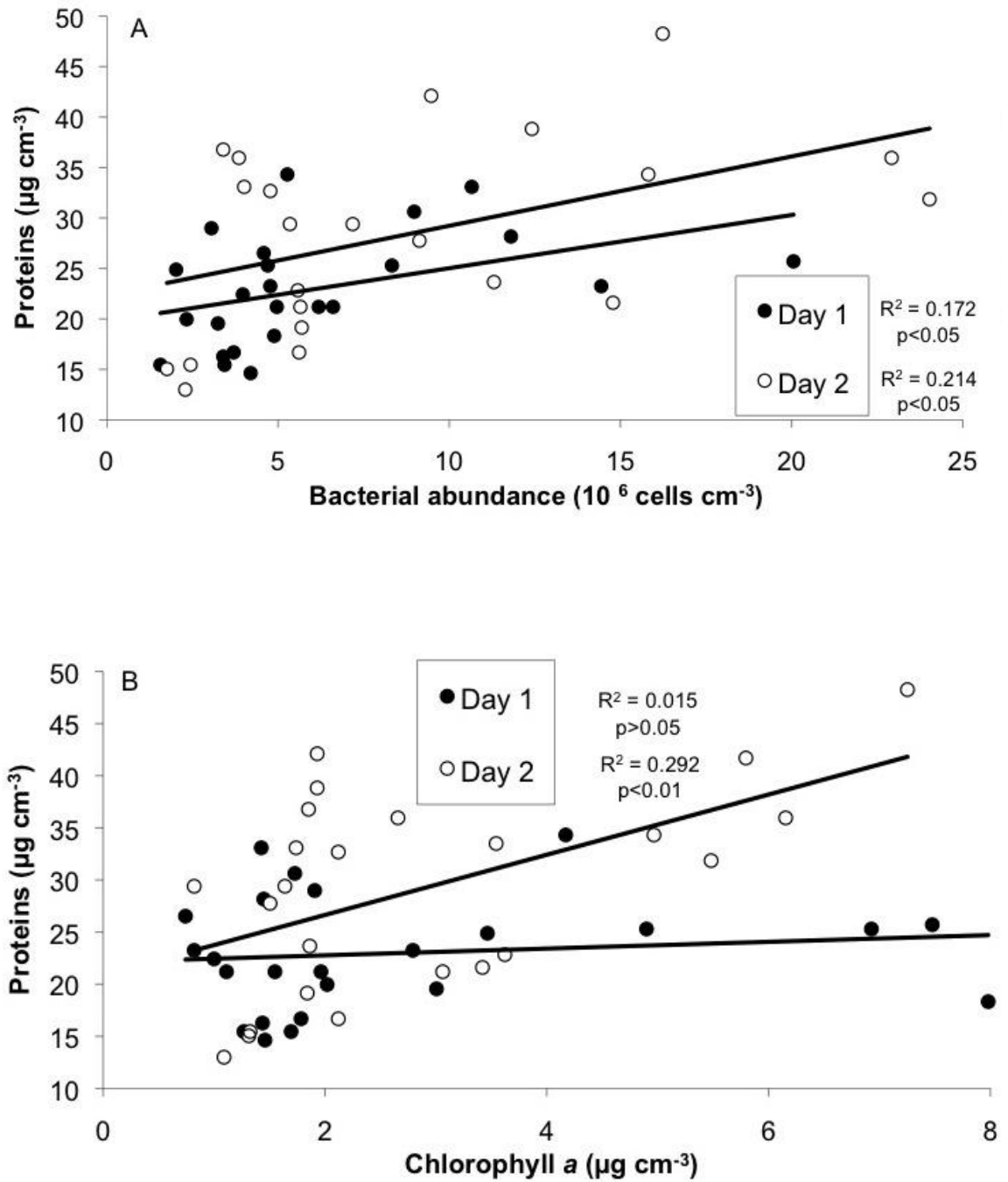
**Figure 4.19:** Simple linear regressions between colloidal proteins and carbohydrates ( $n=24$ ) for the first (day 1) and the second (day 2) sampling dates. The coefficient of determination ( $R^2$ ) and the  $p$ -value are indicated.

On the first sampling day no significant relationship was found between colloidal carbohydrates and bacterial abundance and colloidal carbohydrate and chlorophyll  $a$  (Figure 4.20 A and B). On the second sampling day a strong positive relationship between colloidal carbohydrates and bacterial abundance and chlorophyll  $a$  was observed (Pearson correlation coefficient  $r=0.59$ ,  $p<0.01$  and  $r=0.76$ ,  $p<0.001$ , respectively, Figure 4.20 A and B).



**Figure 4.20:** The relationship between (A) colloidal carbohydrates and bacterial abundance ( $n=24$ ) and (B) chlorophyll *a* ( $n=24$ ) for the first (day 4) and the second (day 10) sampling dates. The coefficient of determination ( $R^2$ ) and the  $p$ -value are indicated.

There was a significant relationship between colloidal proteins and bacterial abundance on the first and second sampling days (Pearson correlation coefficient  $r=0.41$  and  $0.46$ ,  $p < 0.05$ , Figure 4.21 A). Colloidal proteins exhibited strong positive relationship to chlorophyll *a* at the second day of experiment (Pearson correlation coefficient  $r=0.54$ ,  $p < 0.01$ ), but not at the first sampling day (Figure 4.21 B).



**Figure 4.21:** The relationship between (A) colloidal proteins and bacterial abundance ( $n=24$ ) and (B) chlorophyll a ( $n=24$ ) for the first (day 1) and the second (day 2) sampling dates. The coefficient of determination ( $R^2$ ) and the  $p$ -value are indicated.

#### 4.4. Discussion

##### 4.4.1. The coexistence of axenic microalgae culture: growth rate and EPS production.

The results obtained in this experiment, confirmed the main hypothesis, that increasing levels of biodiversity will lead to an increase in microbial biomass. In this experiment, there was no contribution by bacteria in this interaction. The difference between the treatments in terms of biomass and EPS production were addressed only to coexistence of these microalgae species and their preferences to abiotic conditions. Many laboratory studies have shown that the proportion of EPS, produced by algae, varies widely among species and is affected by the physiological status of the algae and by experimental conditions (Smith and Underwood 2000, Goto et al. 2001, Underwood et al. 2004, Cyr and Morton 2006). Values of colloidal EPS components, obtained in present experiment, were in range  $1-5 \mu\text{g cm}^{-3}$ , which is lower than values obtained in previously reported experiment (Yallop et al. 2000, Cyr and Morton 2006, Gerbersdorf et al. 2009). The carbohydrate - protein ratio (1:1) in present experiment were also differing from previously published results (1:2 or 1:5). This may be due to the absence of bacterial culture in this experiment, which may be significant contributor to the overall EPS pool.

The continuous increase of microalgae biomass was observed in treatments where cultures were combined, with more pronounced increase for mixture of three species ANO. At the end of the experiment the treatments ANO and AN showed similar biomass levels as indicated by chlorophyll *a* concentration, however mixture of AO was significantly lower. This may be explained by a density dependent effect, such *Amphora* spp producing a large population of cloned individuals (Davies et al. 1998) and suppressing the growth of *Oscillatoria* spp. The results also confirmed that the biomass of single *Amphora* spp biofilms were higher than in the mixed cultures. In contrast, other single species biofilms (*Navicula* spp and *Oscillatoria* spp) had much lower biomass also their combined cultures which showed a very much lower level of biomass. In fact, the chlorophyll *a* levels in treatment O and NO exhibited a slight increase over the first week of experiment and a rapid decrease thereafter. The slow rate of the development of *Oscillatoria's* biofilm may be a result of low temperature regime (Watermann et al. 1999). The glass beads, which typically simulated sand type

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of the sediment, were used in the present study. Previous investigations (Admiraal 1977, Watermann et al. 1999) suggested that benthic diatoms prefer muddier sediment. In fact, during the present experiment *Navicula*'s biofilm developed very little. This was confirmed by the visual observation of the biofilm along with low concentrations of chlorophyll *a*. This result was mirrored in the analysis of carbohydrate and protein concentrations. Culture conditions such as nutrition, light and temperature etc. also affect the productivity of algal extracellular substances, as has been observed in previous studies (De Philippis and Vincenzini 1998). Examining the sum of EPS across treatments does not give a clear answer. The differences between treatments in terms of carbohydrate production followed microbial biomass. In contrast to this and despite their low biomass, EPS protein produced by *Oscillatoria* culture shown higher concentration than other single culture. This may suggest that (a) *Oscillatoria*'s EPS mainly consist of protein rather than carbohydrate components and (b) *Oscillatoria* species produced larger quantities of EPS proteins than diatom species. The protein concentration in mixed cultures with *Oscillatoria* were higher than proteins produced by mixed diatoms culture AN. The three species ANO had the highest concentration of EPS, at least at the end of the experiment, but the single species A treatment had almost the same concentration of EPS suggesting that *Amphora* produces high levels of EPS, thus may have a higher stabilisation potential than other species.

#### 4.4.2. The species-specific interactions of natural bacteria and microalgae

##### ***The individual and combined microbial assemblages***

The comparison of pure bacterial, axenic microalgal and mixed (bacteria+microalgae) assemblages was designed to provide insights into the individual and combined functional capacity of the heterotrophic and autotrophic biofilm components in terms of substratum properties (Chapter 5). The concentration of colloidal carbohydrates in present experiment were between 50-150  $\mu\text{g cm}^{-3}$ , which is similar to the values reported in previous study (Hirst et al. 2003, Cyr and Morton 2006). Extracellular proteins are rarely measured in marine intertidal flats (Staats et al. 1999, Cyr and Morton 2006). EPS production is also strongly depend of abiotic factors, such as nutrients enrichment, temperature and culture condition (Underwood et al. 2004).

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Thus, it is difficult to perform comparison in EPS values and ratios of EPS components, due to differences in experimental conditions. However, the value of colloidal protein ( $20\text{-}230 \mu\text{g cm}^{-3}$ ) and ratios between carbohydrate and protein (1:1.5) were comparable with values and ratio of carbohydrates and protein 1:2, obtained in by Gerbersdorf et al. (2009).

Separation of the influence of component assemblages of bacteria and diatoms in nature is problematic. In this study the approach was to use assemblages derived from natural systems but manipulated to create the segregation of bacteria and diatoms. However a mixture of antibiotics was used to inhibit bacterial growth which may have raised some potential problems. Chloramphenicol has been reported to suppress the growth of microalgae in general and diatoms in particular (Campa-Cordova et al. 2006, Lai et al. 2009).

It is also known that some microalgae, among them diatoms, require an association with certain bacteria and might be hampered in their metabolic activities of growth and organic matter release otherwise (Fukami et al. 1997, Guerrini et al. 1998, Wichard et al. 2005, Grossart and Simon 2007, Bruckner et al. 2008, Levy et al. 2009). In this study, the microalgal biomass was significantly lower in the axenic diatom assemblage (D) as compared to the assemblage associated with bacteria (BD) which may be an indication of physiological damage and/or species selection by antibiotic treatment or the influence of bacteria/diatom association. In contrast, the bacterial growth was unspoiled in the pure culture without microalgae, showing an impressive increase over the first weeks of the experiment.

It was first hypothesized that the grouping of bacteria and diatoms in the mixed assemblages might result in synergy in community EPS secretion and therefore substratum stabilisation (Chapter 5). The first of these concepts is supported by the data in terms of EPS carbohydrate production but not for EPS protein production.

It is often said that diatoms are promoted by nutrient recycling of the bacteria to enhance their growth rate, cell yield and EPS polysaccharides release (Guerrini et al. 1998, Elifantz et al. 2005, Klug 2005, Grossart and Simon 2007). Over the first 10 days of the experiment, the better development of microalgal biomass in the natural assemblage, as compared to the axenic microalgal culture, seemed to support this possibility. However, with time, the microalgal biomass decreased to comparable levels in both treatments that would not be expected with continuous inorganic

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nutrient supply. Furthermore, the microalgal community composition and the nutrient requirements of the determined species were quite similar over time in both biofilms and thus gave no hints to more or less preferable nutrient conditions. In fact, the natural and axenic microalgal assemblages were both dominated by typical poly- to hypertrophic species found in fresh-brackish waters. In the last week of the experiment, species diversity declined similarly in both biofilms over time until small *Navicula* species remained. This indicates laboratory conditions were not ideal, supporting earlier work on diatom assemblages in laboratory systems (Defew et al. 2002). Surprisingly, the bacterial cell numbers along with the bacterial dividing rates were significantly lower in the mixed assemblage as compared to the pure bacterial culture. In the literature, it is reported that bacteria develop concomitant with benthic microalgae (Bowen et al. 2009) and they adapt quickly to the different organic microalgal exudates with substrate-specific responses regarding enzyme activity and compositional shifts, usually resulting in stimulated bacterial growth and metabolic activity (Schaefer et al. 2002, Haynes et al. 2007). However, the bacteria consortia that developed in this system did not seem to profit from the presence of diatoms. There is a possibility that the bacteria were actively suppressed by the diatoms. It is known that marine bacteria are very sensitive to polyunsaturated aldehydes (PUAs) that are produced by a range of microalgae species (Wichard et al. 2005, Ribalet et al. 2008). This possibility requires further study in benthic systems. In addition, diatoms could have profited better from the initial nutrient concentrations in the inoculums to outcompete bacteria for nutrient in the initial stage of culture. This has been shown for a mixed assemblage with external nutrient supply (Gerbersdorf et al. 2009), although bacteria are usually known as superior nutrients competitors (Jansson 1993). It might be more likely that we are observing a selection/adaptation process as the natural microbial biofilms adapted to culture condition and populations capable of co-existing or exploiting algal/bacterial species were promoted, as has been shown for floodplains and estuaries (Boivin et al. 2007, Haynes et al. 2007). Indeed, the bacterial community showed pronounced composition shifts with the presence of diatoms in the mixed assemblage during the experiment. While the gram negative Proteobacteria continuously constituted the majority of the bacterial community, the percentage of  $\alpha$ ,  $\beta$ ,  $\gamma$ -Proteobacteria changed over time. Members of  $\alpha$ -Proteobacteria were more prominent in the mixed assemblage than in the bacterial culture, although

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the absolute increase over time was similar in the two relevant treatments. Members of  $\alpha$ -Proteobacteria as well as from the *Cytophaga-Flavobacterium-Bacteroides* (CFB) phylum were identified as “satellite bacteria” to marine diatoms (Schaefer et al. 2002). That might explain the preferred association of  $\alpha$ -Proteobacteria with diatoms in the mixed assemblage while hybridization to the CFB phylum was similar to the pure bacterial biofilm over time. In contrast,  $\beta$ -Proteobacteria decreased in both treatments, but this decrease was most pronounced in the natural assemblage where the presence of diatoms might have posed an additional stress factor. Otherwise, their decrease might be related to the constantly high salinity conditions (30 Practical Salinity Units) during the experiment, since  $\beta$ -Proteobacteria are typical for fresh/brackish water habitats (Gockner et al. 1999), where they experience varying salinities in the low-medium range. The  $\gamma$ -Proteobacteria increased solely in the bacterial assemblages and remained unchanged in the mixed biofilm, thus seem to have an inferior role in the presence of diatoms. Hence, the bacterial assemblage seemed to adapt in composition to the presence of diatoms rather than the other way around. Altogether, the data on microbial biomass/cell numbers and community composition gave no evidence for mutual advantages of bacteria and diatoms by their co-existence in the present experiment.

### ***The EPS matrix***

It is generally stated that diatoms produce mainly EPS polysaccharides while bacteria secrete larger proportions of EPS proteins (e.g. Decho 2000, Flemming and Wingender 2001). This is supported by the significantly higher carbohydrate concentrations in the axenic microalgal assemblage as opposed to the bacterial biofilm.

Neither carbohydrates nor proteins are exclusively linked to microalgae or bacterial occurrence and their proportion might not always be as expected from the literature, as could be seen in the pure bacterial and axenic microalgal cultures. Consequently, EPS carbohydrates and EPS proteins in the mixed assemblage were significantly and positively correlated to microalgal biomass *and* bacterial cell numbers; and these relationships were also broadly supported in the pure cultures. In addition, the characteristics of one particular EPS component, carbohydrate or protein, most likely differs between the heterotrophic and autotrophic producers. Thus, the co-bonding of *microalgal* EPS and *bacterial* EPS in the biofilm matrix of the natural assemblage

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might have changed the “quality” of binding as compared to the pure bacterial and microalgal biofilms. The alteration of the EPS concentrations in the mixed assemblage as opposed to the single cultures of bacteria and microalgae were of an additive (proteins), or synergistic (carbohydrates) nature.

#### **4.4.3. The effect of nematodes on microbial growth and EPS production**

##### ***Biofilm development prior to nematode inoculation***

During the experiment, differences between quantity of EPS compounds in treatments which were not inoculated with nematodes B, D and BD, were directly implicated by changes in microbial biomass. Such as the reduction in colloidal carbohydrate in treatments D and BD correlated with a decrease in microalgal biomass in these treatments as indicated by concentrations of Chl *a*. While microalgae secrete mainly polysaccharides (Staats et al. 1999, Stal 2003), bacterial EPS consists of high proportion of proteins (Flemming and Wingender 2001). In fact, increasing bacterial biomass, as determined by flow cytometry was accompanied by an elevation of EPS proteins. The production of such compounds is relatively variable in natural environments and depends strongly on the physiological state of the cells and the environmental conditions (Decho 1990). Thus, inter-comparison between EPS concentration with previous experiment is not realistic, due to differences in experimental conditions and microbial biomass. However, the results obtained in this experiment confirmed previous results which found that when bacteria and diatoms were grown together (BD), they produced much more EPS than when grown separately. This treatment was consistently the highest in EPS concentrations and the highest microbial abundance/biomass as compared with single treatments. Whilst there was a significant relationship between EPS carbohydrate and microbial biomass, the significance level was more pronounced for Chl *a* concentration ( $p < 0.001$ ), than for bacterial cell number ( $p < 0.01$ ). These results suggest that microalgae were more responsible for EPS carbohydrate production than bacteria. The EPS proteins data shows a similar relationship ( $p < 0.05$ ) to chlorophyll *a* and bacterial cell number thus is most like that total protein concentration is a result of both microalgae and bacterial production.

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***Effect of bacterivorous nematodes on microbial growth and exopolymer production***

During locomotion, many nematodes secrete significant amounts of mucus, which may agglutinate sediment particles (Gerlach 1978, Riemann and Schrage 1978), fix eggs to substrata (Moens pers. observ.), or facilitate settlement of specific strains of bacteria (Moens et al. 2005) and life stages of microalgae (Warwick 1981). It has been suggested that these mucus tracks serve to trap bacteria, which will then be grazed upon by the nematodes (mucus-trap hypothesis, Riemann and Schrage 1978, Moens et al. 2005), but this hypothesis has not been confirmed. Nematode mucus secretions contain a substantial share of acid mucopolysaccharides (Riemann and Schrage 1978). However, when grown alone (treatment N), in this experiment, nematodes did not produce high amounts of colloidal EPS (Figure 4.17 B and 4.18 B). This could be due to the low abundances of nematodes, or to the absence of bacterial food in treatment N, which may have negatively impacted nematode activity and movement. The co-occurrence of bacteria and nematodes (treatment BN) significantly increased the EPS production compared to treatment N, but not compared to treatment B (Figure 4.17 B and 4.18 B). Bacterial abundance however was drastically higher in BN than in B. Bacterial grazing by nematodes was clearly not high enough to negatively impact bacterial proliferation. Such top-down controls on bacterial abundance probably only occur at high abundances of nematodes with high grazing rates (De Mesel et al. 2006), and not at the relatively low nematode densities of this experiment. A stimulatory effect of nematodes on bacterial abundance may result from (a) microbioturbation, improving oxygen and nutrient distribution in sediments (Alkemade et al. 1992, Aller and Aller 1992), (b) moderate grazing, preventing bacteria from rapidly reaching carrying capacity and (c) excretion of N-rich compounds by nematodes which stimulate microbial growth (Ingham et al. 1985, Ferris et al. 1998). The first and last explanations appear most plausible for this experiment. Nematode movement may have facilitated oxygen penetration into the sediment. At the same time, bacteria-feeding nematodes generally excrete N assimilated in excess of that required for growth (Ferris et al. 1997, Ferris et al. 1998). This excess N is usually excreted as ammonium and may relieve nutrient limitation for bacteria as well as for diatoms.

The simultaneous presence of diatoms in treatment DN increased the EPS production compared to treatment N (Figure 4.17 B and 4.18 B) as well as the presence of nematodes may stimulate protein production and chlorophyll *a* concentrations

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compared to treatment D. A possible explanation is that the above-mentioned N-excretion by nematodes provided enough nutrients to stimulate diatom growth and protein production.

In the presence of nematodes, the additive effect observed between bacteria and diatoms at the first sampling (Day 1) was even more pronounced. For instance, the carbohydrate concentration increased from the control to BDN (e.g. carbohydrate content in C<D<BD<BDN, Figure 4.17 B). As mentioned before, nematodes probably stimulated bacterial growth and subsequent mineralization/nutrient generation, which may in return have stimulated microagal proliferation. In addition, as hypothesised by Riemann & Helmke (2002), the metabolic activities of mucus-secreting nematodes and the associated bacteria may have complemented each other in this treatment. In this study, nematodes in treatment BDN probably obtained most of their nitrogen from the ingestion and digestion of bacterial cells. In addition, it is possible that, conversely to other treatments, the presence of bacteria helped to fulfil nematode and diatom nitrogen needs by breaking down proteins. This could explain why treatment BDN, which assembled all the studied organisms, displayed the highest polymer and biomass contents.

### **Declaration**

The results presented in this chapter have formed all or part of the following publications:

- HV Lubarsky, C Hubas, M Chocholek, F Larson, W Manz, DM Paterson, SU Gerbersdorf (2010). The stabilisation potential of individual and mixed assemblages of natural bacteria and microalgae. *PLoS One* **5** (e13794) 1-12.
- C Hubas, C Sachidhanandam, H Rybarczyk, HV Lubarsky, A Rigaux, T Moens and DM Paterson (2010). Bacterivorous nematodes stimulate microbial growth and exopolymer production in marine sediments microcosms. *Marine Ecology Progress Series* **419**, 85-94.

## References

- Admiraal, W. 1977. Influence of light and temperature on growth rate of estuarine benthic diatoms in culture. *Marine Biology* **39**:1-9.
- Admiraal, W. and H. Peletier. 1980. Influence of seasonal variations of temperature and light on the growth rate of cultures and natural populations of intertidal diatoms. *Marine Ecology* **2**:35-43.
- Admiraal, W., H. Peletier, and T. Brouwer. 1984. The seasonal succession patterns of diatom species on an intertidal mudflat: an experimental analysis. *Oikos* **42**:30-40.
- Admiraal, W., H. Peletier, and H. Zomer. 1982. Observations and experiments on the population dynamics of epipelagic diatoms from an estuarine mud flat. *Estuarine Coastal and Shelf Science* **14**:471-487.
- Alkemade, R., A. Wielemaker, S. A. Dejong, and A. J. J. Sandee. 1992. Experimental-evidence for the role of bioturbation by the marine nematode *Diplolaimella dievengatensis* in stimulating the mineralization of *Spartina-anglica* detritus. *Marine Ecology-Progress Series* **90**:149-155.
- Aller, R. C. and J. Y. Aller. 1992. Meiofauna and solute transport in marine muds. *Limnology and Oceanography* **37**:1018-1033.
- Barranguet, C., P. M. J. Herman, and J. J. Sinke. 1997. Microphytobenthos biomass and community composition studied by pigment biomarkers: importance and fate in the carbon cycle of a tidal flat. *Journal of Sea Research* **38**:59-70.
- Berland, B. R. and S. Y. Maestrini. 1969. Action de quelques antibiotiques sur le développement de cinq diatomées en culture. *Journal of Experimental Marine Biology and Ecology* **3**:62-75.
- Boivin, M. E. Y., G. D. Greve, J. V. Garcia-Meza, B. Massieux, W. Sprenger, M. H. S. Kraak, A. M. Breure, M. Rutgers, and W. Admiraal. 2007. Algal-bacterial interactions in metal contaminated floodplain sediments. *Environmental Pollution* **145**:884-894.
- Bottcher, M. E., B. Hespeneheide, E. Llobet-Brossa, C. Beardsley, O. Larsen, A. Schramm, A. Wieland, G. Bottcher, U. G. Berninger, and R. Amann. 2000. The biogeochemistry, stable isotope geochemistry, and microbial community structure of a temperate intertidal mudflat: an integrated study. *Continental Shelf Research* **20**:1749-1769.
- Bowen, J. L., B. C. Crump, L. A. Deegan, and J. E. Hobbie. 2009. Increased supply of ambient nitrogen has minimal effect on salt marsh bacterial production. *Limnology and Oceanography* **54**:713-722.
- Bruckner, C. G., R. Bahulikar, M. Rahalkar, B. Schink, and P. G. Kroth. 2008. Bacteria associated with benthic diatoms from lake Constance: phylogeny and influences on diatom growth and secretion of extracellular polymeric substances. *Applied and Environmental Microbiology* **74**:7740-7749.
- Campa-Cordova, A. I., A. Luna-Gonzalez, F. Ascencio, E. Cortes-Jacinto, and C. J. Caceres-Martinez. 2006. Effects of chloramphenicol, erythromycin, and furazolidone on growth of *Isochrysis galbana* and *Chaetoceros gracilis*. *Aquaculture* **260**:145-150.

- Cyr, H. and K. E. Morton. 2006. Distribution of biofilm exopolymeric substances in littoral sediments of Canadian Shield lakes: the effects of light and substrate. *Canadian Journal of Fisheries and Aquatic Sciences* **63**:1763-1776.
- Danovaro, R., M. Armeni, A. Dell'Anno, M. Fabiano, E. Manini, D. Marrale, A. Pusceddu, and S. Vanucci. 2001. Small-scale distribution of bacteria, enzymatic activities, and organic matter in coastal sediments. *Microbial Ecology* **42**:177-185.
- Davies, M. S., R. Dixey, and J. C. Green. 1998. Evaluation of the effects of extremely low frequency electromagnetic fields on movement in the marine diatom *Amphora coffeaeformis*. *Biological Bulletin* **194**:194-223.
- de Deckere, E. M. G. T., T. J. Tolhurst, and J. F. C. de Brouwer. 2001. Destabilization of Cohesive Intertidal Sediments by Infauna. *Estuarine, Coastal and Shelf Science* **53**:665-669.
- De Mesel, I., S. Derycke, T. Moens, K. Van der Gucht, M. Vincx, and J. Swings. 2004. Top-down impact of bacterivorous nematodes on the bacterial community structure: a microcosm study. *Environmental Microbiology* **6**:733-744.
- De Mesel, I., S. Derycke, J. Swings, M. Vincx, and T. Moens. 2006. Role of nematodes in decomposition processes: Does within-trophic group diversity matter? *Marine Ecology-Progress Series* **321**:157-166.
- De Philippis, R. and M. Vincenzini. 1998. Exocellular polysaccharides from cyanobacteria and their possible applications. *FEMS Microbiology Reviews* **22**:151-175.
- de Winder, B., N. Staats, L. J. Stal, and D. M. Paterson. 1999. Carbohydrate secretion by phototrophic communities in tidal sediments. *Journal of Sea Research* **42**:131-146.
- Decho, A. W. 1990. Microbial exopolymer secretions in ocean environments: their role(s) in food webs and marine processes. *Oceanography and Marine Biology: an Annual Review* **28**:73-153.
- Decho, A. W. 2000. Microbial biofilms in intertidal systems: an overview. *Continental Shelf Research* **20**:1257-1273.
- Defew, E. C., D. M. Paterson, and S. E. Hagerthey. 2002. The use of natural microphytobenthic assemblages as laboratory model system. *Marine Ecology-Progress Series* **237**:15-25.
- Defew, E. C., R. G. Perkins, and D. M. paterson. 2004. The light and temperature interactions on a natural estuarine microphytobenthic assemblage. *Biofilms* **1**:21-30.
- Dejong, L. and W. Admiraal. 1984. Competition between 3 estuarine benthic diatom species in mixed cultures. *Marine Ecology-Progress Series* **18**:269-275.
- Dejonge, V. N. and F. Colijn. 1994. Dynamics of microphytobenthos biomass in the Ems estuary *Marine Ecology-Progress Series* **104**:185-196.
- dos Santos, G. A. P., S. Derycke, V. G. Fonsêca-Genevois, L. C. B. B. Coelho, M. T. S. Correia, and T. Moens. 2008. Differential effects of food availability on population growth and fitness of three species of estuarine, bacterial-feeding nematodes. *Journal of Experimental Marine Biology and Ecology* **355**:27-40.
- Elifantz, H., R. R. Malmstrom, M. T. Cottrell, and D. L. Kirchman. 2005. Assimilation of polysaccharides and glucose by major bacterial groups in the Delaware Estuary. *Applied and Environmental Microbiology* **71**:7799-7805.
- Epstein, S. S., D. Alexander, K. Cosman, A. Dompe, S. Gallagher, J. Jarsobski, E. Laning, R. Martinez, G. Panasik, C. Peluso, R. Runde, and E. Timmer. 1997. Enumeration of



- sandy sediment bacteria: Are the counts quantitative or relative? *Marine Ecology-Progress Series* **151**:11-16.
- Falciatore, A. and C. Bowler. 2002. Revealing the molecular secrets of marine diatoms. *Annual Review of Plant Biology* **53**:109-130.
- Ferris, H., R. C. Venette, and S. S. Lau. 1997. Population energetics of bacterial-feeding nematodes: carbon and nitrogen budgets. *Soil Biology & Biochemistry* **29**:1183-1194.
- Ferris, H., R. C. Venette, H. R. van der Meulen, and S. S. Lau. 1998. Nitrogen mineralization by bacterial-feeding nematodes: verification and measurement. *Plant and Soil* **203**:159-171.
- Flemming, H. C. and J. Wingender. 2001. Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects. *Water Science and Technology* **43**:1-8.
- Fukami, K., T. Nishijima, and Y. Ishida. 1997. Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Hydrobiologia* **358**:185-191.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Gerbersdorf, S. U., W. Manz, and D. M. Paterson. 2008. The engineering potential of natural benthic bacterial assemblages in terms of the erosion resistance of sediments. *Fems Microbiology Ecology* **66**:282-294.
- Gerlach, S. A. 1978. Food-chain relationships in subtidal silty sand marine-sediments and role of meiofauna in stimulating bacterial productivity. *Oecologia* **33**:55-69.
- Gockner, F. O., B. M. Fuchs, and R. Amann. 1999. Bacterioplankton compositions of lakes and oceans: a first comparison based on fluorescence in situ hybridization. *Applied and Environmental Microbiology* **65**:3721-3726.
- Goni-Urriza, M., X. de Montaudouin, R. Guyoneaud, G. Bachelet, and R. de Wit. 1999. Effect of macrofaunal bioturbation on bacterial distribution in marine sandy sediments, with special reference to sulphur-oxidising bacteria. *Journal of Sea Research* **41**:269-279.
- Goto, N., O. Mitamura, and H. Terai. 2001. Biodegradation of photosynthetically produced extracellular organic carbon from intertidal benthic algae. *Journal of Experimental Marine Biology and Ecology* **257**:73-86.
- Grossart, H. P. and M. Simon. 2007. Interactions of planktonic algae and bacteria: effects on algal growth and organic matter dynamics. *Aquatic Microbial Ecology* **47**:163-176.
- Guerrini, F., A. Mazzotti, L. Boni, and R. Pistocchi. 1998. Bacterial-algal interactions in polysaccharide production. *Aquatic Microbial Ecology* **15**:247-253.
- Hagerthey, S. E., E. C. Defew, and D. M. Paterson. 2002. Influence of *Corophium volutator* and *Hydrobia ulvae* on intertidal benthic diatom assemblages under different nutrients and temperature regimes. *Marine Ecology Progress Series* **245**:47-59.
- Hansen, K. and E. Kristensen. 1997. Impact of macrofaunal recolonization on benthic metabolism and nutrient fluxes in a shallow marine sediment previously overgrown with macroalgal mats. *Estuarine Coastal and Shelf Science* **45**:613-628.
- Haynes, K., T. A. Hofmann, C. J. Smith, A. S. Ball, G. J. C. Underwood, and A. M. Osborn. 2007. Diatom-derived carbohydrates as factors affecting bacterial community

- composition in estuarine sediments. *Applied and Environmental Microbiology* **73**:6112-6124.
- Heip, C., M. Vincx, and G. Vranken. 1985. The ecology of marine nematodes. *Oceanography and Marine Biology* **23**:399-489.
- Hirst, C. N., H. Cyr, and I. A. Jordan. 2003. Distribution of exopolymeric substances in the littoral sediments of an oligotrophic lake. *Microbial Ecology* **46**:22-32.
- Ingham, R. E., J. A. Trofymow, E. R. Ingham, and D. C. Coleman. 1985. Interactions of bacteria, fungi, and their nematode grazers-effects on nutrient cycling and plant-growth. *Ecological Monographs* **55**:119-140.
- Jain, A., K. K. Nishad, and N. B. Bhosle. 2007. Effects of DNP on the cell surface properties of marine bacteria and its implication for adhesion to surfaces. *Biofouling* **23**:171-177.
- Jansson, M. 1993. Uptake, exchange, and excretion of orthophosphate in phosphate-starved *Scenedesmus quadricauda* and *Pseudomonas* K7. *Limnology and Oceanography* **38**:1162-1178.
- Joseph, P. V. and C. Jacob. 2010. Bacterial, fungal and algal population on pennar river: a fresh water wetland in Kottayam district, Kerala. *Asian Journal of Chemistry* **22**:4286-4290.
- Jung, S. W., B. H. Kim, T. Katano, D. S. Kong, and M. S. Han. 2008. *Pseudomonas fluorescens* HYK0210-SK09 offers species-specific biological control of winter algal blooms caused by freshwater diatom *Stephanodiscus hantzschii*. *Journal of Applied Microbiology* **105**:186-195.
- Kang, Y. H., J. D. Kim, B. H. Kim, D. S. Kong, and M. S. Han. 2005. Isolation and characterization of a bio-agent antagonistic to diatom, *Stephanodiscus hantzschii*. *Journal of Applied Microbiology* **98**:1030-1038.
- Klug, J. L. 2005. Bacterial response to dissolved organic matter affects resource availability for algae. *Canadian Journal of Fisheries and Aquatic Sciences* **62**:472-481.
- Lai, H. T., J. H. Hou, C. I. Su, and C. L. Chen. 2009. Effects of chloramphenicol, florfenicol, and thiamphenicol on growth of algae *Chlorella pyrenoidosa*, *Isochrysis galbana*, and *Tetraselmis chui*. *Ecotoxicology and Environmental safety* **72**:329-334.
- Lee, J. J. 1993. General techniques for the isolation and culture of marine protists from estuarine, littoral, and sublittoral waters. *in* P. F. Kemp, F. B. Sherr, E. B. Sherr, and J. J. Cole, editors. *Handbook of methods in aquatic microbial ecology*. CRC Press.
- Levy, J. L., J. L. Stauber, S. A. Wakelin, and D. F. Jolley. 2009. The effect of bacteria on the sensitivity of microalgae to copper in laboratory bioassays. *Chemosphere* **74**:1266-1274.
- MacIntyre, H. L., R. J. Geider, and D. C. Miller. 1996. Microphytobenthos: The ecological role of the "secret garden" of unvegetated, shallow-water marine habitats .1. Distribution, abundance and primary production. *Estuaries* **19**:186-201.
- Medlin, L. K. 2002. Why silica or better yet why not silica? Speculations as to why the diatoms utilise silica as their cell wall material. *Diatom Research* **17**:453-459.
- Miller, D. C., R. J. Geider, and H. L. MacIntyre. 1996. Microphytobenthos: The ecological role of the "secret garden" of unvegetated, shallow-water marine habitats .2. Role in sediment stability and shallow-water food webs. *Estuaries* **19**:202-212.
-

- Moens, T., G. A. P. dos Santos, F. Thompson, J. Swings, V. Fonseca-Genevois, M. Vincx, and I. De Mesel. 2005. Do nematode mucus secretions affect bacterial growth? *Aquatic Microbial Ecology* **40**:77-83.
- Moens, T. and M. Vincx. 1997. Observations on the feeding ecology of estuarine nematodes. *Journal of the Marine Biological Association of the United Kingdom* **77**:211-227.
- Mu, R. M., Z. Q. Fan, H. Y. Pei, X. L. Yuan, S. X. Liu, and X. R. Wang. 2007. Isolation and algae-lysing characteristics of the algicidal bacterium B5. *Journal of Environmental Sciences-China* **19**:1336-1340.
- Paterson, D. M. and S. E. Hagerthey. 2001. Microhytobenthos in contrasting coastal ecosystems: Biology and dynamics. Pages 105-125 *in* K. Reise, editor. *Ecological comparisons of sedimentary shores*. Springer.
- Raszka, A., M. Chorvatova, and J. Wanner. 2006. The role and significance of extracellular polymers in activated sludge. Part I: Literature review. *Acta Hydrochimica Et Hydrobiologica* **34**:411-424.
- Ribalet, F., L. Intertaglia, P. Lebaron, and R. Casotti. 2008. Differential effect of three polyunsaturated aldehydes on marine bacterial isolates. *Aquatic Toxicology* **86**:249-255.
- Riemann, F. and E. Helmke. 2002. Symbiotic Relations of Sediment-Agglutinating Nematodes and Bacteria in Detrital Habitats: The Enzyme-Sharing Concept. *Marine Ecology* **23**:93-113.
- Riemann, F. and M. Schrage. 1978. The mucus-trap hypothesis on feeding of aquatic nematodes and implications for biodegradation and sediment texture. *Oecologia* **34**:75-88.
- Riera, P., P. Richard, A. Grémare, and G. F. Blanchard. 1996. Food source of intertidal nematodes in the Bay of Marennes-Oléron (France), as determined by dual stable isotope analysis. *Marine Ecology Progress Series* **142**:303-309.
- Round, F. E. 1990. Diatom communities-their response to changes in acidity. *Philosophical Transactions of the Royal Society of London Series B-Biological Sciences* **327**:243-249.
- Schaefer, H., B. Abbas, H. Witte, and G. Muyzer. 2002. Genetic diversity of "satellite" bacteria present in cultures of marine diatoms. *Fems Microbiology Ecology* **42**:25-35.
- Smith, D. J. and G. J. C. Underwood. 1998. Exopolymer production by intertidal epipellic diatoms. *Limnology and Oceanography* **43**:1578-1591.
- Smith, D. J. and G. J. C. Underwood. 2000. The production of extracellular carbohydrates by estuarine benthic diatoms: The effects of growth phase and light and dark treatment. *Journal of Phycology* **36**:321-333.
- Staats, N., B. De Winder, L. J. Stal, and L. R. Mur. 1999. Isolation and characterization of extracellular polysaccharides from the epipellic diatoms *Cylindrotheca closterium* and *Navicula salinarum*. *European Journal of Phycology* **34**:161-169.
- Stal, L. J. 2003. Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. *Geomicrobiology Journal* **20**:463-478.
- Stal, L. J. and A. E. Walsby. 2000. Photosynthesis and nitrogen fixation in a cyanobacterial bloom in the Baltic Sea. *European Journal of Phycology* **35**:97-108.
- Underwood, G. J. C., M. Boulcott, C. A. Raines, and K. Waldron. 2004. Environmental effects on exopolymer production by marine benthic diatoms: Dynamics,
-

- changes in composition, and pathways of production. *Journal of Phycology* **40**:293-304.
- Underwood, G. J. C. and D. M. Paterson. 2003. The importance of extracellular carbohydrate production by marine epipelagic diatoms. *Advances in Botanical Research* **40**:183-240.
- Underwood, G. J. C. and D. J. Smith. 1998. Predicting epipelagic diatom exopolymer concentrations in intertidal sediments from sediment chlorophyll a. *Microbial Ecology* **35**:116-125.
- Villbrandt, M., L. J. Stal, and W. E. Krumbein. 1990. Interactions between nitrogen fixation and oxygenic photosynthesis in a marine cyanobacterial mat. *Fems Microbiology Ecology* **74**:59-72.
- Vu, B., M. Chen, R. J. Crawford, and E. P. Ivanova. 2009. Bacterial extracellular polysaccharides involved in biofilm formation. *Molecules* **14**:2535-2554.
- Warwick, R. M. 1981. Survival strategies of meiofauna. Pages 39-52 in N. V. Jones and W. J. Wolff, editors. *Feeding and survival strategies of estuarine organisms*. Plenum Press, New York.
- Watermann, F., H. Hillebrand, G. Gerdes, W. E. Krumbein, and U. Sommer. 1999. Competition between benthic cyanobacteria and diatoms as influenced by different grain sizes and temperatures. *Marine Ecology-Progress Series* **187**:77-87.
- Wichard, T., S. A. Poulet, and G. Pohnert. 2005. Determination and quantification of alpha, beta, gamma, delta-unsaturated aldehydes as pentafluorobenzyl-oxime derivatives in diatom cultures and natural phytoplankton populations: application in marine field studies. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **814**:155-161.
- Wotton, R. S. 2004. The ubiquity and many roles of exopolymers (EPS) in aquatic systems. *Scientia Marina* **68**:13-21.
- Wynn-Williams, D. D., N. C. Russell, and H. G. M. Edwards. 1997. Moisture and habitat structure as regulators for microalgal colonists in diverse Antarctic terrestrial habitats. A Balkema Publishers, Leiden.
- Yallop, M. L., B. Dewinder, D. M. Paterson, and L. J. Stal. 1994. Comparative structure, primary production and biogenic stabilization of cohesive and noncohesive marine sediments inhabited by microphytobenthos. *Estuarine Coastal and Shelf Science* **39**:565-582.
- Yallop, M. L., D. M. Paterson, and P. Wellsbury. 2000. Interrelationships between rates of microbial production, exopolymer production, microbial biomass, and sediment stability in biofilms of intertidal sediments. *Microbial Ecology* **39**:116-127.

## Chapter 5

### Coexistence of aquatic organisms in terms of sediment stability

#### Abstract

It is recognized that microorganisms inhabiting natural sediments significantly mediate the erosive response of the bed (“ecosystem engineers”) through the secretion of naturally adhesive organic material (EPS: extracellular polymeric substances). However, the relative importance of the different EPS producers on the stabilization of the sediment matrix is still unknown. The aim of the first experiment was to examine the adhesive capacity of mono-species biofilm surfaces of benthic diatoms and cyanobacteria, as well to find out whether the combination of two or three species in a biofilm would lead to any kind of additive or synergistic effect on the adhesive force. Three species, *Navicula hansenii*, *Amphora coffeaeformis* and *Oscillatoria* species, were grown separately or combined on non-cohesive artificial sediment. The adhesive capacity of the biofilm produced by these species was measured by MagPI over a two week experimental period and related to biological data from Chapter 4. In the present experiment the adhesive properties of the biofilm of the three species (ANO) produced continuously higher adhesive values during the experiment than other biofilms. These results support the main hypothesis and quite clearly point in the direction of an increasing level of adhesive force with increasing

level of biodiversity of the biofilms. It was the first attempt to explore earlier unknown ground and increased knowledge of the area of species contribution in biofilms. This study was made possible by using a high resolution experimental set-up Magnetic Particle Induction (MagPI). This knowledge will lead to a deeper understanding of the effect of changing biodiversity on interspecies relationships and related implications for the properties and quality of biofilms.

Since the natural "microalgal mats" is certainly not devoid of heterotrophic bacteria, the question of the functional role and origin of EPS in microbial mats requires further interpretation and can initially be addressed by separate studies of the engineering potential of prokaryotic and eukaryotic assemblages. The aim of the second experiment was to investigate microbial biostabilisation capacity by using natural benthic bacteria and microalgae cultures growing on artificial sediments over 4 weeks. The sediment stability was measured using both a Cohesive Strength Meter (CSM) and a newly developed device Magnetic Particle Induction (MagPI). The results obtained suggest that stabilisation was significantly higher for the bacterial assemblages (up to a factor of 2) than for axenic microalgal assemblages. The EPS concentration and the EPS composition (Chapter 4) were both important in determining stabilisation. The peak of engineering effect was significantly greater in the mixed assemblage as compared to the bacterial (x1.2) and axenic diatom (x1.7) cultures. The possibility of synergistic effects between the bacterial and algal cultures in terms of stability was examined and rejected although the concentration of EPS did show a synergistic elevation in mixed culture. The rapid development and overall stabilisation potential of the various assemblages was impressive (x7.5 and x9.5, for MagPI and CSM, respectively, as compared to controls). This study confirmed the important role of heterotrophic bacteria in "biostabilisation" and highlights the interactions between autotrophic and heterotrophic biofilm consortia.

### **5.1. Introduction**

In intertidal habitats, the cohesive strength of sediments depends on their physicochemical properties such as water content, density, mineralogy, plasticity, salinity and pH (Dade et al. 1992). Benthic communities colonize these habitats and

form pronounced biofilms (de Winder et al. 1999) which can have a large impact on the whole sediment system. The initial step of biofilm formation is normally regarded as the attachment of microbial cells to a surface by the secretion of polymeric substances. In transient biofilms, however, much of the extracellular polymeric substances (EPS) are secreted as a by-product of the locomotive mechanism of diatoms (Consalvey et al. 2004). In recent years it has been shown that benthic biofilms can also act as a protective layer at the sediment surface that can significantly influence erosion and deposition of sediment particles (Underwood and Paterson 2003). Thus investigation into this “biostabilisation” process is very important in terms of the prediction of sediment erosion potential (Perkins et al. 2004). The major mechanism of this microbial biostabilisation is through the production of EPS matrix which is a complex mixture of carbohydrates, proteins and proteoglycans, secreted by biofilms cells. Previous studies on the influence of EPS on sediment stability have been carried out both in the laboratory (Dade et al. 1992, Battin et al. 2003, Droppo et al. 2007) and in the field (Tolhurst et al. 2000, Hirst et al. 2003) using artificially modified sediment (Droppo 2001) and/or natural sediment (Underwood and Smith 1998, Yallop et al. 2000, Perkins et al. 2003, Gerbersdorf et al. 2005). However biological impact is highly variable and difficult to express as one constant factor. Numerous studies have established a positive correlation between sediment stabilization, EPS and microbial biomass. Some studies have attempted to use chlorophyll *a*/microalgae biomass as an indicator of sediment stability, but the relationships were at best site-specific (e.g. Riethmueller et al. 2000, Defew et al. 2002, Le Hir et al. 2007). However, although biostabilisation has been increasingly studied over the last decade, there are still significant gaps in our knowledge.

Motile epipelagic diatoms are recognized as the main EPS producers in intertidal muddy sediments and as the main contributors to biostabilisation (de Brouwer et al. 2005). MPB alter sediment properties (e.g. erodibility) both directly, by forming a mat on the sediment surface, and indirectly by modifying the activities of benthic infauna (Miller et al. 1996). Nevertheless, due to the microalgal influence on the structure and behaviour of sedimentary habitats, they have been put forward as important “ecosystem engineers” (Boogert et al. 2006), irrespective of their small size that is easily compensated by biomass. The adhesion/cohesion mechanism with the EPS matrix the closely related to the biological function of the polymer in nature has been

discussed (Hu et al. 2003). For instance, for most benthic cyanobacteria, the adhesion mechanism with the matrix is due to the surface hydrophobicity of exopolymers (Fattom and Shilo 1984). The biological function of EPS production and characterisation from benthic algae and cyanobacteria were described in detail by Parikh & Madamwar (2006) who suggest that cyanobacterial EPS is composed of a network of macromolecules having different biochemical properties, which may contribute to extracellular functions (Kawaguchi and Decho 2000). Due to different degrees of substitution and different structures of the main chains, EPS producers were characterized as strong or weak species in terms of cohesion stabilization, nevertheless their EPS were similar both in protein content, in monosaccharides composition and linkage types (Hu et al. 2003). However there is still a significant gap in the knowledge of engineering capacity of microalgae species and their individual contributions to the biostabilisation processes. There is evidence that MPB is highly sensitive to changes of environmental conditions and depends from a range of abiotic factor, such as salinity, temperature, UV radiations and presence of pollutants (Dejong and Admiraal 1984). These changes in environmental conditions may have a direct impact on MPB community structure as one species dominates (out competes) another or a species disappears/collapse population. In this context, the knowledge about the contribution of species to biostabilisation is very important. The first part of this study will make a first attempt to resolve the contribution to the adhesion from individual species. The adhesive capacity of the two benthic diatom, *Navicula hansenii* (N), *Amphora coffeaeformis* (A) and *Oscillatoria* species (O) was examined over two week of experimental period by MagPI. These results were related to EPS (spectrophotometric determination of carbohydrates and proteins) and diatom biomass (spectrophotometric determination of chlorophyll *a*) described in previous chapter (see 4.3.1). A further purpose was to test the hypothesis that higher diversity would lead to increased surface adhesion, because most biofilms found in nature show a higher biodiversity than laboratory systems and that the conjunction of all those species may give some advantages for the biofilm and the system.

While biostabilisation by microalgae has been researched extensively in the marine habitat, the ubiquitous heterotrophic bacteria have largely been ignored, even in conceptual models. However, heterotrophic bacteria also secrete copious amounts of EPS and may have a significant influence in the stabilization of sediment (Dade et al.

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1990, Gerbersdorf et al. 2008). Pioneering studies on the entrainment of a clay-water suspension by Dade et al. (1996) and on the stability of experimentally derived biofilms by Leon-Morales et al. (2007) indicate significant effects of bacterial exopolymers on the substratum. In recent works (Gerbersdorf et al. 2008, Gerbersdorf et al. 2009) it has been shown that natural benthic bacterial assemblages from estuarine areas significantly stabilized test substratum, exceeding by far the importance that might be assumed from the dearth of the literature. Despite their importance in marine ecosystems, marine bacteria and their interaction with microalgae are rarely studied in this context (Ribalet et al. 2007).

The aim of the second part of this study was to examine the individual engineering capability of the main biofilm components (heterotrophic bacterial and autotrophic microalgae) in terms of their relative functional contribution to substratum stabilisation. It was hypothesized that the coexistence of bacteria and microalgae will show synergistic effects on their engineering potential to enhance EPS production and stabilize the substratum. For this purpose, stabilisation potential of bacterial assemblages (B), axenic autotrophic microalgal/diatom assemblages (D) and mixed assemblages of both (BD) growing on non-cohesive glass beads were determined over a period of 25 days. The adhesive capacity as well as the cohesive forces, both proxies for sediment stability, were monitored regularly by MagPI and CSM, respectively, and related to microbial growth: bacterial cell numbers, bacterial dividing rate, microalgal biomass and EPS secretion: concentrations/composition of carbohydrates and proteins (described in Chapter 4).

## 5.2. Experimental set-up

Experiments were performed as described in detail in Chapter 4 (see 4.2.1 and 4.2.2). Briefly, for the first experiment of investigation stabilisation capacity of axenic microalgae culture (4.2.1) the microalgae culture of *Navicula hansenii*, *Amphora coffeaeformis* and *Oscillatoria* species were obtained from monospecific laboratory cultures. A layer of 0.5 cm of <math>63\ \mu\text{m}</math> glass beads in total was placed in disposable plastic trays (70Lx70Wx25H in mm) and 50 ml of autoclaved seawater were added in each box. The control (C) contained only glass beads and autoclaved seawater and was treated regularly by the mixture of antibiotics, to prevent bacterial contaminations. Five replicates were established for each treatment and the

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treatment names refer to the first letter(s) of inoculated culture: A for *Amphora coffeaeformis*, N for *Navicula hansenii* and O for *Oscillatoria* species. The adhesive capacity of the microalgae biofilm was monitored regularly by MagPI (2.13.2), over the experimental period of two weeks (measured on days 1, 4, 7, 12, 15).

The second experiment investigates the engineering effects on a non-cohesive test bed as the surface was colonised by natural benthic assemblages (prokaryotic, eukaryotic and mixed cultures). The bacteria and microalgae culture were isolated from natural sediment (as described 2.2.1 and 2.2.2) and were grown both separately and simultaneously on a non-cohesive artificial substratum (Ballotini balls, glass beads). A three cm layer (minimum operation depth of the Cohesive Strength Meter, CSM) of <63  $\mu\text{m}$  glass beads was placed in Rotilab deep-freezes boxes (208Lx208Wx94H in mm) and 2 L of autoclaved seawater were carefully added to each box. Six replicates per treatment were established and treatment names refer to the first letter(s) of the corresponding culture inoculated: B for bacteria, D for diatoms and BD for the mixed culture of bacteria and diatoms. The controls (C) containing only glass beads and seawater were regularly treated with a mixture of antibiotics. The adhesive capacity and the cohesive forces, both proxies for sediment stability, as determined by MagPI (2.13.2) and CSM (2.13.1) respectively, were monitored regularly over the experimental period 7 times in 4 weeks.

### 5.2.1. Statistics

The data violated assumptions of normality and homogeneity of variance (visual assessment of the frequency histogram and normal plot, Kolmogorov-Smirnov and Barlett tests), thus differences between treatments were assessed using a non-parametric Kruskal-Wallis ( $\chi^2$ ) test (KW), followed by the non-parametric Student-Newman-Keuls (SNK) test to correct for multiple comparisons. Additionally, the Mann-Whitney test was used occasionally to compare pairs of treatments.

**5.3. Results**

**5.3.1. Investigation stabilisation potential of axenic microalgae culture using *Navicula hansenii*, *Amphora coffeaeformis* and *Oscillatoria* species**

***The stability of the substratum***

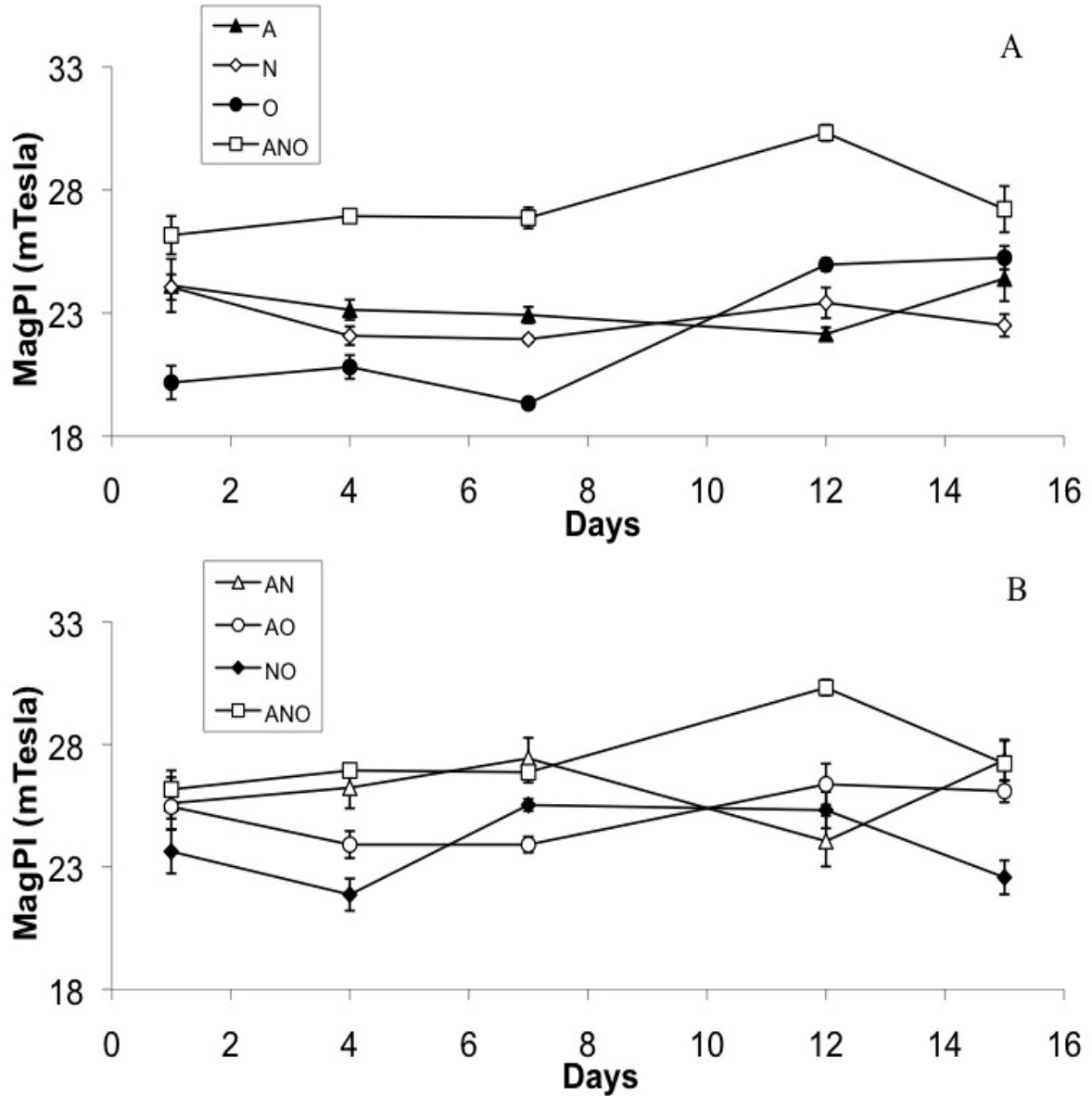
The stability of the sediment surface increased continuously in most treatments up to day 12 (Figure 5.1) and decreased from day 12 until the end of the experimental period. In contrast, there were no significant changes in sediment adhesion/stability for control C sediment, for which the adhesion measurements did not exceed 5 mTesla (to increase the contrast between treatments these data are not presented here). The increase was more pronounced for treatments O and ANO (Figure 5.1 A, Table 5.1).

**Table 5.1:** Differences between the minimum (the first of sampling day) and maximum values reached, as well as differences between mixed assemblages ANO and the given treatments (A, N, O, AN, AO, NO, ANO) both times expressed as quotient/factors for MagPI.

<b>Factor</b>	<b>Treatment</b>	<b>MagPI</b>
Between min and max values	A	1
	N	0.9
	O	1.2
	AN	1.1
	AO	1.0
	NO	1.1
	ANO	1.2
Between ANO and single and combined treatments	A	1.4
	N	1.3
	O	1.2
	AN	1.3
	AO	1.1
	NO	1.2

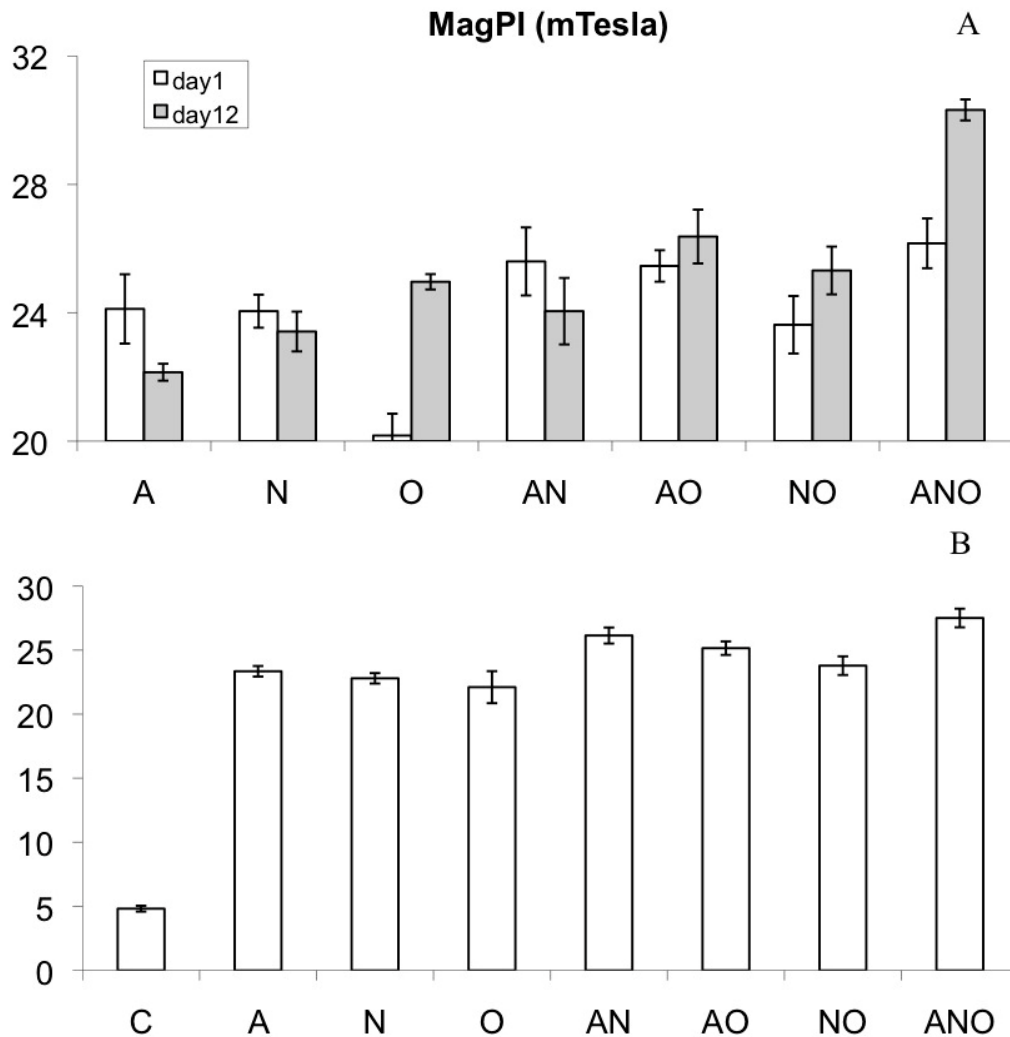
Statistical testing revealed that the differences between the treatments were significant. For example on day 12, stability was significantly different in all treatments (KW,  $\chi^2=30.37$ ,  $df= 6$ ,  $p<0.001$ ). The stability of the biofilm produced by ANO was significantly higher than all treatments ((up to 1.4 times, MW test,  $U=0$ ,  $p<0.001$ ), Figure 5.1, Table5.1). Pair-combined treatment stability was not as high as

the ANO treatment stability but higher than that for the single species cultures, for instance on day 12, treatment AO was significantly higher than treatment A (Mann-Whitney (MW) test,  $U=0$ ,  $p<0.001$ ) and N (MW test,  $U=2$ ,  $p<0.05$ ). Single treatment O was significantly higher than treatment A and N (MW test,  $U=2$ ,  $p<0.05$ ) (Figure 5.1 A).



**Figure 5.1:** Mean values ( $n=5$  per treatment,  $\pm$  SE) of MagPI measurements over the course of the experiment. (A) The different treatments were single culture: ▲ - *Amphora*; ◇ - *Navicula*; ● - *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*. (B) Pairs of mixed cultures: △ - *Amphora* + *Navicula*, ○ - *Amphora* + *Oscillatoria*; ◆ - *Navicula* + *Oscillatoria* and their mixture: □ - *Amphora* + *Navicula* + *Oscillatoria*.

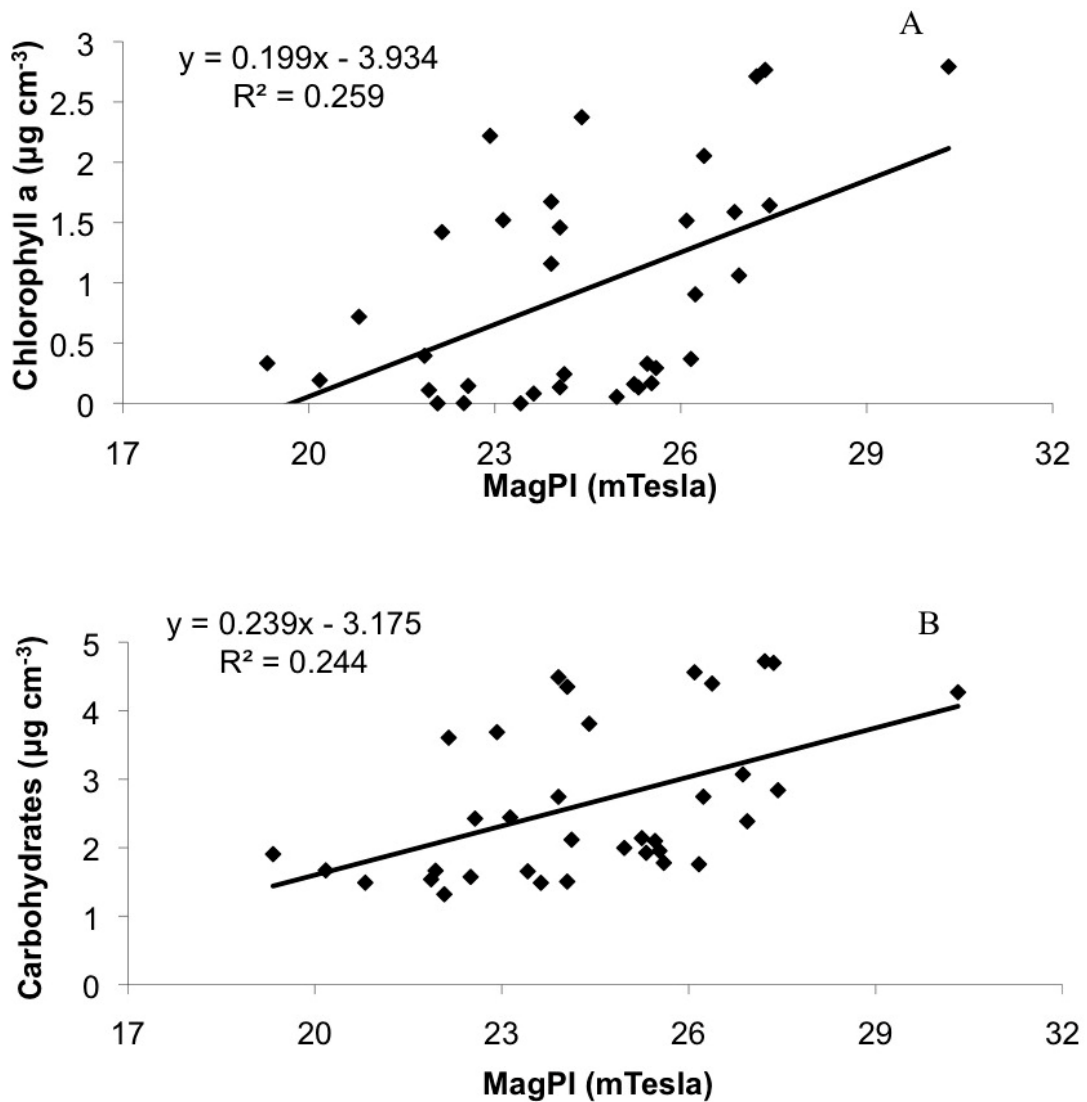
At the day 12, adhesion properties of the biofilm in treatments A, N and AN declined as compared to the first day of the experiment (Figure 5.2 A). Cumulative stability during experimental period was more pronounced for group AN, AO, and ANO (up to 27 mTesla) followed by NO>A>N>O (Figure 5.2 B). The adhesive capacity of the mixed culture biofilm ANO was 5.7 times higher than the control and the single culture was 4.7 times higher than the control (Figure 5.2 B).



**Figure 5.2:** Adhesion capacity as measured by MagPI: (A) between the first sampling day and day 12<sup>th</sup> where most of the variables showed their maximum value. (B) Cumulative adhesion values (n=25) during 2 weeks of experiment. The treatment name (Diatom species) was given according to the first letter of the corresponding culture(s) inoculated: A for Amphora, N for Navicula, O for Oscillatoria and their mixture AN for Amphora and Navicula, AO for Amphora and Oscillatoria, NO for Navicula and Oscillatoria and ANO for Amphora, Navicula and Oscillatoria.

**Relationship between biological variables (described in Chapter 4) and surface adhesion/ stability**

There was a strong positive relationship between adhesion capacity as measured by MagPI and chlorophyll *a* ( $r=0.508$ ,  $N=35$ ,  $p<0.01$ ) and colloidal carbohydrate concentration ( $r=0.492$ ,  $N=35$ ,  $p<0.01$ ) (Figure 5.3 A and B respectively), positive but not significant correlation was found between sediment stability and colloidal proteins concentrations ( $r=0.145$ ,  $N=35$ ,  $p>0.05$ ).



**Figure 5.3:** Relationship between adhesion capacity as measured by MagPI (mTesla) and biological variables ( $n=35$ ). MagPI versus chlorophyll *a* concentrations (A) and MagPI versus colloidal carbohydrates concentrations (B).

**5.3.2. The stabilisation potential of individual and mixed assemblages of natural bacteria and microalgae**

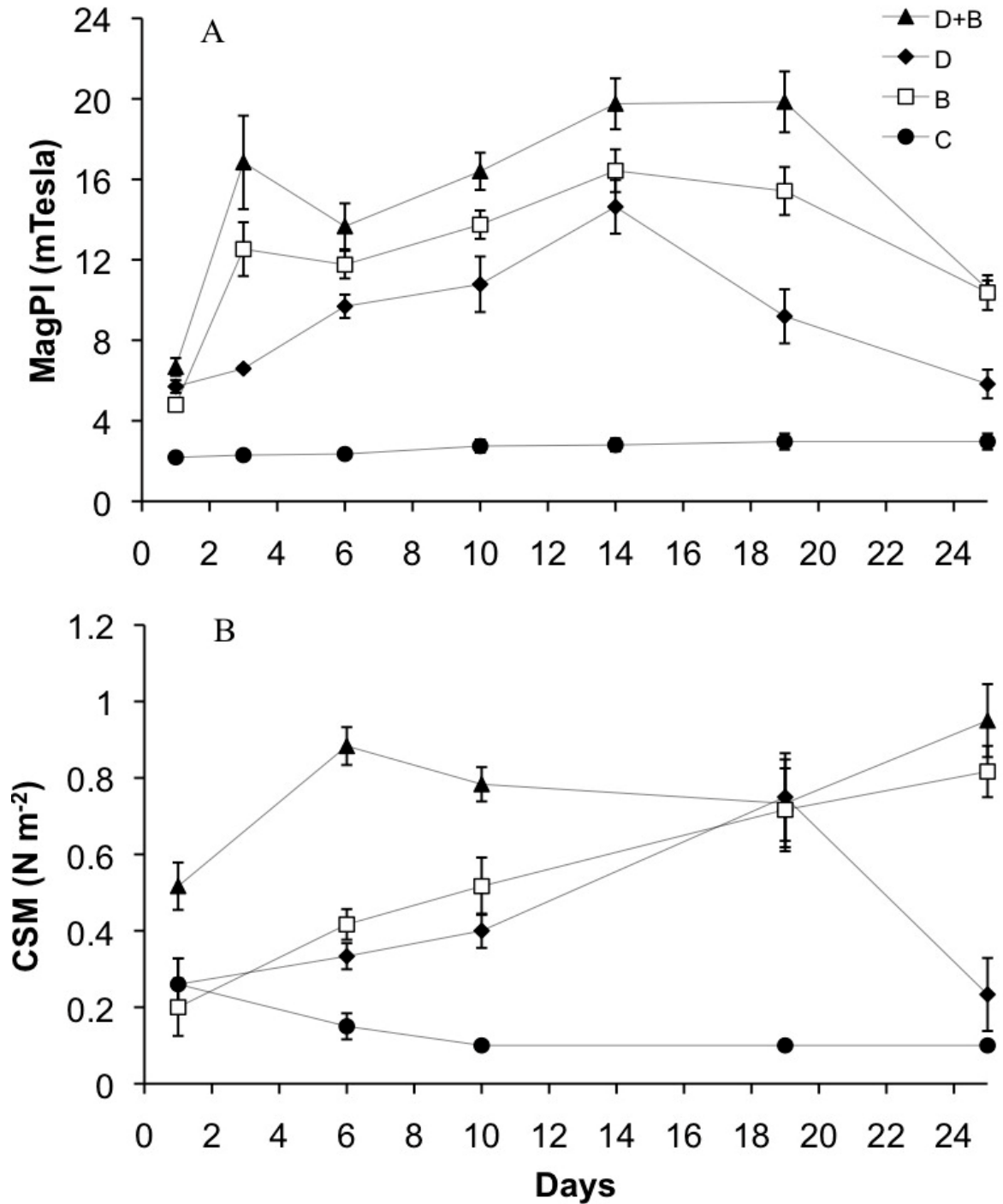
***The stability of the substratum***

The surface adhesion of the substratum, as determined by MagPI, increased for all treatments over time to a maximum value on day 14 (Figure 5.4 A, Table 5.2).

**Table 5.2:** Differences between the first sampling day 1 and day 14 where most of the variables showed their maximum value, as well as differences between the given treatments (mixed: BD, Bacteria B, Diatom D); both times expressed as quotient/factors for MagPI and CSM.

Factors		<i>MagPI</i>	<i>CSM</i>
<b>between day 1-14</b>	<b>B</b>	3.4	4.0
	<b>D</b>	2.6	2.8
	<b>BD</b>	2.9	1.8
<b>between treatments</b>	<b>BD/B</b>	1.4	2.6
	<b>BD/D</b>	2.5	4.1
	<b>B/D</b>	1.7	1.3

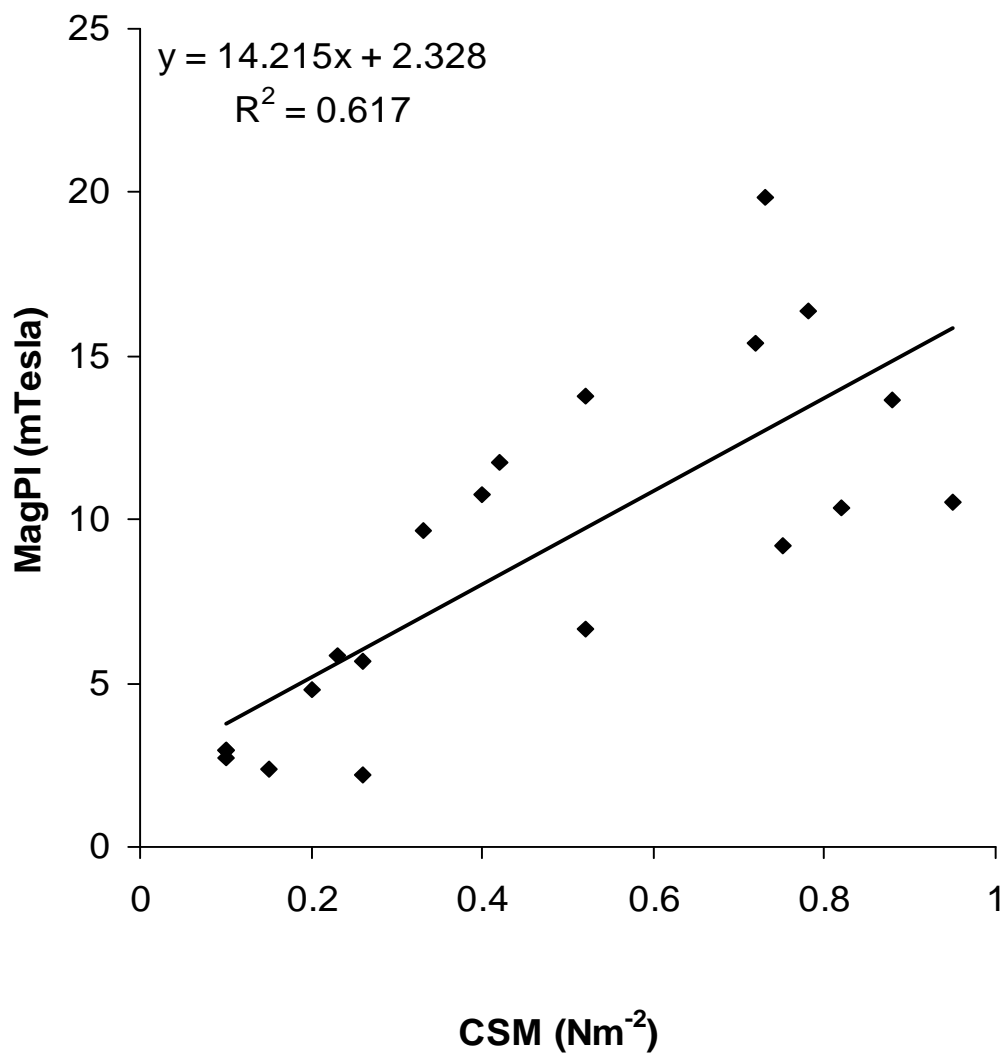
Cohesion of the substratum as indicated by CSM increased continuously for all treatments (Figure 5.4 B, Table 5.2) over the 4 weeks. The control treatments (C) did not show any significant changes in adhesion/stability over the 25 d of the experiment. There was a significant difference in the means of the treatments for the surface adhesion and cohesion ( $p < 0.05$ ) for all dates except at the beginning of experiment. The mixed assemblage (BD) showed the highest surface adhesion of the sediment followed by the bacterial culture (B) and finally, the diatom biofilms (D). The CSM measurements confirmed the MagPI results with significantly higher sediment surface stability in treatment BD followed by B and D (for example, day 24: KW,  $\chi^2 = 10.2$ ,  $df = 3$ ,  $p < 0.05$ , followed by a post hoc SNK test).



**Figure 5.4:** Mean values of sediment stability over the course of the experiment: A by MagPI ( $n=6$ ,  $\pm SE$ ) and B by CSM ( $n=6$ ,  $\pm SE$ ). The different treatments were bacteria and diatoms (BD, ▲), diatoms (D, ◆), bacteria (B, □) and controls (C, ●).

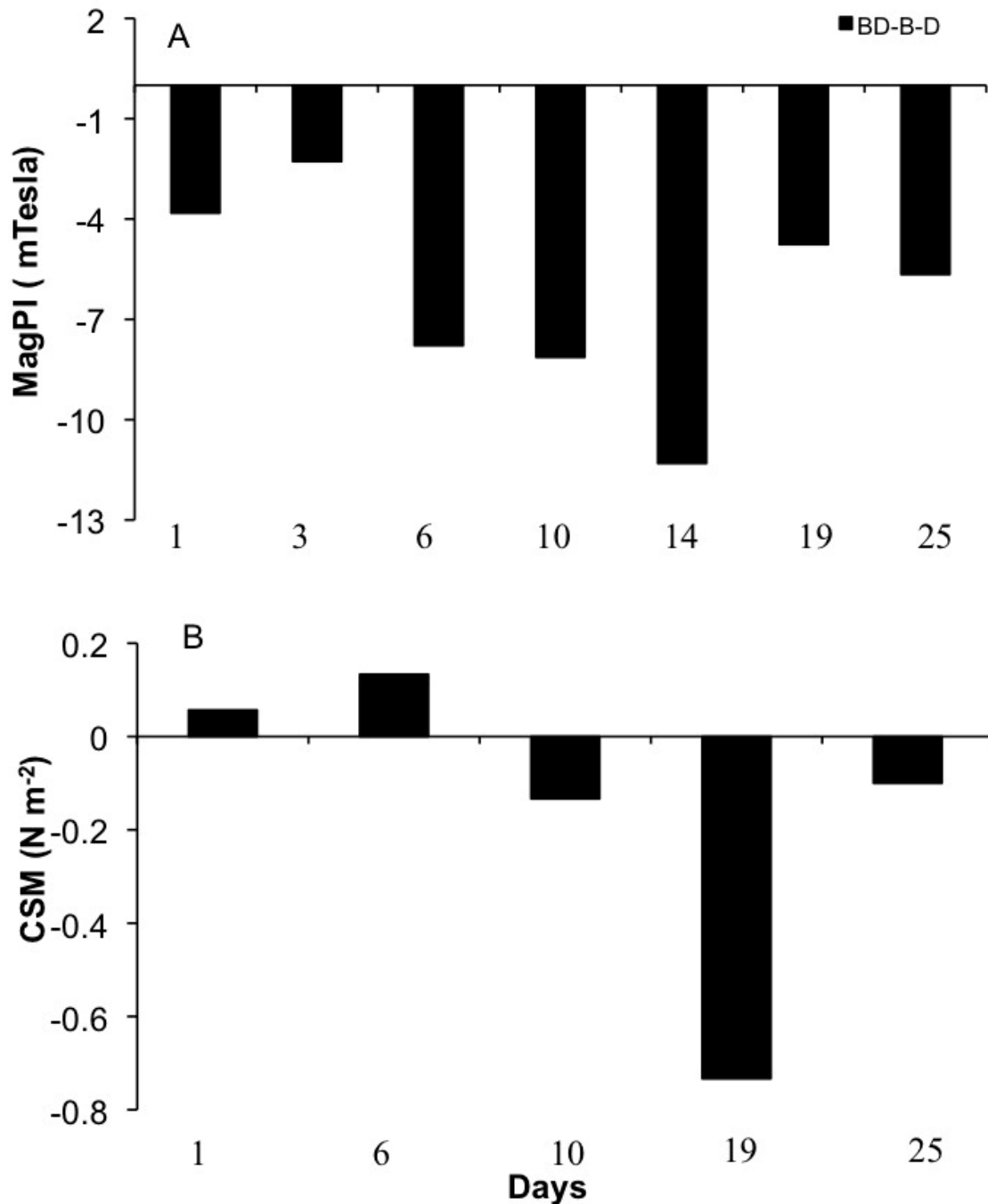
There was a strong linear relationship between CSM (erosion threshold) and MagPI (surface adhesion) (Pearson correlation coefficient:  $r=0.785$ ,  $n=20$ ,  $p<0.001$ , Figure 5.5).





**Figure 5.5:** *The linear relationship between MagPI (mTesla) versus CSM (Nm<sup>-2</sup>).*

In order to visualize possible additive/synergistic effects of bacteria-diatom assemblages for sediment stability, their absolute value of adhesion was compared to the values for the pure bacterial and diatom cultures ([BD]-[B+D], Figure 5.6 A and B). There was a stronger case for interference in the mixed assemblage since the results were much lower than would be expected from the additive effects of the two cultures B and D, as was particularly evident for surface adhesion as determined by MagPI (Figure 5.6 A and B).



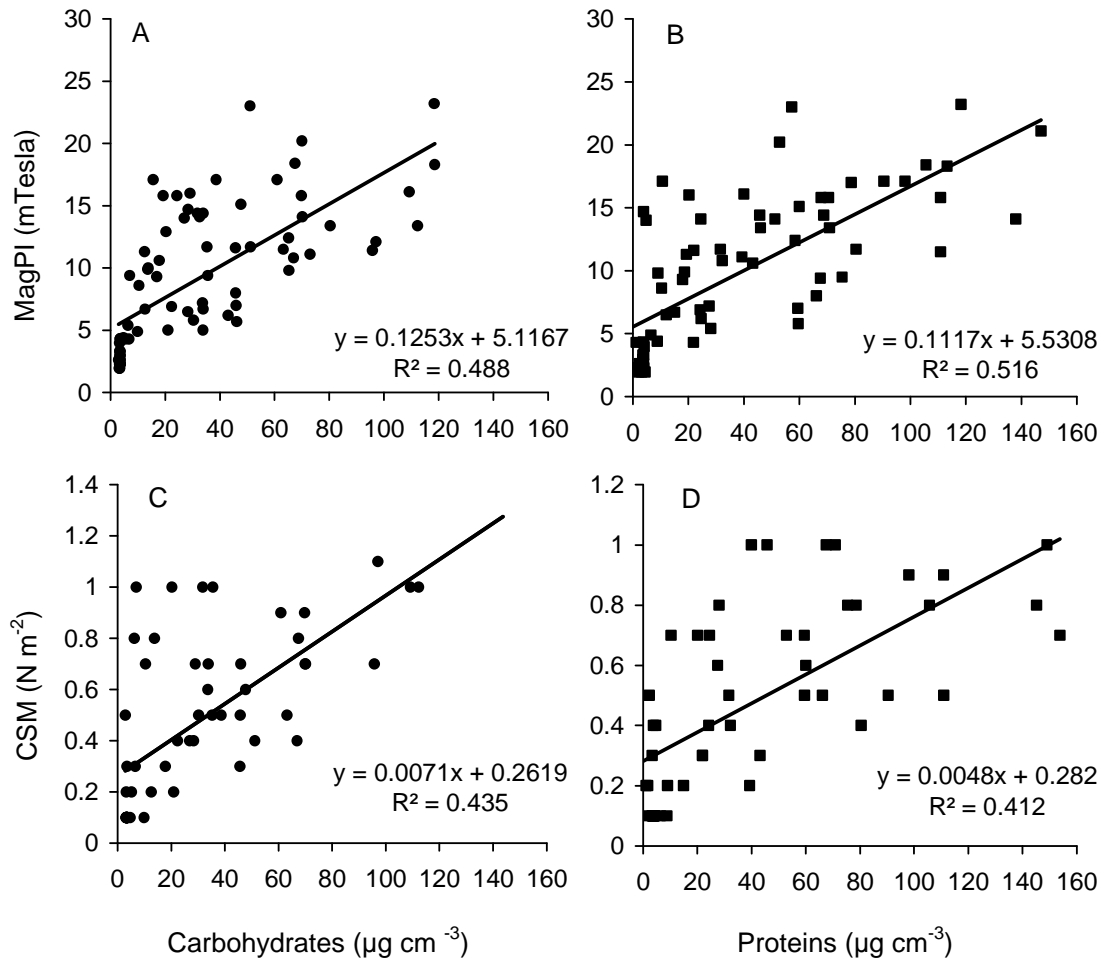
**Figure 5.6:** *The relative assessment between treatments for sediment stability as measured by MagPI (A) and CSM (B). Substratum stability by the mixed BD treatment relative to the stability of the single B and D treatments is given for MagPI (A) and CSM (B). Where the stability created by the mixed culture (BD) exceeds that of the added single cultures (B and D), the value is positive (synergistic effect) and vice versa (inhibitory effect). If the added values of the single cultures equals the mixed cultures then the effect measured is additive.*

**Relationship between biological variables and surface adhesion/ stability**

The data of sediment stability measurement (MagPI and CSM) was addressed to biological variables (see 4.3.2 Chapter 4): chlorophyll *a* concentration, bacterial cell number and EPS concentrations. There was a strong positive relationship between sediment stability measurements and chlorophyll *a* concentrations (MagPI:  $r=0.395$ ,  $p<0.001$ ; CSM:  $r=0.501$ ,  $p<0.001$ ). Similarly, colloidal carbohydrate concentrations were highly significantly correlated with MagPI and CSM measurements for all treatments (Figure 5.7 A and C, Table 5.3). The same applied for the relationship of colloidal protein concentrations to adhesion (MagPI) and cohesion (CSM) of the surface for B and BD, while for D the relationships were not significant (Figure 5.7 B and D, Table 5.3).

**Table 5.3:** Pearson's correlation coefficients between surface adhesion (MagPI) and substratum stability (CSM) and colloidal carbohydrates and proteins per treatment. The significance levels are the following: \*\*\*  $p < 0.001$ . \*\*  $p < 0.01$ . \*  $p < 0.05$ .

<i>Treatments</i>	<i>Techniques</i>	<i>Carbohydrates</i>			<i>Proteins</i>		
Diatom	MagPI	0.882	17	***	-0.189	21	
	CSM	0.869	11	***	0.321	15	
Bacteria	MagPI	0.861	15	***	0.770	14	**
	CSM	0.753	9	*	0.902	10	***
Bacteria + Diatom	MagPI	0.706	15	**	0.741	15	**
	CSM	0.617	12	*	0.494	12	*



**Figure 5.7:** Relationships between sediment stability (MagPI, CSM) and EPS components. A-B. The relationships between surface adhesion (MagPI) and colloidal carbohydrate and protein concentrations. C-D. The relationships between substratum stability (CSM) and colloidal carbohydrates and proteins concentrations.

#### 5.4. Discussion

##### **Biostabilisation potential of axenic microalgae cultures**

How does the level of biodiversity affect adhesive capacity? Numerous studies attempted to use microbial biomass as an indicator of sediment stability and linked sediment stability to EPS (e.g. Riethmueller et al. 2000, Defew et al. 2002, Le Hir et al. 2007). By the end of the experiment, higher adhesive capacity, microbial biomass and EPS carbohydrate concentrations were observed in treatments ANO and AN. The rapid decrease of biomass in treatment AN, on day 12 was mirrored by decrease of sediment stability. To compare with these treatments, AO shows significantly lower

biomass, however similar or higher EPS carbohydrates and protein concentration. As a result, where a lack of biomass is compensated by the quality of EPS, a similar adhesive capacity of the biofilm was determined. There is evidence that sediment stability cannot to be linked only to microbial biomass and highlighted synergistic effect in interaction of EPS carbohydrates and proteins, which might strengthen their binding forces (Costerton et al. 1978, Pennisi 2002). However it may also be due to a different origin of EPS produced by diatom species or cyanobacteria. For instance, in comparison to the single species treatments, higher biomass and EPS concentrations were observed in treatment A to compare with treatment O. However in terms of stabilization treatment O was significantly higher than two single diatom treatments A and N during the second week of the experiment. Higher adhesive capacity exhibited by cyanobacteria biofilms compared to diatom biofilms may be explained by differences of colonisation strategy. The majority of diatoms form biofilms around single grains rather than the filamentous EPS network formed by cyanobacteria (Watermann et al. 1999). The filamentous nature of the cyanobacteria itself may therefore result in different properties of the EPS, thus may have a different impact on sediment stability. Determination of which composition of the carbohydrates exuded from *Oscillatoria* or from the diatoms species require high-resolution chemical analyses of the EPS composition in different species and mixed species treatments. Despite low biomass and EPS concentration the stability in treatment N was significantly higher than O at the first week of the experiment. This is probably explained by the fact that the *Oscillatoria* preferred to grow below the uppermost surface-layer and at the first week of the experiment when the *Oscillatoria* film had grown denser it gave a large quantity of exudates influencing the surface and thereby increasing surface adhesion.

This experiment was targeted to examine the adhesive capacity of the biofilms with different levels of biodiversity. The strength of adhesive properties of the biofilm may vary between the species, due to their different colonisation strategies or various in EPS origin. The ratio and content of polysaccharides, proteins, viscosities, molecular weight and other physical-chemical parameters may also be related to this property. Further this study need to be extended by investigation influence of abiotic factor (e.g.

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temperature, salinity, nutrient level, type of sediment) on co-occurrence of the species and their stabilisation potential.

This is an area where more knowledge can be produced using high-resolution techniques, both physical and chemical. The importance of knowledge of the interspecies relationships in ecosystem services like particle adhesion will be useful in determining effects of disturbances to the systems that changes the biodiversity and relationships between the conjunctive organisms.

***Substratum stabilisation by heterotrophic bacteria and autotrophic microalgae from estuarine sediments.***

This study has shown impressive bio-stabilisation of non-cohesive material by microbial assemblages, as determined by Magnetic Particle Induction (MagPI) and the Cohesive Strength Meter (CSM). These devices determine slightly different surface properties of the test bed. With MagPI, an increase in adhesion (a proxy for particle capture potential and interface stability) was determined from day 1 and this increased with time in all microbial assemblages. MagPI does not require the erosion of the surface and therefore is a non-destructive, repeatable, sub-critical stress measurement with a high sensitivity that has been shown suitable for measuring the surface properties of young, developing biofilms. The CSM is a well-established device to measure erosion resistance; it requires bed failure and can operate over a range of values beyond that of most linear flumes. It does require a surface that has some initial resistance to erosion or the lightest jet pulse causes a 10% reduction in transmission, and therefore it is not as sensitive as MagPI for highly unconsolidated systems. However, these devices were found to complement each other, increasing the range of measurements that could be made and showed a strong correlation in the overlapping range of the data ( $R^2=0.62$ ,  $p<0.001$ ).

***The individual and combined engineering capability of microbial assemblages.***

The comparison of pure bacterial, axenic microalgal and mixed (bacteria+microalgae) assemblages was designed to provide insights into the individual and combined functional capacity of the heterotrophic and autotrophic biofilm components in terms of substratum properties. While this is a limited suite of measurements, they demonstrate the functional development of these assemblages in a new light.

Bacterial assemblages stabilised the substratum significantly more than axenic microalgal assemblages (x2). This work supported the early findings (Gerbersdorf et al. 2009) but is in contrast to most of the literature (Yallop et al. 2000, Lundkvist et al. 2007) where the contribution of bacteria to sediment stabilization is usually regarded as less significant or even negligible as compared with diatom assemblages.

Due to the well-known bacterial-algal interactions, it was first hypothesized that the grouping of bacteria and diatoms in the mixed assemblages might result in mutual advantages which might affect EPS secretion and stabilization positively. The first of these concepts is supported by the data (described in Chapter 4) in terms of EPS carbohydrate production but not for EPS protein production. However, the synergism in EPS carbohydrate was not reflected in surface stability by either method of determination (MagPI, CSM). Indeed, in comparison to the controls, the adhesion capacity and the cohesion forces of the substratum were significantly highest in the natural assemblage with a factor up to 7.5 and 9.5, as determined by MagPI and CSM, respectively. However, the differences to the pure cultures were less than expected: an increase of a factor of 6 and 8 for bacteria and a factor of 5 and 6 for diatoms, by MagPI and CSM, respectively. This may be because the shape of the relationship between EPS concentration and surface stability is not linear and should reach an asymptote as EPS increases. This makes logical sense since by adding more EPS the strength of the surface cannot increase beyond the fundamental binding capacity of the polymer. The improved binding by the mixed culture may reflect the contribution of different types of EPS with varied properties and the nature of the micro-spatial arrangement of the EPS deposited by bacteria (largely attachment to grains) and diatoms (for locomotion) (Figure 5.5).

#### ***The EPS Matrix – key to substratum stabilisation?***

The common suggestion, that diatoms secrete mainly polysaccharide EPS (e.g. Decho 2000, Flemming and Wingender 2001), was supported by our results of significantly higher carbohydrate concentrations in the axenic microalgal assemblage as opposed to the bacterial biofilm. Despite this the stabilization effect of the bacterial assemblage was significantly higher than for the microalgal biofilms, although the EPS protein concentrations were quite similar. This strongly suggests that EPS quantity *per se* cannot be predictive of substratum stabilisation. The ecological function of the

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microbial EPS secretion has to be considered: for instance, bacteria attach firmly to a substratum with the help of EPS while diatoms secrete EPS for locomotion (Edgar and Pickett-Heaps 1983). Thus, it seems logical to suggest that the EPS secreted by bacteria and diatoms must differ in their characteristics and mechanical properties. This variation in properties might explain the unexpectedly greater stabilisation capability of bacterial cultures as compared to the axenic diatom cultures. These findings also support earlier work that suggests proteins play a more significant role in substratum adhesion/cohesion than previously thought (Gerbersdorf et al. 2008, Gerbersdorf et al. 2009). Hydrophobicity, surface charges (Zeta potential) and the free energy of microbial cell surroundings/EPS are crucial factors controlling the “first kiss”, the initial attachment of a microbe to a surface (e.g. Fang et al. 2000). Proteins play a significant role in this first adhesion (Czaczyk and Myszka 2007, Jain et al. 2007), but also contribute towards the binding strength within the developing EPS matrix. This has been demonstrated for marine aggregates, where the incorporation of free protein particles significantly increased stability (Long and Azam 1996). If EPS proteins interact with carbohydrates, they can form a resilient matrix similar to an epoxy resin (Pennisi 2002). The degree of bonding also depends on the lengths of the polymers involved and the degree to which they branch (Pennisi 2002, Wotton 2004). In addition, the characteristics of one particular EPS component, carbohydrates or proteins, most likely differs between the heterotrophic and autotrophic producers. The greatest functional effect, in terms of substratum stabilization, in natural assemblages coincided with significantly higher quantities of microbial produced colloidal carbohydrates and proteins.

The alteration of the EPS concentrations in the mixed assemblage as opposed to the single cultures of bacteria and microalgae were even of additive (proteins), if not synergistic (carbohydrates) nature. However, this did not translate into equivalent adhesion and cohesion capacity, since these proxies for stability did not indicate any additive or synergistic effects in the binding strength of the mixed assemblage. Altogether, the data points to the importance of EPS composition and the interactions of single EPS components that, along with higher EPS concentrations, established the highest substratum stabilization in the natural biofilm.

Although the initial hypothesis of synergistic effects in a combined prokaryotic and eukaryotic biofilm community in terms of stability was not supported, the functional

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capacity for adhesion and cohesion by the liaison between bacteria and microalgae was impressive. This biostabilisation is an important “ecosystem service” since it affects processes beyond the biofilm such as nutrient fluxes, pollutant retention and sediment erosion/transport.

**Declaration**

The results presented in this chapter have formed all or part of the following publication:

- HV Lubarsky, C Hubas, M Chocholek, F Larson, W Manz, DM Paterson, SU Gerbersdorf (2010). The stabilisation potential of individual and mixed assemblages of natural bacteria and microalgae. *PLoS One* 5 (e13794) 1-12.

**References**

- Battin, T. J., L. A. Kaplan, J. D. Newbold, and C. M. E. Hansen. 2003. Contributions of microbial biofilms to ecosystem processes in stream mesocosms. *Nature* **426**:439-442.
- Boogert, N. J., D. M. Paterson, and K. N. Laland. 2006. The implications of niche construction and ecosystem engineering for conservation biology. *Bioscience* **56**:570-578.
- Consalvey, M., D. M. Paterson, and G. J. C. Underwood. 2004. The ups and downs of life in a benthic biofilm: Migration of benthic diatoms. *Diatom Research* **19**:181-202.
- Costerton, J. W., G. G. Geesey, and K. J. Cheng. 1978. How bacteria stick. *Sciens Am* **238**:86-95.
- Czaczyk, K. and K. Myszka. 2007. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Polish Journal of Environmental Studies* **16**:799-806.
- Dade, W. B., J. D. Davis, P. D. Nichols, A. R. M. Nowell, D. Thistle, M. B. Trexler, and D. C. White. 1990. Effect of bacterial exopolymer adhesion on the entrainment of sand. *Geomicrobiology Journal* **8**:1-16.
- Dade, W. B., A. R. M. Nowell, and P. A. Jumars. 1992. Predicting erosion resistance of muds. *Marine Geology* **105**:285-297.
- Dade, W. B., R. L. Self, N. B. Pellerin, A. Moffet, P. A. Jumars, and A. R. M. Nowell. 1996. The effects of bacteria on the flow behavior of clay seawater suspensions. *Journal of Sedimentary Research* **66**:39-42.
- de Brouwer, J. F. C., K. Wolfstein, G. K. Ruddy, T. E. R. Jones, and L. J. Stal. 2005. Biogenic stabilization of intertidal sediments: The importance of extracellular polymeric substances produced by benthic diatoms. *Microbial Ecology* **49**:501-512.
- de Winder, B., N. Staats, L. J. Stal, and D. M. Paterson. 1999. Carbohydrate secretion by phototrophic communities in tidal sediments. *Journal of Sea Research* **42**:131-146.
- Decho, A. W. 2000. Microbial biofilms in intertidal systems: an overview. *Continental Shelf Research* **20**:1257-1273.
- Defew, E. C., T. J. Tolhurst, and D. M. Paterson. 2002. Site-specific features influence sediment stability of intertidal flats. *Hydrology and Earth System Sciences* **6**:971-981.
- Dejong, L. and W. Admiraal. 1984. Competition between 3 estuarine benthic diatom species in mixed cultures. *Marine Ecology-Progress Series* **18**:269-275.
- Droppo, I. G. 2001. Rethinking what constitutes suspended sediment. *Hydrological Processes* **15**:1551-1564.
- Droppo, I. G., N. Ross, M. Skafel, and S. N. Liss. 2007. Biostabilization of cohesive sediment beds in a freshwater wave-dominated environment. *Limnology and Oceanography* **52**:577-589.
- Edgar, L. A. and J. D. Pickett-Heaps. 1983. The mechanism of diatom locomotion. I. An ultrastructural study of the motility apparatus. *Proceedings of the Royal Society B-Biological Sciences* **218**:331-343.

- Fang, H. H. P., K. Y. Chan, and L. C. Xu. 2000. Quantification of bacterial adhesion forces using atomic force microscopy (AFM). *Journal of Microbiological Methods* **40**:89-97.
- Fattom, A. and M. Shilo. 1984. Hydrophobicity as an adhesion mechanism of benthic cyanobacteria. *Applied and Environmental Microbiology* **47**:135-143.
- Flemming, H. C. and J. Wingender. 2001. Relevance of microbial extracellular polymeric substances (EPSs)-Part I:Structural and ecological aspects. Pages 1-8 *Water Science and Technology*, Milheim,Germany.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Gerbersdorf, S. U., T. Jancke, and B. Westrich. 2005. Physico-chemical and biological sediment properties determining erosion resistance of contaminated riverine sediments - Temporal and vertical pattern at the Lauffen reservoir/River Neckar, Germany. *Limnologica* **35**:132-144.
- Gerbersdorf, S. U., W. Manz, and D. M. Paterson. 2008. The engineering potential of natural benthic bacterial assemblages in terms of the erosion resistance of sediments. *Fems Microbiology Ecology* **66**:282-294.
- Hirst, C. N., H. Cyr, and I. A. Jordan. 2003. Distribution of exopolymeric substances in the littoral sediments of an oligotrophic lake. *Microbial Ecology* **46**:22-32.
- Hu, C. X., Y. D. Liu, B. S. Paulsen, D. Petersen, and B. Klaveness. 2003. Extracellular carbohydrate polymers from five desert soil algae with different cohesion in the stabilization of fine sand grain. *Carbohydrate Polymers* **54**:33-42.
- Jain, A., Y. Gupta, R. Agrawal, P. Khare, and S. K. Jain. 2007. Biofilms - A microbial life perspective: A critical review. *Critical Reviews in Therapeutic Drug Carrier Systems* **24**:393-443.
- Kawaguchi, T. and A. W. Decho. 2000. Biochemical characterization of cyanobacterial extracellular polymers (EPS) from modern marine stromatolites (Bahamas). *Preparative Biochemistry & Biotechnology* **30**:321-330.
- Le Hir, P., Y. Monbet, and F. Orvain. 2007. Sediment erodability in sediment transport modelling: Can we account for biota effects? *Continental Shelf Research* **27**:1116-1142.
- Leon-Morales, C. F., M. Strathmann, and H. C. Flemming. 2007. Role of biofilms on sediment transport - investigations with artificial sediment columns. *in* B. Westrich and U. Foerstner, editors. *Sediment dynamics and pollutant mobility in rivers*. Springer-Verlag, pp. 358-386, Berlin Heidelberg New York.
- Long, R. A. and F. Azam. 1996. Abundant protein-containing particles in the sea. *Aquatic Microbial Ecology* **10**:213-221.
- Lundkvist, M., M. Grue, P. L. Friend, and M. R. Flindt. 2007. The relative contributions of physical and microbiological factors to cohesive sediment stability. *Continental Shelf Research* **27**:1143-1152.
- Miller, D. C., R. J. Geider, and H. L. MacIntyre. 1996. Microphytobenthos: The ecological role of the "secret garden" of unvegetated, shallow-water marine habitats .2. Role in sediment stability and shallow-water food webs. *Estuaries* **19**:202-212.
- Parikh, A. and D. Madamwar. 2006. Partial characterization of extracellular polysaccharides from cyanobacteria. *Bioresource Technology* **97**:1822-1827.
- Pennisi, E. 2002. Materials science - Biology reveals new ways to hold on tight. *Science* **296**:250-251.
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- Perkins, R. G., C. Honeywill, M. Consalvey, H. A. Austin, T. J. Tolhurst, and D. M. Paterson. 2003. Changes in microphytobenthic chlorophyll a and EPS resulting from sediment compaction due to de-watering: opposing patterns in concentration and content. *Continental Shelf Research* **23**:575-586.
- Perkins, R. G., D. M. Paterson, H. Sun, J. Watson, and M. A. Player. 2004. Extracellular polymeric substances: quantification and use in erosion experiments. *Continental Shelf Research* **24**:1623-1635.
- Ribalet, F., J. A. Berges, A. Ianora, and R. Casotti. 2007. Growth inhibition of cultured marine phytoplankton by toxic algal-derived polyunsaturated aldehydes. *Aquatic Toxicology* **85**:219-227.
- Riethmuller, R., M. Heineke, H. Kuehl, and R. Keuker-Ruediger. 2000. Chlorophyll a concentration as an index of sediment surface stabilisation by microphytobenthos? *Continental Shelf Research* **20**:1351-1372.
- Tolhurst, T. J., R. Riethmuller, and D. M. Paterson. 2000. In situ versus laboratory analysis of sediment stability from intertidal mudflats. *Continental Shelf Research* **20**:1317-1334.
- Underwood, G. J. C. and D. M. Paterson. 2003. The importance of extracellular carbohydrate production by marine epipellic diatoms. *Advances in Botanical Research* **40**:183-240.
- Underwood, G. J. C. and D. J. Smith. 1998. Predicting epipellic diatom exopolymer concentrations in intertidal sediments from sediment chlorophyll a. *Microbial Ecology* **35**:116-125.
- Watermann, F., H. Hillebrand, G. Gerdes, W. E. Krumbein, and U. Sommer. 1999. Competition between benthic cyanobacteria and diatoms as influenced by different grain sizes and temperatures. *Marine Ecology-Progress Series* **187**:77-87.
- Wotton, R. S. 2004. The ubiquity and many roles of exopolymers (EPS) in aquatic systems. *Scientia Marina* **68**:13-21.
- Yallop, M. L., D. M. Paterson, and P. Wellsbury. 2000. Interrelationships between rates of microbial production, exopolymer production, microbial biomass, and sediment stability in biofilms of intertidal sediments. *Microbial Ecology* **39**:116-127.

## Chapter 6

### Effect of triclosan on bacterial stabilisation potential

#### Abstract

The importance of bacteria on biostabilisation processes through the secretion of organic glue (EPS: extracellular polymeric substances) has been previously recognized (Gerbersdorf et al. 2008). However, investigation of bacterial engineering capacity under stress, such as presence of contaminants has never been performed. This study investigates the stabilisation potential of natural benthic bacterial assemblages from the Eden Estuary (Scotland, UK) on non-cohesive glass beads over 17 days in the presence of a toxin. The toxin employed was triclosan (TCS) which is a potent biocide that is included in a diverse range of products.

A range of triclosan (TCS) concentrations, relevant to environmental occurrence (2-100  $\mu\text{g l}^{-1}$ ) was used. The adhesive capacity of the biofilm produced by bacteria was determined by MagPI (Magnetic Particle Induction). This technique has been successfully used in a number of experiments and shows high sensitivity in determination of the response of complex communities to a range of stresses (such as nutrient-depleted condition) and the associated changes in sediment properties of the biofilm. The stabilisation potential of bacteria (treatment CB) was up to 2 times higher than treatments of bacteria with triclosan. Substratum stability was closely

related to bacterial cell number ( $R^2=0.47$ ) and EPS carbohydrates concentrations ( $R^2=0.53$ ) but less strongly related to bacterial dividing rate ( $R^2=0.13$ ) and EPS protein concentrations ( $R^2=0.17$ ). TCS exposure reduced microbial biomass and EPS production and as result had a negative effect on bacteria stabilisation. This negative effect was more pronounced with increasing concentration of TCS. The data presented in this chapter significantly contributes to the investigation of “ecosystem functioning” and biostabilisation processes under natural conditions. This work is the first investigation of microbial stabilisation potential under the stress of a xenobiotic toxin.

### 6.1. Introduction

***Triclosan—a recent chemical introduction to aquatic habitats.*** Triclosan(TCS) (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a broad-spectrum antibacterial and antifungal compound that has been widely used in pharm personal care products (PPCPs), textiles, cleaning supplies, toys and computer equipment since 1972 (Singer et al. 2002). About 96% of triclosan (TCS) originating from consumer products is disposed of through residential drains (Adolfsson-Erici et al. 2002), leading to considerable loads of the chemical in waters entering waste-water treatment plants (WWTP). While biological sewage treatment was regarded as an effective barrier for TCS due to removal efficiencies of 98% in the aqueous phase, Heidler & Halden (2007) proved that the particle-associated TCS was sequestered into waste-water residuals and accumulated in the sludge with less than half of the total mass being biotransformed or lost. Consequently, substantial quantities of the chemical can be transferred into soils and groundwater by sludge recycling (Heidler and Halden 2007) or directly enters rivers with estimated concentrations between 11-98 ng l<sup>-1</sup> (Singer et al. 2002) and with one report of up to 2.7 µg l<sup>-1</sup> (Chalew and Halden 2009). In the aqueous phase, the transformation of TCS into a variety of polychlorinated dibenzo-p-dioxins under the exposure of sunlight and especially at high pH values becomes problematic (Mezcua et al. 2004); and the last twenty years has seen a rapid increase in the use of triclosan-containing products (Schweizer 2001). Water testing studies by the U.S. Geological Survey have found that triclosan is among the top 10 persistent contaminants in US rivers, streams, lakes, and underground aquifers (Kolpin et al.

2002, Halden and Paull 2005). McAvoy et al. (2002) suggests that TCS is readily biodegradable under aerobic conditions, but not under anaerobic conditions therefore accumulation in sediments even more likely than in the water column.

The environmental occurrence of TCS is of interest to environmental scientists and the results of determination of TCS in water samples and sediment have been reported in numerous studies (Okumura and Nishikawa 1996, Kolpin et al. 2002, Lindstrom et al. 2002, McAvoy et al. 2002, Singer et al. 2002) and further summarized by Chalew and Halden (2009). Due to a photodegradation it was suggested a seasonal dependence of the TCS concentration (lower in summer, higher in winter) (Lindstrom et al. 2002, Singer et al. 2002). Analytical data from environmental samples in several countries demonstrate different range of concentrations for rivers, lakes and streams. For instance, McAvoy et al. (2002) reported that TCS concentration in wastewater ranged from 3.8 to 16.6  $\mu\text{g l}^{-1}$ , however Aguera et al. (2003) presented concentrations of 37.8  $\mu\text{g l}^{-1}$  of TCS in samples coming from urban wastewater treatment plants. Some studies report concentrations in sediment samples situated in the range 0.27 to 130.7  $\mu\text{g kg}^{-1}$  (Aguera et al. 2003), and the highest concentrations have been found under anaerobic conditions (McAvoy et al. 2002). These findings varied between countries and sampling sites (Okumura and Nishikawa 1996).

**Effects of triclosan on bacteria.** TCS is a broad-spectrum antimicrobial agent, which may have two major actions: stopping microbial reproduction or killing microorganism, which have been well investigated for bacteria in the laboratory. Thus, it has been shown that perturbation of bacterial membranes and functions were a consequence of the specific inhibition of fatty acid biosynthesis by triclosan (Heath et al. 1999). TCS specifically inhibits the enzyme enoyl-acyl carrier protein reductase (ENR) by mimicking its natural substrate, thus blocking the final, regulatory FabI step in the fatty-acid synthesis cycle (Levy et al. 1999). Consequently, bacterial cells can become resistant to TCS as has been shown for several strains of *Escherichia coli* (McMurry et al. 1998, Escalada et al. 2005a). Schweizer (2001) reported that some bacterial strains (such as gram-negative bacteria) use a multiple triclosan resistance mechanism, including active efflux from cells where bacteria pump TCS actively out of their cell. Although it has been discussed whether the inhibition of the metabolic pathway via ENR can solely explain the complex mode of action and lethality of TCS

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for bacteria (Escalada et al. 2005a), other impairments of bacterial functions by TCS have not yet been investigated.

The effects of TCS on bacteria may vary according to the concentration of the chemical, the exposure time, its bioavailability, the physiology of the target organisms and the targeted species. For instance, Russell (2004) reported that TCS affects many, but not all types of Gram-positive and Gram-negative bacteria. Inactive bacteria seem to be more resilient to the lethal effects of TCS possible due to a reduced metabolism and an enhanced physical barrier against TCS caused by debris and dead cells in the stationary growth phase (Escalada et al. 2005b). Recent research (Suller and Russell 1999, 2000, Escalada et al. 2005b) showed that at low concentrations (0.02–0.5  $\mu\text{g ml}^{-1}$ ) TCS appears bacteriostatic and affected the growth of several bacteria, while higher TCS concentrations (10  $\text{mg l}^{-1}$  and above) were bactericidal regardless of the growth phase (Escalada et al. 2005b). At higher concentrations, TCS seems to act rapidly and with damaging effects on multiple cytoplasmic and membrane targets, resulting in leakage of intracellular material (Villalain et al. 2001). However, in natural samples, lethal effects of TCS were observed at much lower concentrations of environmental relevance, by using the bioluminescence assay of *Vibrio fischeri*. For instance, DeLorenzo et al. (2008) reported an  $\text{EC}_{50}$  of 53  $\mu\text{g l}^{-1}$  for estuarine samples and Farré et al. (2008) determined an  $\text{EC}_{50}$  of 280  $\mu\text{g l}^{-1}$  in waste-waters while Ricart et al. (2010) observed mortality within a river biofilm at 0.21  $\mu\text{g l}^{-1}$  TCS. The same is true for acute toxic effects of TCS exposure on co-occurring non-target components, especially for microalgae (Wilson et al. 2003, Lawrence et al. 2009, Ricart et al. 2010) and for higher organisms (e. g. shrimps, Orvos et al. 2002). This indicates that the relatively low TCS concentrations currently measured in the aquatic habitats can have a profound effect on the inhabiting organisms.

#### ***Does TCS impair ecosystem services of bacterial biofilms?***

Microbial consortia are important contributors towards the functional capabilities of natural benthic ecosystems. Microbial biofilms represent the dominant microbial life forms in many aquatic systems and drive a number of important “ecosystem functions” (Cyr and Morton 2006). Possible effects of TCS on microbial biofilm were addressed only in terms of biomass and EPS production. Therefore there are still



significant gaps in our knowledge concerning the possible effect of TCS on their “ecosystem functionality”. Research has shown that the presence of TCS may influence both the structure and the function of microbial communities (Lawrence et al. 2009). These changes could result in shifts in evolutionary and ecological processes such as gene flow, in nutrient processing capacity and the natural food web structure (Wilson et al. 2003). One interesting ecosystem service is biostabilisation where the microorganisms modify the response of aquatic sediments to erosive forces (flow velocity, turbulence). Microbial communities release organic compounds associated with the binding of particles and the retention of pollutants (Bellin and Rao 1993, Wolfaardt et al. 1998). The importance of bacteria as ecosystem engineers with broad range of effect (Jones et al. 1994) including biostabilisation has been confirmed (Gerbersdorf et al. 2008, Gerbersdorf et al. 2009) and enhanced by recent work (Lubarsky et al. 2010) demonstrating that natural benthic bacterial assemblages from estuarine areas can significantly stabilised test substrata. Due to the importance of bacteria in the biostabilisation processes, the investigation of the possible effect of TCS on bacterial stabilisation capacity is required. It was hypothesized that TCS may have a negative effect on bacterial population, EPS secretion and hence bacterial stabilisation potential.

The present study investigated for the first time the effects of TCS exposure on the stabilization potential of natural bacterial biofilms using different TCS concentrations (ranging from 2–100  $\mu\text{g l}^{-1}$ ). Over the course of 2 weeks, the adhesive capacity, a proxy for sediment stability, was determined with a newly developed device, as described in Chapter 3 (MagPI, Larson et al. 2009). In parallel, bacterial cell numbers, division rates, species composition and EPS (proteins, carbohydrates) secretion were monitored and related to the adhesive capacity. Knowledge of the biostabilisation capacity of biofilms and its impairment by pollutant exposure is of high significance for sediment management strategies in waterways and coastal regions.

## **6.2. Experimental set-up and triclosan spiking**

Since triclosan (TCS) is highly absorptive, the use of plastic or Perspex had to be avoided. Thus, glass boxes were used (in mm 105Lx105Wx55H) in which a 1 cm layer

of <63  $\mu\text{m}$  glass beads was added as a non-cohesive substratum for biofilm growth (Figure 6.1). The boxes were gently filled with 300 ml of autoclaved seawater (controls) that had been spiked with defined TCS concentrations (treatments). For the latter, the stock solution of TCS was prepared by dissolving the commercially available powder (Irgasan, Sigma-Aldrich C.N 72779) in seawater with the help of a magnetic stirrer (STUART GB) for 4 h. The stock solution was diluted with seawater to gain the defined concentrations of 2  $\mu\text{g l}^{-1}$ , 10  $\mu\text{g l}^{-1}$ , 20  $\mu\text{g l}^{-1}$ , 50  $\mu\text{g l}^{-1}$ , and 100  $\mu\text{g l}^{-1}$  of triclosan. TCS is a highly photodegradable compound. Taking into account this fact, the experiment has been performed in dark condition to eliminate degradation and maintain required concentration. However, due to water evaporation, it was predicted accumulation of TCS on water column and sediment and, as a result, the actual concentrations of TCS may be different from the targeted concentration.

The bacterial culture was isolated from natural sediment (as described 2.2.1). The glass boxes were further inoculated by 10 ml of bacterial stock solution to initiate biofilm growth, with the exception of the negative controls.

The following treatments were established with four replicates each:

1. bacterial culture + 2  $\mu\text{g l}^{-1}$  of triclosan (T1)
2. bacterial culture + 10  $\mu\text{g l}^{-1}$  of triclosan (T2)
3. bacterial culture + 20  $\mu\text{g l}^{-1}$  of triclosan (T3)
4. bacterial culture + 50  $\mu\text{g l}^{-1}$  of triclosan (T4)
5. bacterial culture + 100  $\mu\text{g l}^{-1}$  of triclosan (T5)
6. negative control (CT): no triclosan, no bacterial culture
7. positive control (CB): no triclosan, plus bacterial culture

The negative control (CT) containing only glass beads and seawater and was treated once a week with a mixture of antibiotics (150  $\mu\text{g l}^{-1}$  streptomycin and 20  $\mu\text{g l}^{-1}$  chloramphenicol, final concentrations) to prevent bacterial colonisation. All treatments were gently aerated and kept at constant temperature (15°C) in the dark, over the experimental period of 2 week.



**Figure 6.1:** *Experimental setup.*

### ***Sampling strategy***

Sampling took place every second day during the experiment. For each replicate ( $n=4$ ) of the treatments and the controls, 4 cores (2 mm depth) were removed using a cut-off syringe 10 mm diameter (see 2.3.1). The cores were immediately processed for the determination of bacterial cell numbers (described in 2.7) and division rates (described in 2.8) or frozen at  $-80^{\circ}\text{C}$  for further analysis of extracellular polymeric substances (EPS)(described in 2.4) and DNA extractions for bacterial community analysis (described in 2.9.1). To monitor triclosan concentrations over time, samples of water and substratum (additional cores of 5 mm depth) were taken at the beginning (sampling day 1), in the middle (sampling day 4) and at the end of the experiment (sampling day 7) from each box. The, 4 substratum cores per treatment were pooled within a 15 ml Apex centrifuge tube to account for spatial heterogeneity and stored for future analysis at  $-80^{\circ}\text{C}$  (as described in 2.11.1). The adhesive capacity as proxy for sediment stability, of the bacterial biofilm, was monitored every second day by MagPI (2.13.2), over the experimental period of two week.

### 6.2.1. Statistics

The data did not pass Kolmogorov-Smirnov normality test and Bartlett test for homogeneity of variance. Thus differences between treatments were addressed using a non-parametric PERMANOVA (999 permutations) test followed by the non-parametric post-hoc Student-Newman-Keuls (SNK) test to compare pairs of treatments.

All the measured variables were analysed by Principal Component Analysis (PCA) with R©2.9.0 using the `dudi.pca` function of the “ade4” package. Briefly, Eigen value decomposition of a data covariance matrix was performed from a data frame containing the variables colloidal EPS (proteins and carbohydrates), bacterial cell numbers, substratum adhesion (MagPI) and bacterial division rates as well as the objects treatments and sampling dates. The aim of the numerical decomposition was to generate principal components (PC1 and PC2) that explain the majority of the total variance of the whole dataset. The calculation was performed with centred and scaled parameters after deleting rows that contained missing values. Scores (coordinates of the objects) were plotted twice, either clustered according to the treatment or the sampling date. Loadings (coordinates of the variables) were visualized in the correlation circle. Both, scores and loadings were plotted separately for a better readability.

## 6.3. Results

### 6.3.1. Triclosan concentrations

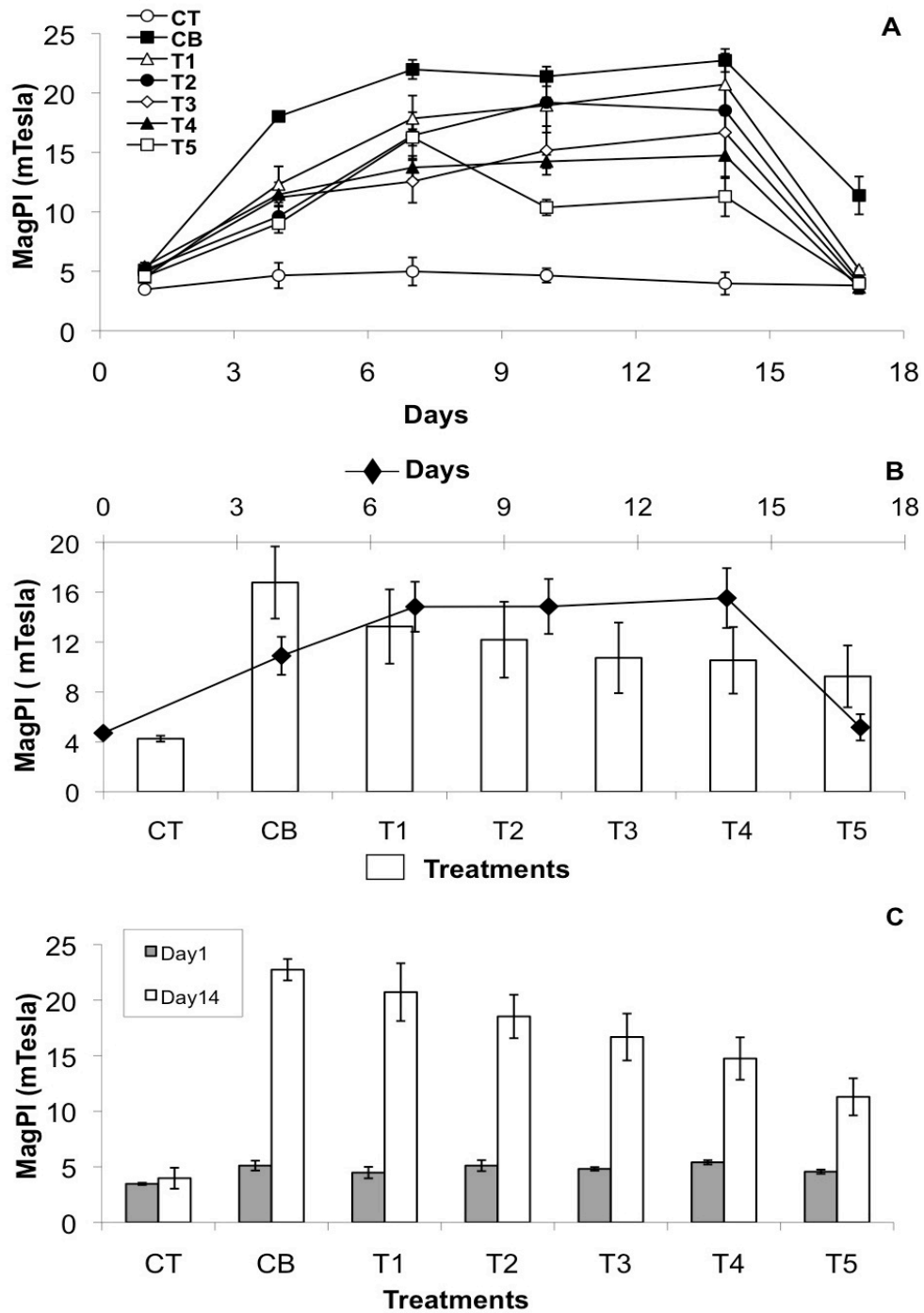
Triclosan concentrations within the substratum were about two times higher than the intended concentrations (measured over the whole spiking range:  $4 \mu\text{g l}^{-1}$ – $200 \mu\text{g l}^{-1}$  (from the lowest to the highest value)). The determined triclosan concentrations in the overlaying water were again two times higher than the intended concentrations in the low range (up to  $4 \mu\text{g l}^{-1}$ ), but similar to the spiking concentrations in the medium range ( $49 \mu\text{g l}^{-1}$ ) and even lower in the high range ( $30 \mu\text{g l}^{-1}$ ). Over the experimental period, the water within the glass boxes evaporated to a noticeable degree (water height dropped from 4 cm to 3 cm).

### 6.3.2. The stability of the substratum

The adhesion of the substratum surface increased continuously in all treatments with biofilms up to day 14 and decreased afterwards (Figure 6.2 A, B). In contrast, the negative control (CT) did not show any significant changes in adhesion over the experimental time (Figure 6.2 A, B). The stability increase by the biofilms was most pronounced for the treatments CB and T1 (up to 4.6) followed by T2 and T3 (up to 3.6) as well as T4 and T5 (up to 2.7) (Figure 6.2 A, B, C, Table 6.1). Accordingly, the positive control without triclosan showed the highest surface adhesion of the sediment (CB) (22.73 mTesla), that was otherwise declining in the bacterial cultures with increasing TCS exposure: T1 (20.7 mTesla)>T2 (18.53 mTesla)>T3 (16.7 mTesla)>T4 (15.7 mTesla)>T5 (14.7 mTesla) (Figure 6.2 C, Table 6.1). Statistical testing generally revealed significant differences between the treatments. For example on day 14, the stability of the biofilm without TCS (CB) was significantly higher than T3, T4, and T5 (PERMANOVA  $p < 0.0001$ , followed by a non-parametric SNK test,  $p < 0.05$ ).

**Table 6.1:** A. Ratio for different variables between the first day (minimum) and day 14 (maximum) of the experiment. B. Ratio for different variables between the positive control "CB" and the treatments ("T1, T2, T3, T4, T5").

	<i>Treatment</i>	<i>Adhesion MagPI</i>	<i>EPS Carbohydrates</i>	<i>EPS Proteins</i>	<i>Bacterial cell numbers</i>	<i>Bacterial division rates</i>
<b>Ratio A</b>	CB	4.4	3.5	2.2	2.0	8.3
	T1	4.6	3.2	1.4	1.9	1.9
	T2	3.6	2.6	1.8	1.4	3.1
	T3	3.5	1.7	1.6	1.5	1.1
	T4	2.7	2.9	1.8	1.2	4.0
	T5	2.5	3.5	1.1	1.7	3.7
<b>Ratio B</b>	T1	1.1	1.3	1.4	0.9	1.4
	T2	1.2	1.4	1.4	1.5	1.2
	T3	1.3	1.8	1.7	1.4	1.3
	T4	1.5	1.1	1.0	2.5	1.0
	T5	2.0	1.6	1.3	1.9	1.0



**Figure 6.2:** Adhesion as a proxy for stability, measured by MagPI, over the course of the experiment. (A) Mean values ( $n=4$  per treatment,  $\pm SE$ ): positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu g l^{-1}$ , △), T2 (TCS:  $10 \mu g l^{-1}$ , ●), T3 (TCS:  $20 \mu g l^{-1}$ , ◇), T4 (TCS:  $50 \mu g l^{-1}$ , ▲), T5 (TCS:  $100 \mu g l^{-1}$ , □). (B) Mean values per day ( $n=7$ ,  $\pm SE$ , ◆) and per treatment ( $n=6$ ,  $\pm SE$ , bar plots). (C) Mean values ( $n=4$ ,  $\pm SE$ ) shown for the different treatments on the first day (grey bars) as opposed to the day 14 (white bars).

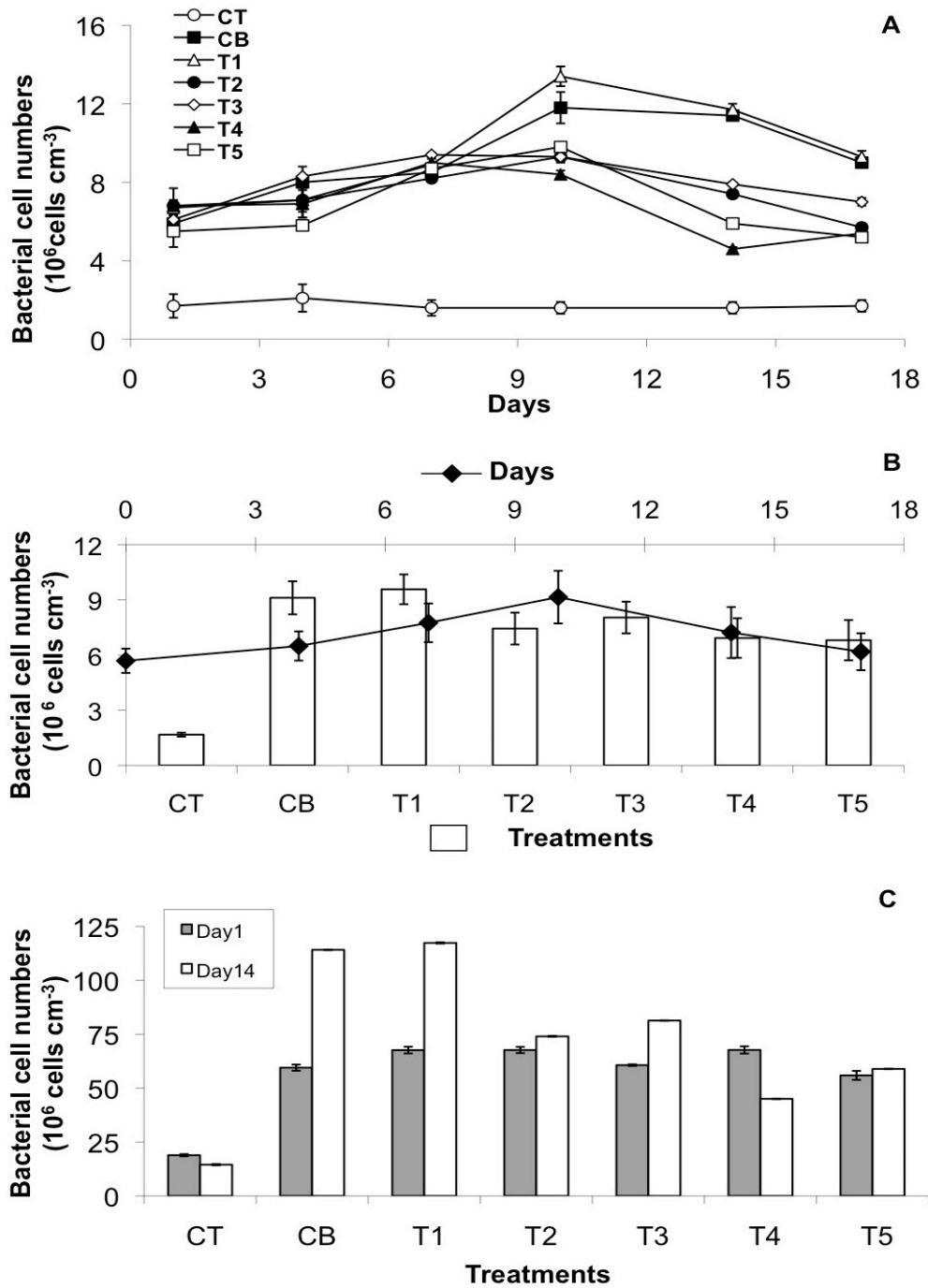
### 6.3.3. Bacterial cell numbers and growth rate

In the first experimental week, the bacterial cell numbers increased in all treatments up to day 10 (Figure 6.3 A, B). The increase was more pronounced for the treatments CB and T1 (up to 2 times) with bacterial cell numbers ranging from  $5.9 \times 10^6$  to  $12 \times 10^6$  cells  $\text{cm}^{-3}$  and  $6.7 \times 10^6$  to  $13 \times 10^6$  cells  $\text{cm}^{-3}$ , respectively (Figure 6.3 A, C, Table 6.1). Generally, the other treatments showed significantly lower bacterial cell numbers. For examples on day 14, both treatments CB and T1 were significantly higher than T4 and T5 (PERMANOVA  $p < 0.0001$ , followed by a non-parametric SNK test,  $p < 0.05$ ). A general decrease of bacterial cell numbers along with increasing TCS concentrations was observed, except for T1 which was quite similar to the positive control (Figure 6.3 C).

The bacterial division rates of the community were highly variable within the treatments over time (Table 6.2). Still, the biofilm without triclosan (CB) showed a more consistent and pronounced increase in the bacterial division rates as compared to the TCS treatments (Table 6.1). No significant relations could be determined between bacterial cell numbers and bacterial division rates in the different treatments. Like for the bacterial cell numbers, the bacterial division rates were negligible in the negative controls.

**Table 6.2:** Bacterial division rates in treatments over the experimental time ( $10^6$  cells  $\text{cm}^{-3} \text{h}^{-1}$ )

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6
CB	0.64	2.30	5.13	3.48	5.33	1.53
T1	2.04	0.91	0.24	1.37	3.89	1.47
T2	1.41	4.14	2.77	4.03	4.46	1.30
T3	3.81	4.23	2.72	2.85	4.01	1.32
T4	2.06	2.91	8.43	2.76	4.86	1.46
T5	1.33	0.11	4.61	3.72	4.95	0.64



**Figure 6.3:** Bacterial cell numbers over the course of the experiment. (A) Mean values ( $n = 4$  per treatment,  $\pm SE$ ): positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu\text{g l}^{-1}$ , △), T2 (TCS:  $10 \mu\text{g l}^{-1}$ , ●), T3 (TCS:  $20 \mu\text{g l}^{-1}$ , ◇), T4 (TCS:  $50 \mu\text{g l}^{-1}$ , ▲), T5 (TCS:  $100 \mu\text{g l}^{-1}$ , □). (B) Mean values per day ( $n=7$ ,  $\pm SE$ , ◆) and per treatment ( $n=6$ ,  $\pm SE$ , bar plots). (C) Mean values ( $n=4$ ,  $\pm SE$ ) shown for the different treatments on the first day (grey bars) as opposed to the day 14 (white bars).

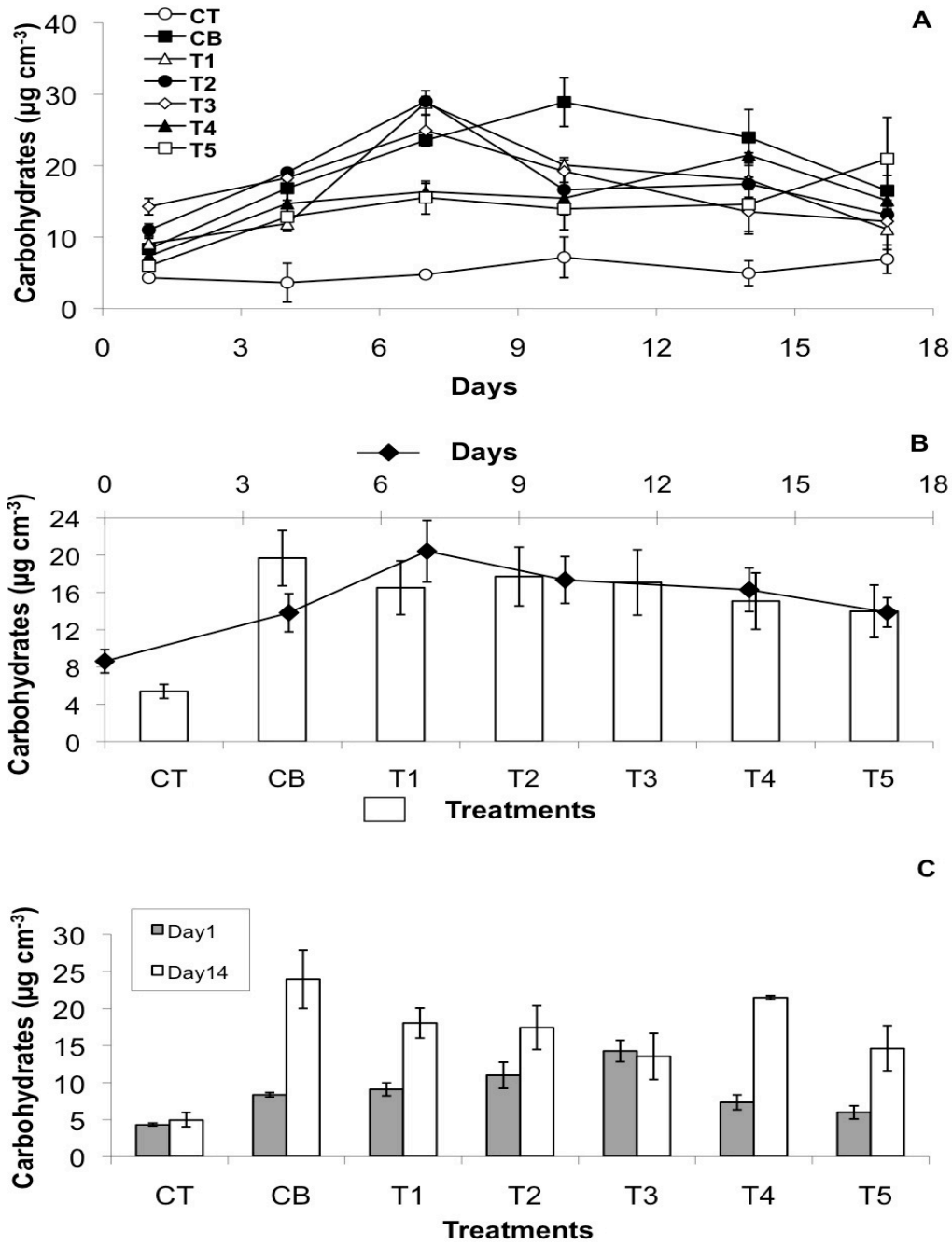


#### 6.3.4. Changes in colloidal EPS components

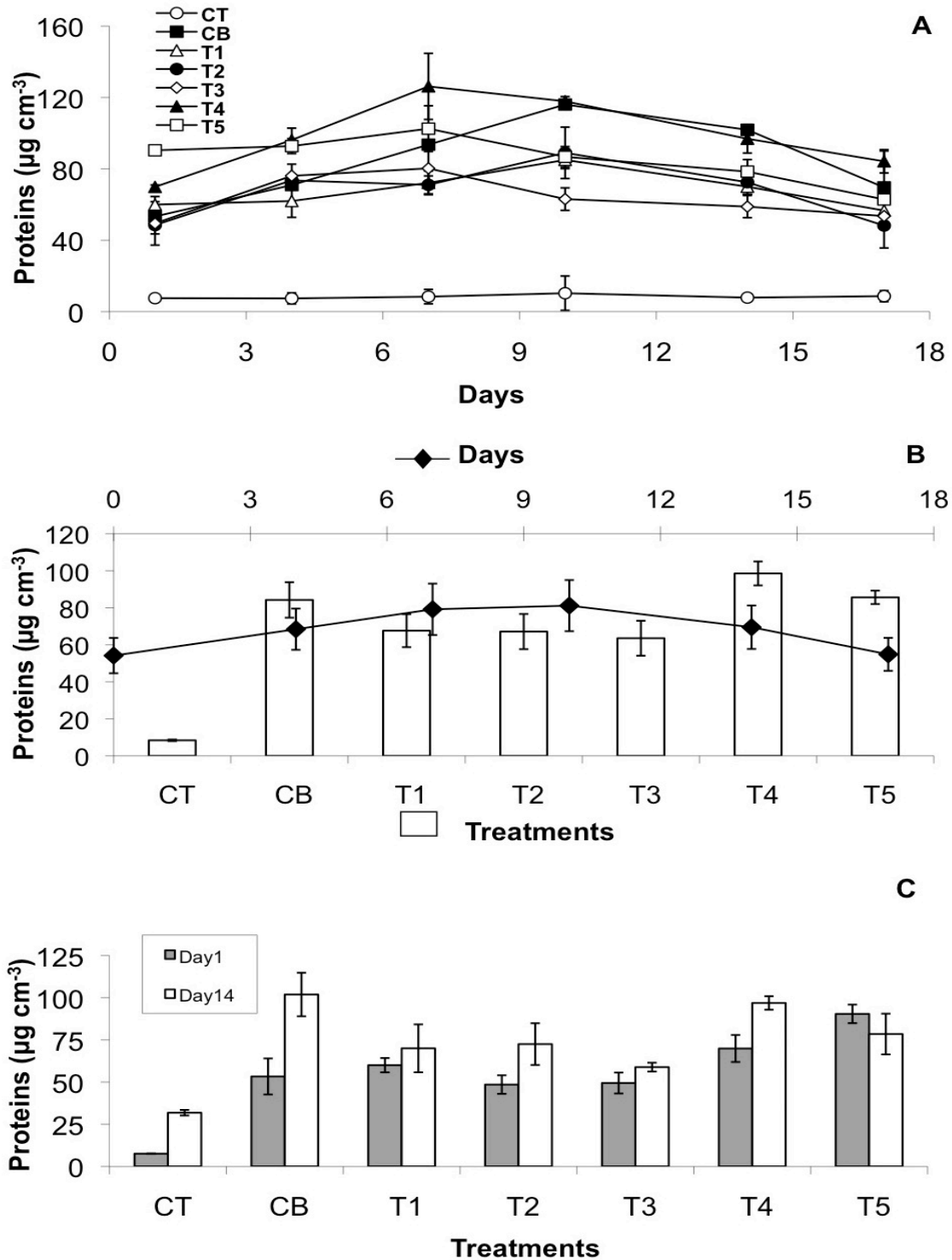
The colloidal EPS carbohydrate concentrations increased up to the middle of experiment and gradually decreased thereafter in the positive control and the treatments with low TCS concentrations (Figure 6.4 A, B). In contrast, the treatments T4 and T5 with the highest TCS concentrations showed a much lower increase over the first week, however at the end of experiment (day 10 and day 12) carbohydrate concentration in these treatments increased and the concentration value was comparable with positive control CB. Thus, the relative increase in EPS colloidal carbohydrates from the starting point to its maximum was finally similar between all treatments (about 3-3.5 times, except T3, Table 6.1 and Figure 6.4 C). Still, CB, T1 as well as T2 showed the highest carbohydrate concentrations as compared to the other treatments, with ranges between 8.35–28.9  $\mu\text{g cm}^{-3}$ , 9.09–28.8  $\mu\text{g cm}^{-3}$ , 11–29.01  $\mu\text{g cm}^{-3}$ , respectively (Figure 6.4 A). For instance, on day 7, CB and T1 were significantly higher than T3, T4 and T5 (PERMANOVA  $p < 0.0001$ , followed by a non-parametric SNK test,  $p < 0.05$ ). At the same time, T3 (range 14.27–24.9  $\mu\text{g cm}^{-3}$ ) was significantly higher than T4 and T5 (range 7.34–21.5  $\mu\text{g cm}^{-3}$  and 5.98–20.96  $\mu\text{g cm}^{-3}$ , respectively) (PERMANOVA  $p < 0.0001$ , followed by a non-parametric SNK test,  $p < 0.05$ ) (Figure 6.4 A). The negative controls without biofilms showed negligible concentrations of EPS carbohydrates.

The water-extractable proteins exhibited a clear increase over the first half of the experiment and a decrease thereafter; in all treatments (Figure 6.5 A, B). Still, the relative increase in EPS proteins from starting point to maximum was most pronounced for the biofilm without TCS (up to 2.2 times, ranged between 53.3–116  $\mu\text{g cm}^{-3}$ , Figure 6.5 C, Table 6.1). Consequently, the positive control had significantly higher EPS protein concentrations on most of the sampling days as compared to T1 (range 60–85  $\mu\text{g cm}^{-3}$ ), T2 (range 48.5–89  $\mu\text{g cm}^{-3}$ ) and T3 (49.4–80.3  $\mu\text{g cm}^{-3}$ ) (PERMANOVA  $p < 0.0001$ , followed by a non-parametric SNK test,  $p < 0.05$ , Figure 6.5 A). However, the treatments with the highest TCS concentrations (T4, T5) started with higher protein concentrations that, over the course of the experiment, were in a similar range to the positive control (between 69.9–126.2  $\mu\text{g cm}^{-3}$  and 90.4–102.5  $\mu\text{g cm}^{-3}$ , respectively) (Figure 6.5 B, C, Table 6.1). Accordingly, there were no significant differences between CB and T4 as well as T5.

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**Figure 6.4:** EPS carbohydrate concentrations, over the course of the experiment. (A) Mean values ( $n=4$  per treatment,  $\pm SE$ ): positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu g l^{-1}$ , Δ), T2 (TCS:  $10 \mu g l^{-1}$ , ●), T3 (TCS:  $20 \mu g l^{-1}$ , ◇), T4 (TCS:  $50 \mu g l^{-1}$ , ▲), T5 (TCS:  $100 \mu g l^{-1}$ , □). (B) Mean values per day ( $n=7$ ,  $\pm SE$ , ◆) and per treatment ( $n=6$ ,  $\pm SE$ , bar plots). (C) Mean values ( $n=4$ ,  $\pm SE$ ) shown for the different treatments on the first day (grey bars) as opposed to the day 14 (white bars).



**Figure 6.5:** EPS protein concentrations, over the course of the experiment. (A) Mean values ( $n=4$  per treatment,  $\pm SE$ ): positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu g l^{-1}$ , △), T2 (TCS:  $10 \mu g l^{-1}$ , ●), T3 (TCS:  $20 \mu g l^{-1}$ , ◇), T4 (TCS:  $50 \mu g l^{-1}$ , ▲), T5 (TCS:  $100 \mu g l^{-1}$ , □). (B) Mean values per day ( $n=7$ ,  $\pm SE$ , ◆) and per treatment ( $n=6$ ,  $\pm SE$ , bar plots). (C) Mean values ( $n=4$ ,  $\pm SE$ ) shown for the different treatments on the first day (grey bars) as opposed to the day 14 (white bars).

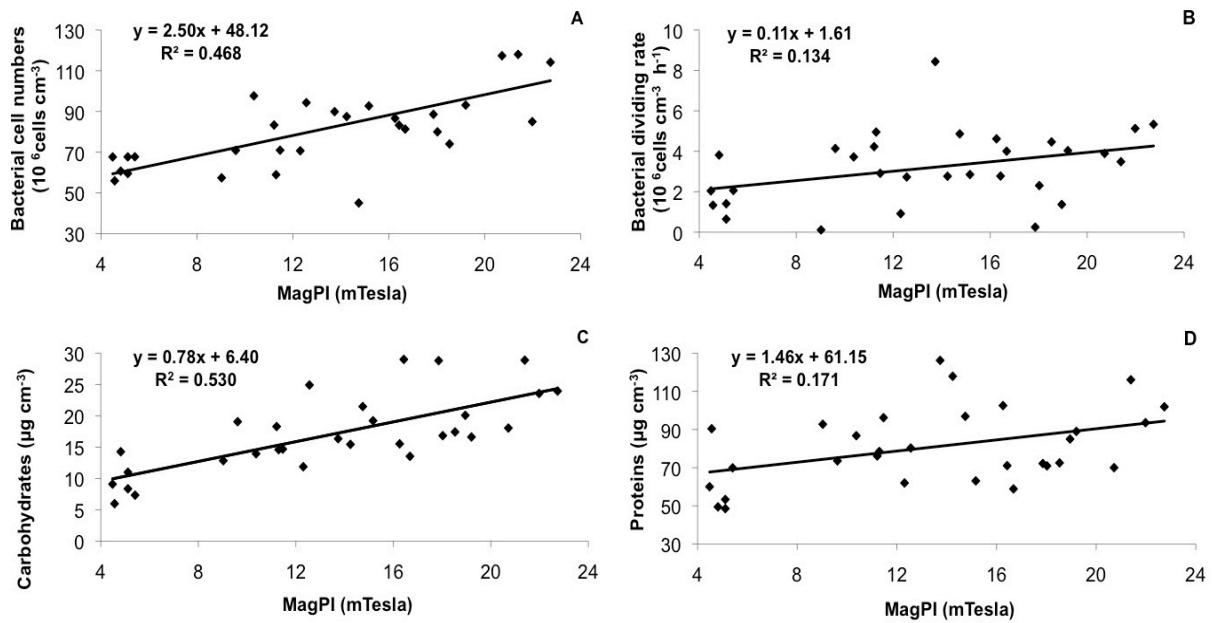
A strong relationship was determined between EPS colloidal carbohydrates and EPS colloidal proteins for all treatments except T5 (CB:  $R^2=0.748$ ; T1:  $R^2=0.523$ ; T2:  $R^2=0.542$ , T3:  $R^2=0.560$ ; T4:  $R^2=0.508$ ;  $p<0.05$ ). The colloidal carbohydrates and proteins both showed a significant positive relation to the bacterial division rate for the positive control and the treatments with highest TCS concentration (CB:  $R^2=0.834$ , T4:  $R^2=0.632$ , T5:  $R^2=0.799$ ,  $p<0.01$ , for carbohydrates; CB:  $R^2=0.590$ , T4:  $R^2=0.672$ , T5:  $R^2=0.468$ ,  $p<0.05$ , for proteins). The relations between EPS components and bacterial cell numbers were positive in tendency, but not significantly different.

### 6.3.5. Relation between biological variables, surface adhesion/stability and triclosan exposure

Considering the whole dataset, positive relationships were determined between substratum adhesion/stability and bacterial cell numbers (Figure 6.6 A) as well as to bacterial division rates (Figure 6.6 B). Substratum adhesion was also closely related to EPS colloidal carbohydrates (Figure 6.6 C) and, to a lesser extent, to EPS proteins (Figure 6.6 D). Focusing on the single treatments separately, the strongest correlations between adhesion/stability and the biological parameters (bacteria, EPS) were generally determined for the treatments with no or lower triclosan exposure (Table 6.3).

**Table 6.3.** Pearson's correlation coefficients between surface adhesion (MagPI) and EPS carbohydrates and proteins bacterial cell number and bacterial division rate per treatment. The significance levels are the following: \*\*\*  $p<0.001$  \*\*  $p<0.0$  \*  $p<0.05$ .

Treatment	<i>Carbohydrates</i>			<i>Proteins</i>			<i>Bacterial cell</i>			<i>Bacterial dividing rate</i>		
CB	0.774	20	**	0.795	20	**	0.528	20	*	0.834	13	**
T1	0.634	20	**	0.595	18	**	0.497	29	*	-0.154	14	
T2	0.542	16	*	0.548	20	*	0.537	16	*	0.626	12	*
T3	0.011	18		0.135	18		-0.233	18		0.094	12	
T4	0.667	20	**	0.483	20	*	0.438	16		0.642	12	*
T5	0.610	20	**	0.096	20		0.465	18	*	0.617	14	*



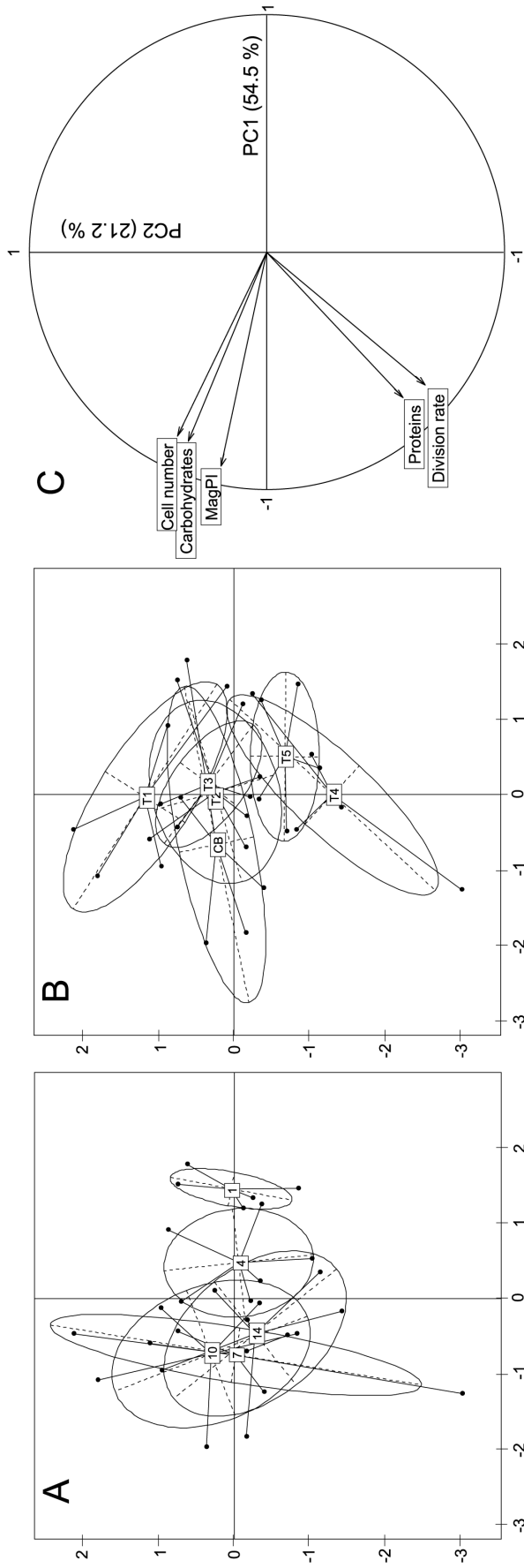
**Figure 6.6:** Relationship ( $n=30$ ) between bacterial biofilm adhesion expressed by MagPI (mTesla) versus bacterial cell numbers (A), bacterial dividing rates (B), EPS carbohydrate concentrations (C) and EPS protein concentrations (D).

The first and second principal components (PC1 and PC2) explained about 75% of the total variability (inertia), respectively 54.5 and 21.2%. Objects (rows) of the original data frame were grouped by plotting their ellipse inertia (which indicated the size of the group) along with their gravity centre. The projection of the objects in the plan formed by PC1 and PC2 showed that the gravity centres are distributed differently depending on whether they are grouped according to the sampling dates (Figure 6.7 A) or the treatments (Figure 6.7 B). Despite a relatively high variability within the groups (especially in Figure 6.7 B), the sampling dates gravity centres were clearly distributed along PC1 with the first dates at the right end of the graph and the last sampling dates at the left end. Treatments were mostly distributed along PC2 with lowest triclosan concentrations located at the top and highest TCS concentrations located at the bottom. Two groups of variables were identified (Figure 6.7 C): within the PC1, substratum adhesion (MagPI), EPS carbohydrates and bacterial cell numbers were strongly correlated (i.e. Spearman Rank correlation coefficient  $p=0.71$  and  $0.70$  between MagPI vs. EPS carbohydrates and cell numbers, respectively,  $n=30$ ,  $p < 0.001$ ) and they accounted for 75% of the variance (MagPI, EPS carbohydrates,

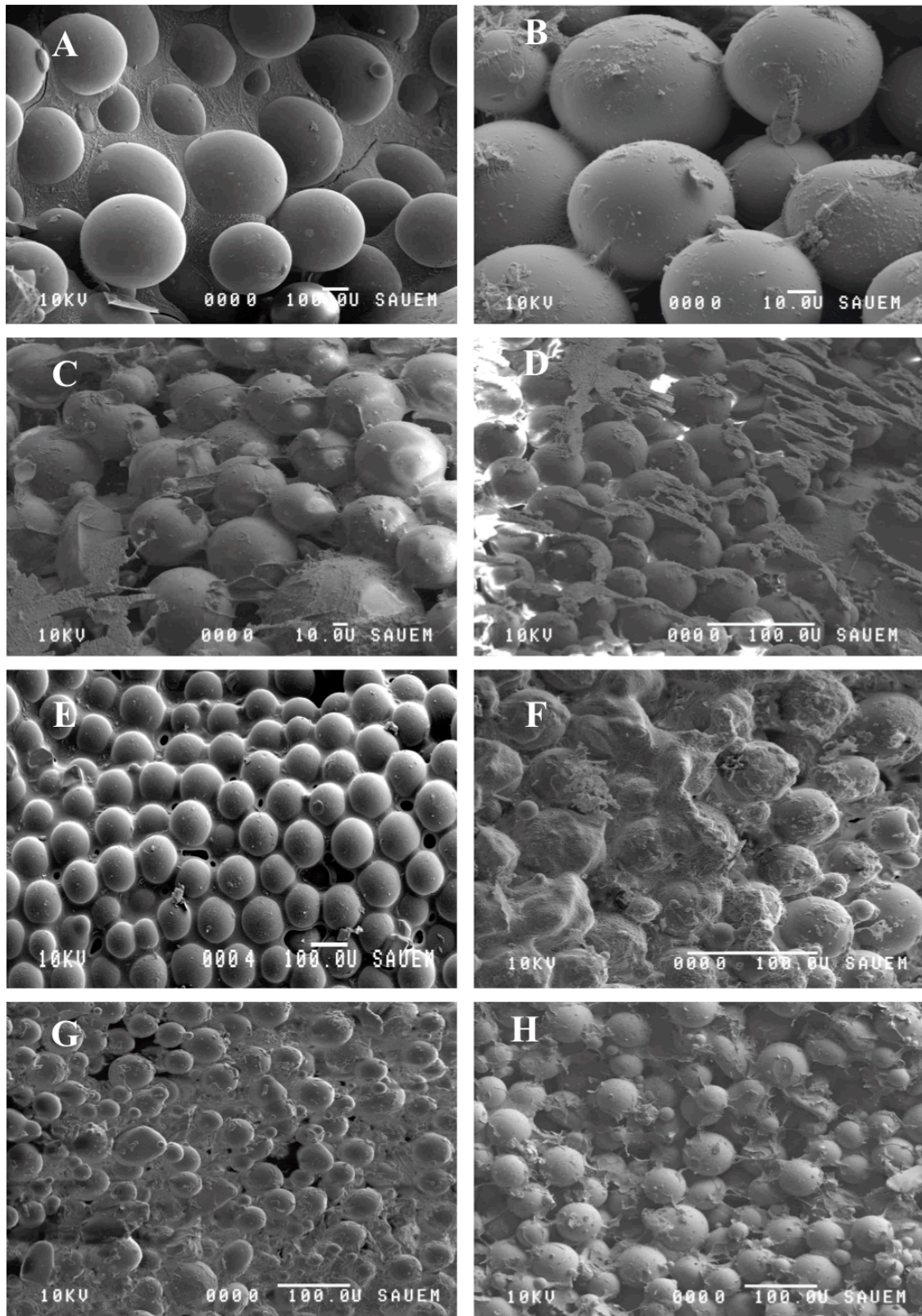
bacterial cell numbers each 30, 23 and 22%, respectively). Within PC2, the bacterial division rates (42%) and EPS proteins (31%) contributed to 73% of the variance and were correlated to each other (i.e.  $p=0.41$ ,  $n=30$ ,  $p<0.05$ ).

The analysis suggested that carbohydrates, sediment stability and cell number increased with time, but also that they tend to decrease with increasing the triclosan concentrations. Simultaneously bacterial division rate and protein concentration tend to increase with increasing time but also with increasing triclosan concentrations.

The distribution of the different treatments along the second axis (PC2), suggested that the triclosan concentration explained more than 20% of the total inertia of the dataset.



**Figure 6.7:** PCA. The projection of the objects in the plane formed by PC1 and PC2 showed that the gravity centres are distributed differently depending on whether they are grouped according to the sampling dates (A) or the treatments (B). (C) Circle of correlation for variables and projection of the variables in the factorial plane PC1 - PC2.



**Figure 6.8:** *LTSEM (low-temperature scanning electron microscopy) images of the biofilms: A-B: controls (negative and positive, higher magnification) at day 1; C-D: T 2*



and T5 at day 1; E–F: controls (negative and positive, lower magnification) at day 7; G–H: T2 and T5 at day 7. Note the water has been frozen around the clean glass beads of the control without biofilm (A, E). In the presence of bacteria (F–H), the secreted bacterial EPS matrix is visible, covering the glass beads and becoming denser by the end of the incubation (F–H) and permeating the intermediate space. However, at day 7, the biofilm EPS matrix exposed to higher TCS concentrations (H) appeared less as compared to the low TCS concentration treatment.

#### 6.4. Discussion

##### ***Impairment of the bacterial stabilisation potential by triclosan***

This is the first study to investigate the effect of triclosan (TCS) on bacterial stabilization and compares the effect of different concentrations of triclosan (relevant to environmental occurrence). The biofilm was established in all of the treatments and the bacterial colonization resulted in the development of a biofilm which significantly stabilized the test substratum. Since the chosen substratum was composed of non-cohesive glass beads, the binding force must have been entirely due to bacterial attachment and the secretion of a polymeric matrix (Figure 6.8) (Gerbersdorf et al. 2008). The stabilisation effect as determined by MagPI was significantly more pronounced for the positive control CB without TCS, than for the treatments with TCS exposure and was over 5 times higher than negative control CT. Initial adhesion of the pure cultures depends on a range of factors including selected pharmaceuticals and their concentration (Schreiber and Szewzyk 2008). In present experiment the overall stability was increased over time in all treatments, suggesting that environmental concentrations of triclosan would not stop development of the bacterial biofilm. However different “slope of increase” of stabilisation indicate that TCS does inhibit the adhesive properties of the biofilm. These results suggest the impairment of the bacterial stabilisation by TCS exposure were more pronounced along with TCS gradient.

It has been reported that sensitivity of bacteria to TCS depends on culture condition (Johnson et al. 2009). The present experiment was performed under nutrient depleted condition and a decrease in stabilisation was observed in all treatments including the positive control, after two week of experiment. This decrease may be due to a “batch

culture” effect. However, such a decrease was already observed after one week of experiment in treatments with higher TCS concentration it may indicate the highest sensitivity of bacterial culture to presence of TCS in nutrient-depleted conditions.

The negative control, without bacterial biofilm CT does not show an increase in stability and also does not show variation over time. This indicates that the contribution of gravity forces on the overall stabilization in all of the treatments is negligible.

***What is the cause of stabilization in TCS treatments?***

In recent years it has been highlighted that EPS production may significantly stabilize the sediment (Perkins et al. 2003, Underwood and Paterson 2003). In fact increasing stability was mirrored by increasing EPS concentrations in the first week of the experiment in all of the treatments. After the first week of experiments a rapid decrease in EPS concentration for both carbohydrate and proteins were observed in almost all TCS treatments in contrast to a continuous increase in CB. Indeed, the positive control CB demonstrates higher stabilisation effect together with higher EPS carbohydrate and protein concentration than treatments with TCS exposure. Despite that treatments with higher TCS concentration (T4 and T5) demonstrate rapid increase in EPS carbohydrate production at the end of experiment (Figure 6.4 C), the cumulative EPS carbohydrate concentration in these treatments was still significantly lower than other treatments (Figure 6.4 B). Consequently due to the quite similar pattern of stability and EPS production, highly significant relationships between sediment stability and EPS carbohydrate ( $r=0.728$ ,  $n=29$ ,  $p<0.001$ ) were determined. However due to the highly variability between treatments in EPS protein concentration relationship between stability and EPS protein concentration was less pronounced, but still significant ( $r=0.414$ ,  $n=29$ ,  $p<0.05$ ). These variations in EPS values are likely to have direct implications for the adhesion potential and confirm that EPS matrix is responsible for stability. So does less EPS mean less stability? Different tendencies in stability and EPS production were observed in treatments with lower and higher TCS concentrations. For the lower range of TCS concentrations, parallel decreases in stability and EPS concentration were observed. However in treatments with higher TCS (T4 and T5) the EPS concentrations were also higher and comparable to the positive control CB. This is in opposition to stability data, which

shows a continual decrease with increasing TCS concentration. This may be explained by:

a) As a bactericidal effect of TCS. The TCS toxicity-mediated cell lysis may augment EPS and suggest that some EPS compounds may be relative to intracellular pools.

As has been previously shown, the density of bacteria in biofilms can be affected by chemical compounds (TCS concentration  $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ M) (Dobretsov et al. 2007). The presence of TCS could promote both structural and functional changes in the bacterial membrane (Villalain et al. 2001). The latest observations indicate that in low concentration, triclosan working as bacteriostatic agent, inhibit bacterial growth and reproduction (Suller and Russell 1999, 2000, Escalada et al. 2005b, Tabak et al. 2007). At the higher concentrations, triclosan become bactericidal and is likely to damage the bacterial membrane (Villalain et al. 2001). Our results corroborate these latest observations, whereby at low concentrations, bacterial growth was severely affected, but at the high concentration (i. e. T4, T5) the bactericidal effect was rapid, indicating a more damaging effect such as membrane activity. Indeed the bacterial cell number in highest TCS concentration T4, T5 after 2 weeks of experiment (day 14) were significantly lower as compare to the control and lowest TCS treatments (Figure 6.3). These results corroborate the previous observation that the highest TCS concentration ( $<100 \mu\text{g l}^{-1}$ ) interacted with the cell envelopes and may cause bacterial lysis and subsequent fatal loss of intracellular material (Villalain et al. 2001, Escalada et al. 2005b)

b) EPS quantity *per se* is not always decisive for the binding capacity of sediments (Gerbersdorf et al. 2009), thus for the substratum stabilization and also depends on abiotic and environmental condition and on the particular organism from which they are produced (Decho 1990). In many earlier studies, polysaccharides were considered to be main constituents of EPS, however, when more extensive analyses of EPS were performed, proteins were frequently shown to be abundant in the EPS from pure cultures of Gram-negative and Gram-positive bacteria (Flemming et al. 1999). Bacteria need to attach firmly to a substratum with the help of EPS and proteins play a significant role in this first adhesion (Czaczyk and Myszka 2007, Jain et al. 2007), but also contribute towards the binding strength within the developing EPS matrix. Due to high proportions of the hydrophobic amino acid alanine, extracellular proteins contribute to hydrophobic properties of EPS. If proteins intertwine with

carbohydrates, they form heteropolymers of EPS (Jorand et al. 1998) with net negative or positive charges (Flemming and Wingender 2001). This may enhance and strengthen the EPS matrix (Pennisi 2002) by involving the electrostatic and hydrophobic interaction between different macromolecules (Flemming and Wingender 2001). In addition, the characteristic quantity of one particular EPS component, carbohydrates or proteins, most likely differs between the different group of bacteria, and so changes in bacterial community composition may lead to changes in EPS quality *per se*.

In summary, the interaction of triclosan with the bacterial cell is complex. There is evidence that TCS exposure affects bacterial biomass and EPS production and this affect was more pronounced with increasing TCS concentration. This study demonstrates that TCS concentration, relevant to environmental occurrence not stop development of the bacterial biofilm. However, significant impairment the bacterial stabilisation potential along with TCS gradient was suggested. Further studies should examine the effect of TCS on stabilisation capacity of the natural microbial community (bacteria and microalgae). These results will have a wider implication for optimisation sediment /pollutant management strategies.

#### **Declaration**

The results presented in this chapter have formed all or part of the following publication:

- HV Lubarsky, C Hubas, S Behrens, F Riccardi, DM Paterson, SU Gerbersdorf (2011). Impairment of the bacterial stabilisation potential by triclosan. (*Submitted for publication to Aquatic Toxicology*).

**References**

- Adolfsson-Erici, M., M. Pettersson, J. Parkkonen, and J. Sturve. 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**:1485-1489.
- Aguera, A., A. R. Fernandez-Alba, L. Piedra, M. Mezcua, and M. J. Gomez. 2003. Evaluation of triclosan and biphenylol in marine sediments and urban wastewaters by pressurized liquid extraction and solid phase extraction followed by gas chromatography mass spectrometry and liquid chromatography mass spectrometry. *Analytica Chimica Acta* **480**:193-205.
- Bellin, C. A. and P. S. C. Rao. 1993. Impact of bacterial biomass on contaminant sorption and transport in a subsurface soil. *Applied and Environmental Microbiology* **59**:1813-1820.
- Chalew, T. E. A. and R. U. Halden. 2009. Environmental exposure of aquatic and terrestrial biota to triclosan and triclocarban. *Journal of the American Water Resources Association* **45**:4-13.
- Cyr, H. and K. E. Morton. 2006. Distribution of biofilm exopolymeric substances in littoral sediments of Canadian Shield lakes: the effects of light and substrate. *Canadian Journal of Fisheries and Aquatic Sciences* **63**:1763-1776.
- Czaczyk, K. and K. Myszka. 2007. Biosynthesis of extracellular polymeric substances (EPS) and its role in microbial biofilm formation. *Polish Journal of Environmental Studies* **16**:799-806.
- Decho, A. W. 1990. Microbial exopolymer secretions in ocean environments-their role(s) in food webs and marine processes. *Oceanography and Marine Biology* **28**:73-153.
- DeLorenzo, M. E., J. M. Keller, C. D. Arthur, M. C. Finnegan, H. E. Harper, V. L. Winder, and D. L. Zdankiewicz. 2008. Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environmental Toxicology* **23**:224-232.
- Dobretsov, S., H. U. Dahms, Y. L. Huang, M. Wahl, and P. Y. Qian. 2007. The effect of quorum-sensing blockers on the formation of marine microbial communities and larval attachment. *Fems Microbiology Ecology* **60**:177-188.
- Escalada, M. G., J. L. Harwood, J. Y. Maillard, and D. Ochs. 2005a. Triclosan inhibition of fatty acid synthesis and its effect on growth of *Escherichia coli* and *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **55**:879-882.
- Escalada, M. G., A. D. Russell, J. Y. Maillard, and D. Ochs. 2005b. Triclosan-bacteria interactions: single or multiple target sites? *Letters in Applied Microbiology* **41**:476-481.
- Farre, M., D. Asperger, L. Kantiani, S. Gonzalez, M. Petrovic, and D. Barcelo. 2008. Assessment of the acute toxicity of triclosan and methyl triclosan in wastewater based on the bioluminescence inhibition of *Vibrio fischeri*. *Analytical and Bioanalytical Chemistry* **390**:1999-2007.
- Flemming, H. C. and J. Wingender. 2001. Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects. *Water Science and Technology* **43**:1-8.

- Flemming, H. C., J. Wingender, R. Moritz, W. Borchard, and C. Mayer. 1999. Physico-chemical properties of biofilms - A short review. Pages 1-12 in C. W. Keevil, A. Godfree, D. Holt, and C. Dow, editors. *Biofilms in the Aquatic Environment*.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Gerbersdorf, S. U., W. Manz, and D. M. Paterson. 2008. The engineering potential of natural benthic bacterial assemblages in terms of the erosion resistance of sediments. *Fems Microbiology Ecology* **66**:282-294.
- Halden, R. U. and D. H. Paull. 2005. Co-occurrence of triclocarban and triclosan in US water resources. *Environmental Science & Technology* **39**:1420-1426.
- Heath, R. J., J. R. Rubin, D. R. Holland, E. L. Zhang, M. E. Snow, and C. O. Rock. 1999. Mechanism of triclosan inhibition of bacterial fatty acid synthesis. *Journal of Biological Chemistry* **274**:11110-11114.
- Heidler, J. and R. U. Halden. 2007. Mass balance assessment of triclosan removal during conventional sewage treatment. *Chemosphere* **66**:362-369.
- Jain, A., K. K. Nishad, and N. B. Bhosle. 2007. Effects of DNP on the cell surface properties of marine bacteria and its implication for adhesion to surfaces. *Biofouling* **23**:171-177.
- Johnson, D. R., K. Czechowska, N. Chevre, and J. R. van der Meer. 2009. Toxicity of triclosan, penconazole and metalaxyl on *Caulobacter crescentus* and a freshwater microbial community as assessed by flow cytometry. *Environmental Microbiology* **11**:1682-1691.
- Jones, C. G., J. H. Lawton, and M. Shachak. 1994. Organisms as ecosystem engineers. *Oikos* **69**:373-386.
- Jorand, F., F. Boue-Bigne, J. C. Block, and V. Urbain. 1998. Hydrophobic/hydrophilic properties of activated sludge exopolymeric substances. *Water Science and Technology* **37**:307-315.
- Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, and H. T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology* **36**:1202-1211.
- Larson, F., H. Lubarsky, D. M. Paterson, and S. U. Gerbersdorf. 2009. Surface adhesion measurements in aquatic biofilms using magnetic particle induction: MagPI. *Limnology and Oceanography: Methods* **7**:490-497.
- Lawrence, J. R., B. Zhu, G. D. W. Swerhone, J. Roy, L. I. Wassenaar, E. Topp, and D. R. Korber. 2009. Comparative microscale analysis of the effects of triclosan and triclocarban on the structure and function of river biofilm communities. *Science of the Total Environment* **407**:3307-3316.
- Levy, C. W., A. Roujeinikova, S. Sedelnikova, P. J. Baker, A. R. Stuitje, A. R. Slabas, D. W. Rice, and J. B. Rafferty. 1999. Molecular basis of triclosan activity. *Nature* **398**:383-384.
- Lindstrom, A., I. J. Buerge, T. Poiger, P. A. Bergqvist, M. D. Muller, and H. R. Buser. 2002. Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. *Environmental Science & Technology* **36**:2322-2329.
- Lubarsky, H. V., C. Hubas, M. Chocholek, F. Larson, W. Manz, D. M. Paterson, and S. U. Gerbersdorf. 2010. The stabilisation potential of individual and mixed assemblages of natural bacteria and microalgae. *Plos One* **5**:e13794.

- McAvoy, D. C., B. Schatowitz, M. Jacob, A. Hauk, and W. S. Eckhoff. 2002. Measurement of triclosan in wastewater treatment systems. *Environmental Toxicology and Chemistry* **21**:1323-1329.
- McMurry, L. M., M. Oethinger, and S. B. Levy. 1998. Triclosan targets lipid synthesis. *Nature* **394**:531-532.
- Mezcua, M., M. J. Gomes, I. Ferrer, A. Aguera, M. D. Hernando, and A. R. Fernandez-Alba. 2004. Evidence of 2,7/2,8-dibenzodichloro-p-dioxin a photodegradation product of triclosan in water and wastewater samples. Pages 241-247 in 8th International Symposium on Analytical Methodology in the Environmental Fiels/13th Meeting of the Spanish-Society-of-analytical- Chemistry. *Analytica Chimica Acta*, University A Coruna, Spain.
- Okumura, T. and Y. Nishikawa. 1996. Gas chromatography mass spectrometry determination of triclosans in water, sediment and fish samples via methylation with diazomethane. *Analytica Chimica Acta* **325**:175-184.
- Orvos, D. R., D. J. Versteeg, J. Inauen, M. Capdevielle, A. Rothenstein, and V. Cunningham. 2002. Aquatic toxicity of triclosan. *Environmental Toxicology and Chemistry* **21**:1338-1349.
- Pennisi, E. 2002. Materials science - Biology reveals new ways to hold on tight. *Science* **296**:250-251.
- Perkins, R. G., C. Honeywill, M. Consalvey, H. A. Austin, T. J. Tolhurst, and D. M. Paterson. 2003. Changes in microphytobenthic chlorophyll a and EPS resulting from sediment compaction due to de-watering: opposing patterns in concentration and content. *Continental Shelf Research* **23**:575-586.
- Ricart, M., H. Guasch, D. Barcelo, R. Brix, M. H. Conceicao, A. Geiszinger, M. J. L. de Alda, J. C. Lopez-Doval, I. Munoz, C. Postigo, A. M. Romani, M. Villagrasa, and S. Sabater. 2010. Primary and complex stressors in polluted mediterranean rivers: Pesticide effects on biological communities. *Journal of Hydrology* **383**:52-61.
- Russell, A. D. 2004. Whither triclosan? *Journal of Antimicrobial Chemotherapy* **53**:693-695.
- Schreiber, F. and U. Szewzyk. 2008. Environmentally relevant concentrations of pharmaceuticals influence the initial adhesion of bacteria. *Aquatic Toxicology* **87**:227-233.
- Schweizer, H. P. 2001. Triclosan: a widely used biocide and its link to antibiotics. *Fems Microbiology Letters* **202**:1-7.
- Singer, H., S. Muller, C. Tixier, and L. Pillonel. 2002. Triclosan: Occurrence and fate of a widely used biocide in the aquatic environment: Field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environmental Science & Technology* **36**:4998-5004.
- Suller, M. T. E. and A. D. Russell. 1999. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcus. *Journal of Hospital Infection* **43**:281-291.
- Suller, M. T. E. and A. D. Russell. 2000. Triclosan and antibiotic resistance in *Staphylococcus aureus*. *Journal of Antimicrobial Chemotherapy* **46**:11-18.
- Tabak, M., K. Scher, E. Hartog, U. Romling, K. R. Matthews, M. L. Chikindas, and S. Yaron. 2007. Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms. *Fems Microbiology Letters* **267**:200-206.

- Underwood, G. J. C. and D. M. Paterson. 2003. The importance of extracellular carbohydrate production by marine epipelagic diatoms. *Advances in Botanical Research* **40**:183-240.
- Villalain, J., C. R. Mateo, F. J. Aranda, S. Shapiro, and V. Micol. 2001. Membranotropic effects of the antibacterial agent triclosan. *Archives of Biochemistry and Biophysics* **390**:128-136.
- Wilson, B. A., V. H. Smith, F. Denoyelles, and C. K. Larive. 2003. Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environmental Science & Technology* **37**:1713-1719.
- Wolfaardt, G. M., J. R. Lawrence, R. D. Robarts, and D. E. Caldwell. 1998. In situ characterization of biofilm exopolymers involved in the accumulation of chlorinated organics. *Microbial Ecology* **35**:213-223.



## Chapter 7

### Effect of triclosan on a natural freshwater biofilm

#### **Abstract**

The introduction of herbicidal and pesticidal compounds onto the consumer market has rapidly increased over the last decade and as a result, the level of organic compounds found in surface water and sediments have increased (Ricart et al. 2010). This creates a potential risk to aquatic communities and their functionality, such as biostabilisation processes described in this thesis. To assess the potential environmental risk of such contaminants on biostabilisation, the establishment of a natural biofilm community was used as a suitable model of their community ecotoxicology. The response of a natural freshwater biofilm, under exposure to a concentration series of triclosan in a range relevant to environmental occurrence, was investigated. The biofilm was grown for 3 weeks in flow-through glass channels (mini-flumes) before the start of the experiment and artificial glass beads served as the inert non-cohesive substratum. The TCS (triclosan) was loaded using impregnated silicon rods (Bandow et al. 2009a, Bandow et al. 2009b) and TCS concentrations were checked regularly over experimental time. Assessment of the effects of TCS on natural freshwater biofilm was conducted over a two week period following the 3 weeks of biofilm development. The biostabilisation potential of natural biofilm under TCS

exposure was assessed using the sensitive new method of MagPI (Magnetic Particle Induction) (see Chapter 3) which can be used to determine the adhesive capacity of the biofilm. The results suggest an inhibitory effect of TCS on microbial biostabilisation. The positive control (CB) without TCS exposure was up to 2 times more adhesive than treatments with TCS concentrations. This data was followed up by examining the biological properties of the biofilms, such as microbial biomass and quantity of EPS compounds (carbohydrates and proteins) production. The results suggest that changes in the biofilm have a direct implication to their stabilisation potential. The bacterial growth demonstrated a delayed response to the TCS as determined by flow cytometry, and reveal that the decrease in bacterial cell number was more pronounced with increasing TCS concentrations. TCS was observed to inhibit microalgae photosynthetic activity (PSII) along with TCS gradient. In contrast, higher EPS carbohydrate quantity was found in treatments with higher TCS concentration.

TCS had an inhibitory effect on microbial stabilisation processes, which was found to be more pronounced along the TCS gradient. Biostabilisation potential cannot be addressed simply by the quality and quantity of extracellular polymeric substances, but also needs to take account of context and the structural parameters of the biofilm and their interactions. The present study raises the unexpected effects of using toxins in consumer products. The data presented will have wider implications for optimisation of sediment/pollutant management strategies and provides significant contribution to the investigation of biostabilisation process as a very important ecosystem function.

## **7.1. Introduction**

### ***Triclosan–persistence in environment and effect on aquatic organisms.***

Toxic pollution of aquatic systems and the associated effects on aquatic communities is of major environmental concern (Schmitt-Jansen and Altenburger 2008). The previous studies of toxicity assessment were focused on single diatom species or bacterial strains. However, neither microalgae nor bacteria exist independently in natural sediment (Gerbersdorf et al. 2009), and their interactions are complex and highly species-specific. Thus, the investigation effect of toxins on natural assemblages is great of the interest. Toxicity assessment was based on biomass, EPS components or

shift in community composition; however, the effect of pollution on ecological functionality of aquatic organisms, such as biostabilisation is still unknown. For this propose, a set of physiological variables of microbial community were observed and further addressed in terms of biostabilisation capacity of the microorganism, growing under environmental stress, such as presents of the triclosan.

Triclosan (TCS) (5-chloro-2-(2,4-dichlorophenoxy)phenol) is a synthetic, broad spectrum antimicrobial pesticide used to destroy or suppress the growth of harmful microorganisms, such as bacteria and fungi. TCS has use has expanded markedly into the consumer marked over the last 30 years and is present in soap (0.10-1.00%), toothpastes, deodorants, shaving creams, mouthwashes and other products. Over the last decade, there has been a rapid increase in the use of TCS-containing products. About 96 percent of TCS from consumer products are disposed of in residential drains, leading to large loads of the chemical in water entering wastewater treatment plants (Ciba 1998). TCS is a stable, lipophilic compound ( $\log K_{ow}=4.8$ ) which forms low chlorinated dioxins on incineration and under the influence of sunlight (Adolfsson-Erici et al. 2002). It has been observed that TCS itself is relatively non-toxic to humans and other mammals (Bhargava and Leonard 1996), however TCS degradation products, such as dioxin, are highly carcinogenic and can cause health problems (Glaser 2004). On the other hand, TCS is non-degradable under anaerobic conditions (McAvoy et al. 2002), and as TCS is disposed of predominantly down residential drains (Reiss et al. 2002), it can reach groundwater and accumulated on sediment surface. Halden & Paull (2005) suggested a propensity of TCS to persist in various environmental compartments with predicted half-lives ranging from 0.75 days in air to 540 days in sediment. A U.S. Geological Survey study of organic wastewater contaminants in water samples, found TCS ranking in the top 10 in occurrence rate and in the top 20 in maximum concentration among 96 organic pollutants (Kolpin et al. 2002, Halden and Paull 2005), due to its continuous replenishment and its accumulation within the sediments. Analytical data from environmental samples in several countries demonstrate concentration levels in rivers, lakes and streams in the range 18-2300ng l<sup>-1</sup> (Okumura and Nishikawa 1996, Kolpin et al. 2002, Lindstrom et al. 2002, McAvoy et al. 2002, Singer et al. 2002). Digested sludge concentrations of TCS ranged from 0.5 to 15.6 µg g<sup>-1</sup> (dry weight), where the lowest value was from an aerobic digestion process and the highest value was from an anaerobic digestion

process (McAvoy et al. 2002). Measurable concentrations of TCS first appeared in sediment cores around 1964 (Cantwell et al. 2010), the measured vertical concentration profile of TCS in a lake sediment core (Singer et al. 2002), reflects its increased use over 30 years and presence of TCS in sediment samples was found in concentrations 0.27 to 130.7  $\mu\text{g kg}^{-1}$  (McAvoy et al. 2002, Aguera et al. 2003). According to Reiss et al. (2002) the mean percentage of triclosan sorbed to suspended sediment and solids was 14.3 ( $\pm$ ) 7.3 (range 3.6-86.9 ppb).

Triclosan acts as a biocide, with multiple cytoplasmic and membrane targets, causing leakage of intracellular material (Villalain et al. 2001). However at low concentrations TCS appears bacteriostatic and associated with an inhibition of membrane biogenesis through a specific target, the enoyl reductase enzyme, which is involved in the synthesis of fatty acids (McDonnell and Pretzer 1998, McMurry et al. 1998, Escalada et al. 2005) and affects reproduction in bacteria. The minimum inhibitory concentration (MIC) of TCS for clinically important bacteria were found to be as high as 1  $\mu\text{g ml}^{-1}$  (Suller and Russell 1999).

Triclosan has a broad range of activity that encompasses many, but not all, types of Gram-positive and Gram-negative non-sporulating bacteria (Russell 2004). Bacteria may form resistance to TCS, due to mutation and/or overproduction of enoyl reductase (Escalada et al. 2005). These aspects of the adaptation of bacteria to TCS have since been examined extensively (Schweizer 2001, Escalada et al. 2005, Tabak et al. 2007) and other research shows that minimum inhibitory concentration of triclosan for different bacteria strains may reach 3000  $\mu\text{g l}^{-1}$  (Bhargava and Leonard 1996). DeLorenzo et al. (2008) found that the presence of sediment decreased TCS toxicity and determined that bacteria were sensitive to TCS with 15 min of aqueous TCS value of 53  $\mu\text{g l}^{-1}$  and a 15 min spiked sediment TCS value of 616  $\mu\text{g kg}^{-1}$ . In natural sediment, bacteria coexist with other phototrophic and heterotrophic microorganisms. There is evidence that triclosan is also acutely and chronically toxic to other aquatic organisms (Orvos et al. 2002, Ishibashi et al. 2004, DeLorenzo et al. 2008), of which microalgae are the most sensitive to TCS (Reiss et al. 2002, Neumegen et al. 2005). Biofilms can be used as an early warning system for detection of the effect of toxicants on aquatic systems (Sabater et al. 2007) and microphytobenthos have been shown to be a suitable model in community ecotoxicology (Schmitt-Jansen and Altenburger 2005a, 2008). Using natural algal communities sampled both upstream

and downstream Wilson et al. (2003) shows shifts in the community structure and suggests that toxin exposure may potentially influence both the structure and the function of algal communities.

Experiments to assess the potential risks of toxicants on aquatic organisms (community) have been previously performed using various strategies. The toxicity of TCS was evaluated using microalgae systems varying in biological complexity, under different periods of TCS exposure and including various abiotic parameters (such as nutrient enrichments). Depending on species composition varying ranges of TCS concentration were found to be toxic to microalgae: Tabak et al. (2007) observed an eight-fold log reduction of exponentially growing cell with  $1000 \mu\text{g ml}^{-1}$  triclosan within 10 min, Franz et al. (2008) found the inhibition of photosynthetic efficiency of various microalgae system after 24 h TCS exposure with concentrations  $3.7 \mu\text{g l}^{-1}$  for a chlorophyte suspension and  $900 \mu\text{g l}^{-1}$  for periphyton communities, DeLorenzo et al. (2008) examined range of marine species, and suggest the phytoplankton species were the most sensitive species tested, with a 96 h  $\text{EC}_{50}$  value of  $3.55 \mu\text{g l}^{-1}$ , Orvos et al. (2002) found inhibited growth of algae in presence of TCS in concentration ranged between  $3.4\text{-}13 \mu\text{g l}^{-1}$  over 9 d of experiment, especially for the diatom *Skeletonema costatum* ( $>66 \mu\text{g l}^{-1}$  96 h). Lawrence et al. (2009) determined a significant reduction of algae biomass as result of TCS exposure in concentration  $10 \mu\text{g l}^{-1}$  over 8 weeks of experiment. There is evidence that TCS maybe even more harmful for algae than bacteria (Johnson et al. 2009). Tatarazako et al. (2004) shows that some microalgae species were 30-80 fold ( $\text{IC}_{25}=0.0034 \text{ mg l}^{-1}$  triclosan) more sensitive to triclosan toxicity than bacteria.

#### ***Natural communities may improve cell tolerance to toxins?***

The central purpose of environmental risk assessment is the protection of ecosystem from adverse impacts of chemicals (McClellan et al. 2008). Due to presence of microorganisms and their functional importance, microbial test is widely used in toxicity assessment. These investigations were mainly based on single species testing requiring extrapolation routines to estimate community-level effects (Schmitt-Jansen and Altenburger 2008).

Community composition might better reflect the effects of the toxicant(s), because they may cause a shift from a sensitive to a progressively more tolerant community

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(Schmitt-Jansen and Altenburger 2005b, Sabater et al. 2007). In contrast to this, Johnson et al (2009) showed that a natural fresh water microbial community was more sensitive to TCS than a culture of the freshwater bacterium *Caulobacter crescentus*. In natural sediment, organisms do not exist independently, and their interactions, especially between bacteria and microalgae are complex and range from symbiosis to parasitism (Cole 1982). Some algal cells constitute niches for bacterial species (Schaefer et al. 2002) by providing a substantial pool of organic carbon available to the bacteria as source of food (Bell et al. 1974, Haynes et al. 2007), as the result elevating the numbers of bacterial cells (Wang and Priscu 1994). On the other hand, bacterial remineralisation of organic nutrients may increase algae growth (Grossart 1999). However the inhibition and sometimes lethal effect (Imai et al. 1993) of these interactions between microorganisms have also been described in detail. Bacteria may compete with algae for nutrients and can inhibit algae growth (Grossart 1999). To prevent this, microalgae have a capability to produce antibacterial substances against both gram-positive and gram-negative bacterial strains which inhibit bacterial activities (Sastry and Rao 1994). According to Schaefer et al. (2002) algal diversity might be an important factor in explaining the enormous bacterial diversity and vice versa. Thus, bacteria-microalgae interactions are highly species-specific and presence of toxins can alter these interactions drastically, causing a shift in total community structure and knock-on effects on ecological functioning of the biofilm.

As described above, the biofilms consist of layers of microorganisms embedded in an extracellular polymeric matrix. In natural biofilms the EPS is the net result of prokaryotic and eukaryotic communities. EPS may fulfil a variety of functions (Wotton 2004), for instance bacteria produce EPS for attachment to a substratum (Costerton et al. 1999) and diatoms mostly for locomotion (Underwood and Paterson 2003). To enhance survival, both of these organisms produce EPS for protection of individual cells (Roberson and Firestone 1992, Sutherland 2001b, a). The EPS matrix can also buffer microbial colonies from some effects of desiccation (Roberson and Firestone 1992), may reduce diffusion and hence contribute to enhanced resistance of the biofilm-associated cells (Tabak et al. 2007). Recent investigation of effect of pollutants on EPS secretion shows a significant increase of EPS-polysaccharide formation in the presence of toluene, a toxic hydrocarbon (Schmitt et al. 1995), elevated extracellular

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carbohydrates and proteins under chromium (Fang et al. 2002, Iyer et al. 2004, Priester et al. 2006), and cadmium (White and Gadd 1998) exposure. Lawrence et al. (2009) indicate that TCS treatments result in significant changes in the composition of the EPS matrix and suggest the significant alteration in community composition from one dominated by autotrophic processes to one dominated by heterotrophic processes. However, interaction of EPS and TCS may depend on the quality and quantity of EPS, which in turn varies with the coexisting bacterial-microalgae assemblages. In the past, microalgae were considered as main EPS producers (reviewed in Stal and Walsby 2000, Underwood and Paterson 2003) with polysaccharides as their main product (Staats et al. 1999, Paterson et al. 2000, Stal 2003), however bacteria act not only as decomposers of the organic matrix (Goto et al. 2001), but also may secrete a copious amount of EPS (Decho 1990) with a high proportion of proteins (Flemming and Wingender 2001a, Gerbersdorf et al. 2008). Bruckner et al. (2008) showed that the presence of different bacteria strongly influenced carbohydrate secretion by the alga and changed monomer composition of extracellular polysaccharides. Previous studies have shown that in mixed assemblages (bacteria+microalgae) the EPS concentrations were significantly higher than in single culture assemblages (Lubarsky et al. 2010). Taking these aspects into account it is hypothesized that natural assemblages of microorganisms may provide biofilms with a high potential of tolerance to toxicants.

***Biofilm formation under toxicant exposure.***

In recent years, microbial EPS have been linked to a number of important ecological functions (Cyr and Morton 2006), such as accumulating pollutants and biostabilisation processes. The process of biostabilisation was intensively investigated (Paterson 1989, Dade et al. 1990, de Winder et al. 1999, Paterson et al. 2000), and related to microbial biomass (microalgae and bacteria) and EPS compounds (carbohydrates and protein) (Yallop et al. 2000, Gerbersdorf et al. 2009, Lubarsky et al. 2010). Secreting EPS into the surrounding sediment matrix may further aid organisms to attach to surfaces (adhesion) (Stal 2003) that can result in sediment cohesion and the increased stability of the sediment. It has been suggested that, by inhibiting initial adhesion, biofilm formation might be prevented (Cerca et al. 2005). Investigations into the effect of a range of pharmaceuticals on microbial adhesion

capacity have been performed using both bacterial cultures and natural microbial community. The results suggest that pharmaceuticals at environmentally relevant concentrations can influence the initial adhesion of bacteria, this was especially noted in the drinking water community which exhibited a decreased adhesion in the presence of the pharmaceuticals regardless of adhesion surface (Furneri et al. 2003, Schreiber and Szewzyk 2008). However, effect of toxins on biostabilisation capacity of natural biofilm is mostly unknown.

The ability of aquatic organisms, especially certain algal species, to continuously bioaccumulate toxic compounds into aquatic food webs were previously described (Zaranko et al. 1997, Jabusch and Swackhamer 2004, Coogan et al. 2007). There was evidence that the presence of toxins can significantly increase the formation of carboxyl groups thus increasing the number of negatively charged groups and consequently, the sorption capacity of the biofilm (Schmitt et al. 1995), which may result an increasing bioaccumulation capacity of biofilm.

To summarise, toxins may have a negative effect on microbial biomass, but on other hand, the presence of toxins may elevate EPS production and increase bioaccumulation capacity of the microorganisms. The prediction of possible effect of toxins on biostabilisation is complex and must be investigate extensively.

For this propose the flow-through channels approach applied in this study represents a more realistic scenario for biofilm development and colonization of substratum. The use of silicon rods provided an improved strategy of TCS spiking and equilibrium and produced a better approximation between calculated and actual concentrations of TCS than aqueous addition. Structural parameters (e.g. microbial biomass) and functional parameters (e. g photosynthetic capacity and EPS production), together with assessment of substratum stability will be used to demonstrate the effect of triclosan on biostabilisation potential of freshwater microbial community.

## **7.2. Experimental set-up and triclosan spiking**

To simulate the natural scenarios of development of the biofilm in river system, the flow-through channels containing Petri dishes that were filled with glass beads were used in this experiment. This creates potential problems with pseudoreplicates. Despite this fact this experiment has been chosen in order to increase range of



concentrations that may appear in aquatic system. Thus the experiment was conducted in seven flow through channels (LxWxH in cm 86x11.5x10) filled with water from the river Parthe which joins the White Elster in north-western Leipzig, Germany (51021'39''N 12020'32''E). Small glass beads in the size range 0.04-0.07 mm (Ballotini balls, Jenson) were used as an artificial, non-cohesive substratum, supporting development of biofilm. A 1 cm layer of glass beads was placed in glass Petri dishes ( $\varnothing$  10 cm, 2 cm height) and 6 Petri dishes were placed in each channel (Figure 7.1).



**Figure 7.1:** Experimental setup: Prior to experiment (top) and during experiment (bottom). Triclosan rods and air pump system are marked with A and B respectively.

Natural fresh water (3.5 l) was carefully added to each channel, after placing a layer of buoyant plastic onto the surface of the artificial sediment to protect the bed from erosion (Gerbersdorf et al. 2009). To simulate the natural scenarios of development of the biofilm in river system, the flow in the pumped system (Tubing Pump BVP Standard, ISMATEC) was set to a rate of 2 l min<sup>-1</sup>. The biofilms grew for 18 d under laboratory conditions, were illuminated at 80±5 μmol photons s<sup>-1</sup> m<sup>-2</sup> from a neon tube with a light/dark cycle of 14/10 h, and the temperature was kept constant (16°C). During the growth phase, the fresh river water was replaced regularly (once per week). Development of the biofilm was checked regularly by visual observation and it was observed that the biofilm was equally distributed in each channel. Each channel was initially inoculated by adding biofilm cultivated for four weeks on glass slides in an aquarium (under similar conditions). The biofilm was scraped from the glass slides, mixed with relevant amount of fresh water, homogenised and added at 100 ml per channel.

Prior to the experiment, an UV-filter (UV CL SR HPR, WIPA Technik, Germany) was used to avoid UV-degradation of TCS during the experiment and the flow system was replaced by an air pump system (Koi Air KA25, max output 25 l min<sup>-1</sup>, max pressure BLAGDON, Dorking, Surrey, England, Figure 7.1 B). Following the system design criteria of Wicke et al. (2007) all reactor materials, namely the solid support for the biomass, were polar to reduce hydrophobic interaction and sorption of the analytes. Only glassware was used and air supply tubing was fitted with glass tips (15 cm length and 0.5 cm diameter).

Stock solution of TCL (150 μg l<sup>-1</sup>) was prepared as described in 2.11.2 and working solutions were obtained by further dilution of stock solution. The following treatments were established aiming to produce concentrations of triclosan at 2 μg (T1), 20 μg l<sup>-1</sup> (T2), 50 μg l<sup>-1</sup> (T3), 100 μg l<sup>-1</sup> (T4), 150 μg l<sup>-1</sup> (T5), the positive control (CB) contained only natural biofilm culture, glass beads and fresh water. The negative controls (CT) contained glass beads, autoclaved fresh water and was not inoculated with biofilm. To exclude possible effects of TCS and dimethylsulfoxide (DSMO) on sediment stabilisation, 150 μg l<sup>-1</sup> of triclosan dissolved in 1% DSMO was additionally added to this channel. Additionally, silicon rods were prepared as described in 2.11.2

and served as a passive dosing tool in order to keep concentrations stable throughout the 10 d period of the experiment. Starting concentration of the analytes should not exceed the microgram per litre range (Wicke et al. 2007), which are concentrations similar to those one can expect to occur in the environment. Thus, the amount of TCS necessary to reach equilibrium concentrations on the levels mentioned above were calculated, and silicon rods were loaded accordingly. Then, the loaded rods were placed in the channels below the water surface (Figure 7.1 A). Prior to the experiment the fresh river water was replaced and kept at a constant level to account for evaporation over the course of the experiment, the missing volume was replenished by fresh river water (4 times~600 ml per channel). The pH-regime was checked regularly each day of experiment, using the universal measuring device Multi 340i (WTW GmbH, Weilheim, Germany) and kept stable by adding phosphate buffer (NaH<sub>2</sub>PO<sub>4</sub>, 4mM) according to Clark et al. (1981).

### ***Sampling strategy***

Prior to the experiments (day 0) all variables were measured to obtain the baseline readings, after which all channels were treated with appropriated level of TCS. First sampling took place the day after the first TCS treatment (day 1) and was repeated every second day for the two weeks of experiment. For each treatment, 3 Petri dishes were randomly selected and sampled in turn at each measurement. From each Petri dish, 3 sediment cores of 2 mm depth were sampled with a cut-off syringe 10 mm diameter (see 2.3.1). The 2 sediment cores were immediately frozen at -80°C until further analysis of EPS (described in 2.4) and the additional core was fixed with a glutaraldehyde solution (1% final concentration) and stored for future analysis of bacterial cell numbers (described in 2.7). For determination of a possible shift in microbial community, 2 additional cores were taken, fixed by adding 2.5% glutaraldehyde for determination of microalgae community composition (described in 2.9.2) and 3.7% formaldehyde for bacterial community composition (described in 2.9.1) and stored for future analyses. The functional parameter photosynthesis as proxy of microalgae biomass was measured by PAM fluorescence (described in 2.6) and determination of the sediment stability by MagPI (2.12.2) was measured for each Petri dish at every second day during the experimental period.

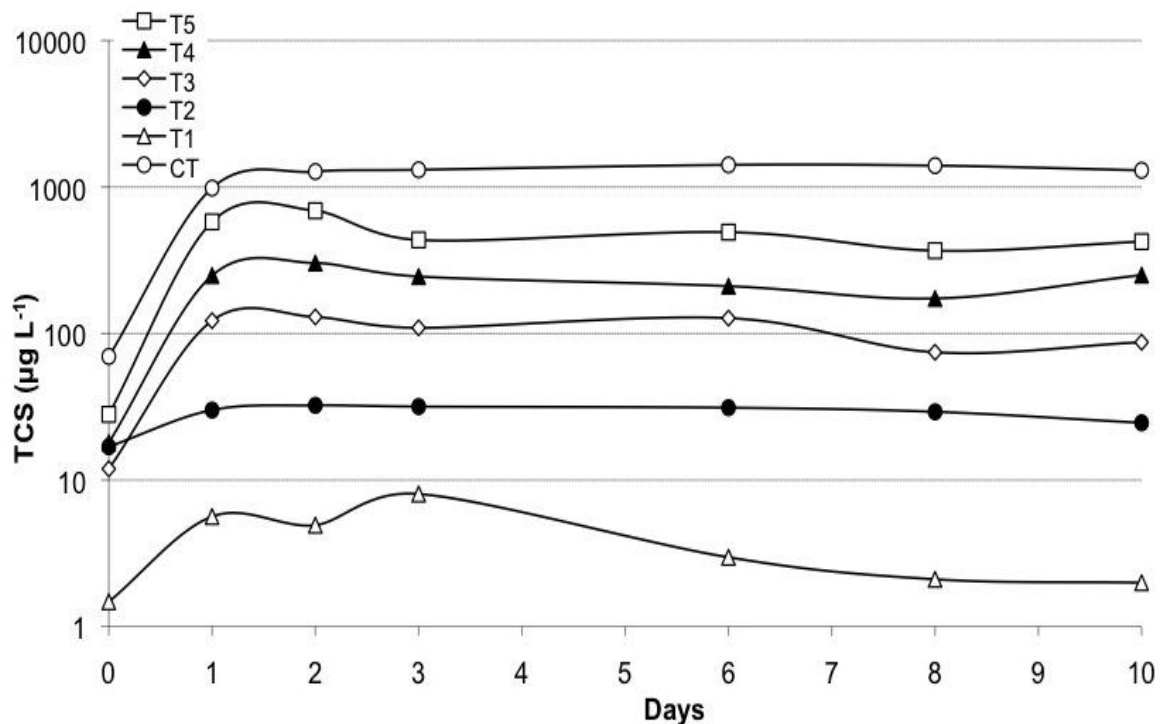
### 7.2.1. Statistics

The majority of the data did not pass the Kolmogorov-Smirnov normality test and Bartlett test for homogeneity of variance. Thus differences between treatments were addressed using a non-parametric Kruskal-Wallis test of variance followed by the non-parametric post-hoc Student-Newman-Keuls (SNK) test to compare pairs of treatments.

## 7.3. Results

### 7.3.1. Triclosan concentrations

The actual triclosan concentrations in water samples did not increase over time (Figure 7.2), but were about two times higher than the spiked concentrations. The actual average TCS concentration in the overlaying water determined were  $3.9 \mu\text{g l}^{-1}$  for T1,  $28 \mu\text{g l}^{-1}$  for T2,  $94.7 \mu\text{g l}^{-1}$  for T3,  $207.1 \mu\text{g l}^{-1}$  for T4 and  $430.7 \mu\text{g l}^{-1}$  for T5. In the negative control CT, the TCS concentration exceeded  $1000 \mu\text{g l}^{-1}$  and there were no significant changes over the experimental period.



**Figure 7.2:** Triclosan concentration over the experimental period.

Surprisingly, the minimum concentration of TCS ( $3 \mu\text{g l}^{-1}$ ) was also determined in positive control CB, this may be due to the actual concentration of TCS in the river

Parthe. At the end of the experiment the concentration of TCS was measured in the sediment and biofilm. The results suggest that TCS accumulated more in biofilm than in sediment (Table 7.1). The accumulation of the TCS was increased, along the gradient of triclosan. Similar to the water samples, the minimum concentration of the TCS was determined in sediment for treatment CB.

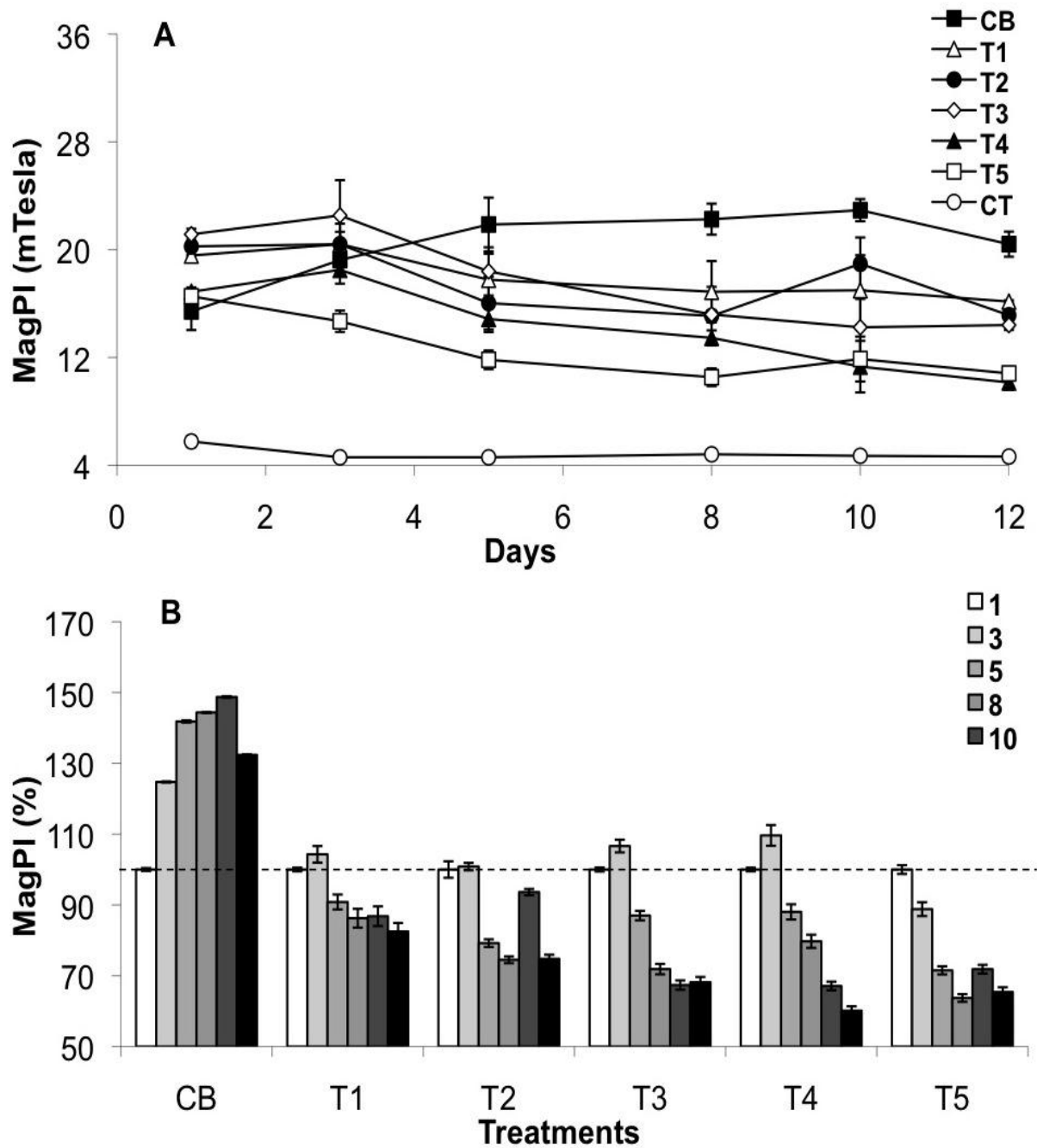
**Table 7.1.** Concentration of TCS in glass beads and in biofilm per treatment.

Treatment	<b>TCS concentration in sediment <math>\mu\text{g/g}</math></b>	<b>TCS concentration in biofilm <math>\mu\text{g/g}</math></b>
CB	0.006	0.27
T1	0.005	0.53
T2	0.013	2.71
T3	0.073	7.50
T4	0.119	15.14
T5	0.170	18.64

### 7.3.2. The stability of the substratum

The biofilm in all treatments was developed during a 3-week period prior to the start of the experiment and on the first sampling day (before TCS spiking) the stabilisation effect on the substratum with biofilm was significantly higher (up to 4.8 times) in than for the negative control (CT) without the biofilm (Kruskal-Wallis (KW) test,  $\chi^2=15.4$ ,  $\text{df}=6$ ,  $p<0.05$ ). The adhesive capacity of the negative control (CT) was below 5 mTesla and did not show any significant changes in adhesion/stability over the experimental time (Figure 7.3 A). On the first day of the experiment (before TCS spiking), no significant differences were found between all of the treatments, suggesting that the biofilm was equally developed before it was treated with TCS. On sampling day 3 (after TCS spiking), all treated cultures exhibited a slight increase (~6%) in the adhesion of the substratum, except the treatment with higher TCS concentration T5, which showed a rapid decrease of stability by 12% (Figure 7.3 B). In contrast to that positive control CB was increased by 24%. After TCS spiking a decrease in substratum stabilisation was observed for all treated cultures (Figure 7.3 A), and the overall decrease was more pronounced for the treatments with higher and medium concentration of TCS, followed by low concentration of the TCS T1 and T2 (Figure 7.3

B). In contrast, in the positive control CB (without triclosan), the stability of the sediment surface increased continuously up to day 10 (Figure 7.3 A), exhibiting an overall increase up to 49% as compared to the first day of the experiment (Figure 7.3 B) and was in average 1.4 times higher than T1, T2, T3 and 2 times higher than T4 and T5. There was no significant difference between the TCS treatments including positive control CB, in the day 1 and day 3 of experiment. However, after this time there was a significant difference between treatments for example day 8 (KW,  $\chi^2=27.5$ ,  $df=6$ ,  $p<0.001$ ) and day 10 (KW,  $\chi^2=32.8$ ,  $df=6$ ,  $p<0.001$ ). The positive control CB was significantly higher than other treatments with triclosan for example day 8 (KW, followed by post-hoc SNK test  $p<0.01$ ). The treatment with higher TCS concentration T5 was significantly lower than other treatments for all of this time (KW,  $p<0.01$  followed by post-hoc SNK test), except the day 10 and 12, where no significant difference was found between treatment T5 and T4. On the rest of the experiment treatment with low TCS concentrations, T1 and T2, were significantly higher than treatments T4 and T5, for example day 8 (KW, followed by post-hoc SNK test  $p<0.05$ ).



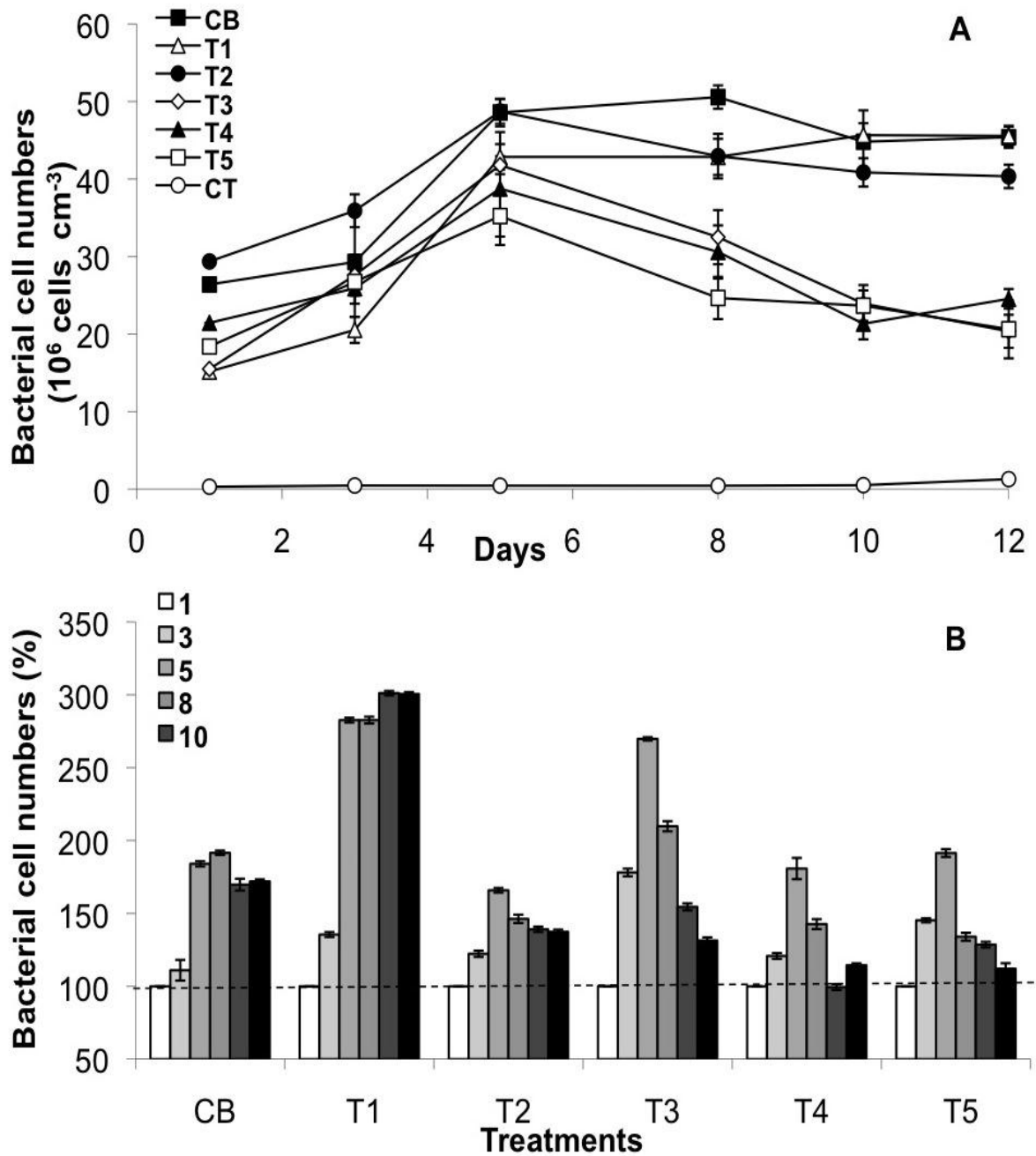
**Figure 7.3:** Biofilm adhesion, measured by MagPI, over the course of the experiment. (A) Mean values ( $n=6$  per treatment,  $\pm SE$ ): positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu\text{g l}^{-1}$ , △), T2 (TCS:  $20 \mu\text{g l}^{-1}$ , ●), T3 (TCS:  $50 \mu\text{g l}^{-1}$ , ◇), T4 (TCS:  $100 \mu\text{g l}^{-1}$ , ▲), T5 (TCS:  $150 \mu\text{g l}^{-1}$ , □). (B)-Changes in biofilm adhesion in relation to the first day (100%) represented as a dashed line, mean values ( $n=6$ ,  $\pm SE$ ).

### 7.3.3. Microbial biomass

#### **Bacterial cell number**

From the beginning of experiment the bacterial cell numbers in all treatments determined by flow cytometry were significantly higher than negative control CT (KW,  $\chi^2=20$ ,  $df=6$ ,  $p<0.01$ ), which did not show any significant changes over the experimental time. In the first experimental week the bacterial cell numbers continuously increased in all treatments up to day 5 (Figure 7.4 A) and decreased afterwards, however, the bacterial cell numbers in the positive control (CB) and treatment with low TCS concentration T1 increased or remained stable until the end of experiment. The increase was most pronounced for the treatments T1 and T3 (up to 180%), in other treatments the overall increase was quite similar and did not exceed 91% (Figure 7.4 B). The highest bacterial cell numbers were determined in the treatments CB and T1 and ranged between  $26.4 \times 10^6$ - $50.6 \times 10^6$  cells  $\text{cm}^{-3}$  as well as  $15.2 \times 10^6$ - $45.6 \times 10^6$  cells  $\text{cm}^{-3}$  respectively, followed by T2 and T3 with range  $29.4 \times 10^6$ - $48.7 \times 10^6$  cells  $\text{cm}^{-3}$  as well as  $15.5 \times 10^6$  - $41.8 \times 10^6$  cells  $\text{cm}^{-3}$  respectively and finally T4 and T5 with range 21.4-38.8 cells  $\text{cm}^{-3}$  as well as 18.4-35.2 cells  $\text{cm}^{-3}$  respectively. There was no significant difference between the treatments at the beginning of experiment, however by the second week of experiment differences between treatments became significant. For example from day 5 treatments CB, T1 and T2 were significantly higher than T4 and T5 (KW,  $\chi^2=15.6$ ,  $df=6$ ,  $p<0.01$  followed by post-hoc SNK test). By day 8 the positive control CB was significantly higher compared with T1 and T2 (KW, followed by post-hoc SNK test  $p<0.01$ ), however, there was no significant difference between these treatments at the end of experiment. From day 8 and until to the end of experiment, treatments CB, T1 and T2 were significantly higher than T3, T4 and T5 (KW,  $\chi^2=18.2$ ,  $df=6$ ,  $p<0.01$ , followed by post-hoc SNK). After day 8 treatments T3 and T4 were rapidly decreased and were not significant different from T5.





**Figure 7.4:** Bacterial cell numbers over the course of the experiment. (A) Mean values ( $n=3$  per treatment,  $\pm SE$ ): positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu\text{g l}^{-1}$ , △), T2 (TCS:  $20 \mu\text{g l}^{-1}$ , ●), T3 (TCS:  $50 \mu\text{g l}^{-1}$ , ◇), T4 (TCS:  $100 \mu\text{g l}^{-1}$ , ▲), T5 (TCS:  $150 \mu\text{g l}^{-1}$ , □). (B)-Changes in bacterial cells number in relation to the first day (100%), represented as a dashed line, mean values ( $n=6$ ,  $\pm SE$ ).

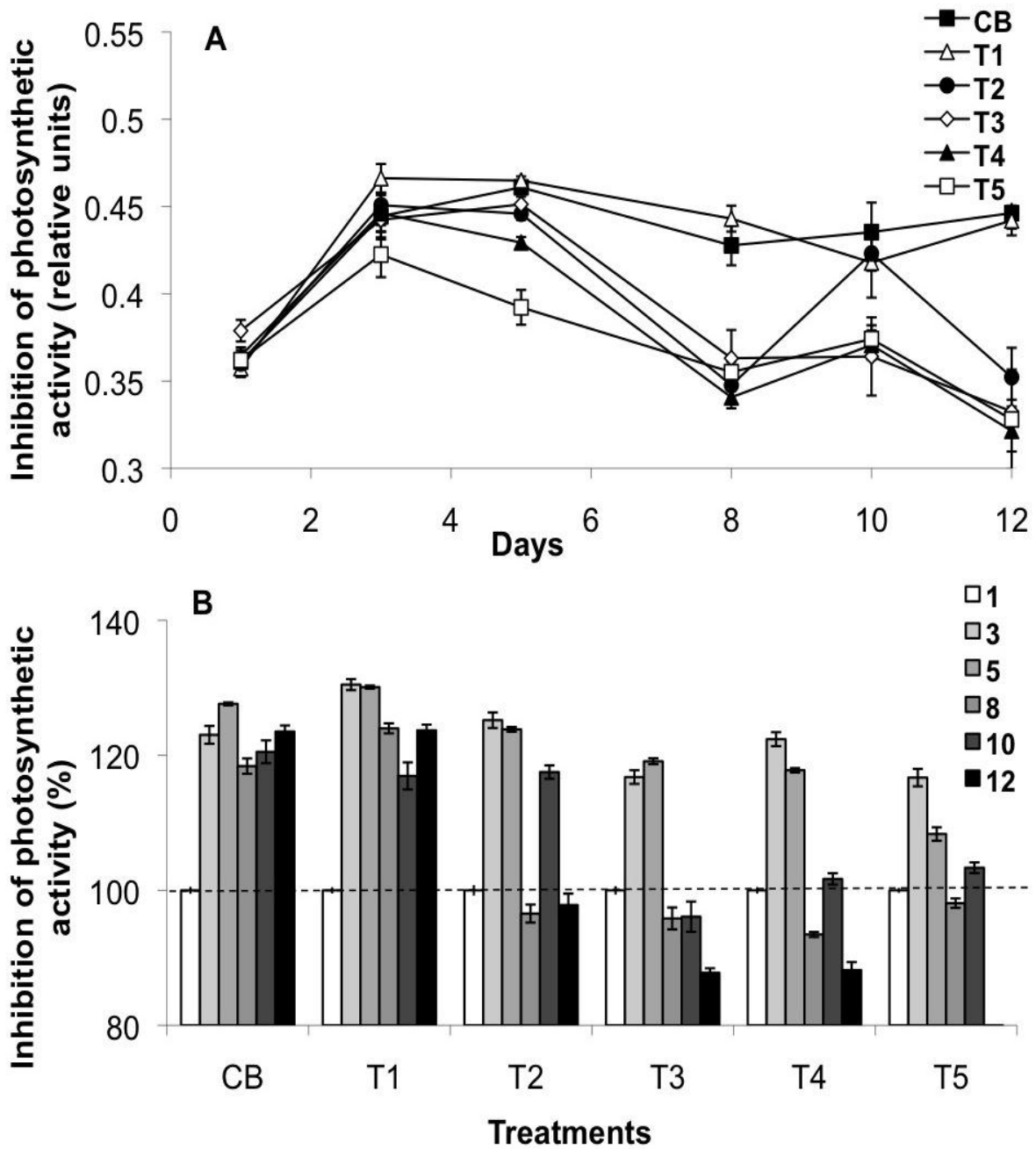
**Microalgae biomass and the effective quantum yield (PSII)**

The changes of biomass as indicated by  $Fo^5$  were highly variable within the treatments over time (Table 7.2), although the minimum fluorescence of the biofilm without triclosan (CB) increased throughout the experiment, in contrast to all TCS treatments.

**Table 7.2** Biomass development, measured as minimum Chl *a* fluorescence ( $Fo^5$ ) in treatments over experimental time (relative units).

<b>Fo</b>	<i>Day 1</i>	<i>Day 2</i>	<i>Day 3</i>	<i>Day 4</i>	<i>Day 5</i>	<i>Day 6</i>
CB	0.926	0.910	0.940	0.910	0.944	0.993
T1	0.943	0.904	0.955	0.915	0.968	0.940
T2	0.903	0.904	0.962	0.899	0.963	0.933
T3	0.947	0.931	0.899	0.965	0.933	0.931
T4	0.895	0.902	0.903	0.996	0.927	0.897
T5	0.899	0.932	0.904	0.994	0.898	0.911

The effective quantum yield did not differ significantly between treatments at the beginning of the experiment (day 1 and day 3) (Figure 7.5 A). However, after TCS spiking, there were major differences in the inhibition of the photosynthetic yield PSII, which were more pronounced along the increasing TCS gradient (Figure 7.5 B). The effective quantum yield in treatments CB and T1 increased in comparison with the first day of the experiment and remained stable until the end of the experiment. Effective quantum yield of samples in CB and T1 treatments were significantly higher than other treatments for example day 8 (KW,  $\chi^2=16.7$ ,  $df=6$ ,  $p<0.01$ , followed by post-hoc SNK test) except on day 10, where no significant difference was found between CB, T1 and T2. The negative controls without biofilms were undetectable by multi-wavelength-excitation PAM fluorometry.

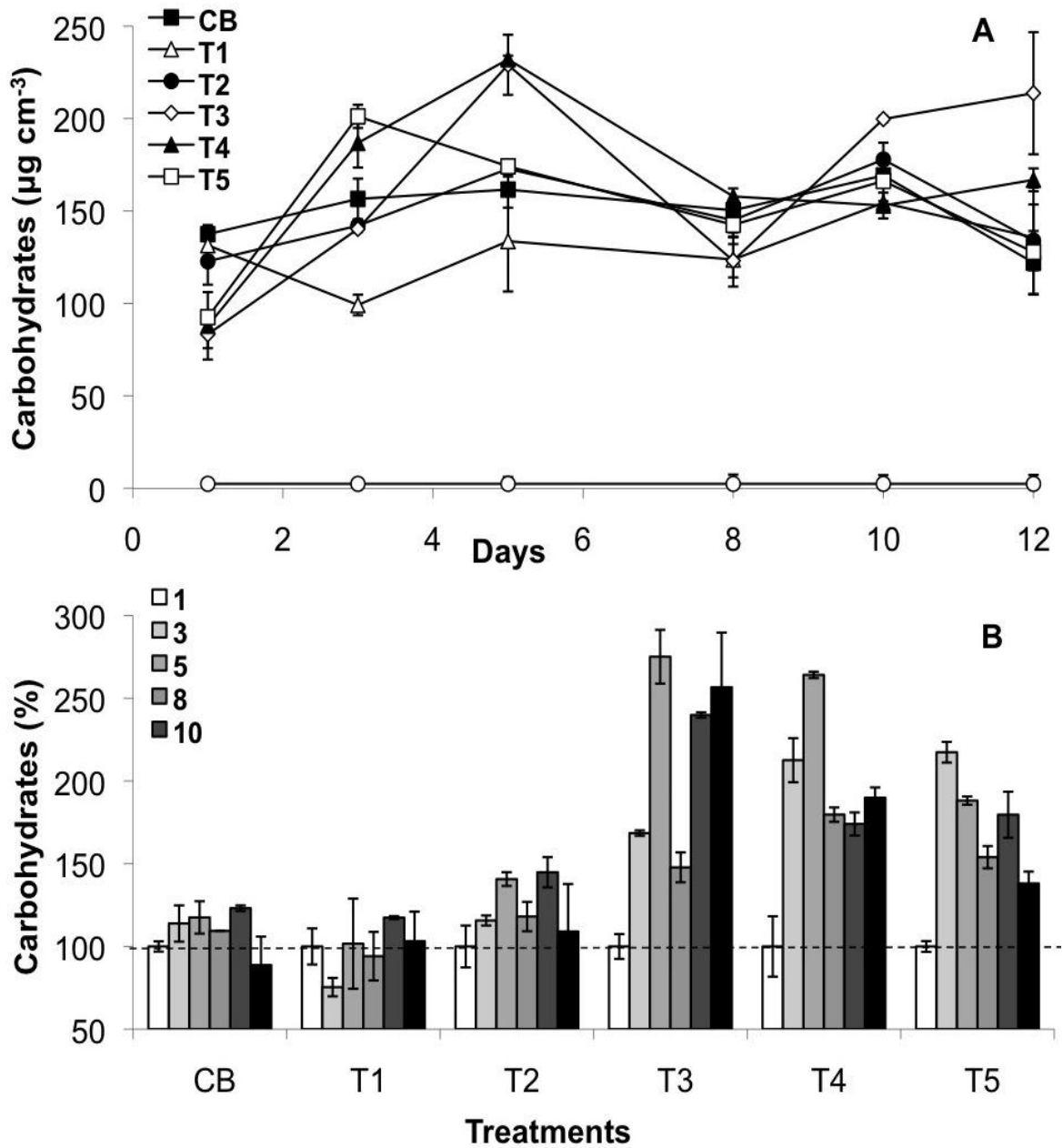


**Figure 7.5:** The inhibition of photosynthesis ( $n=3$  per treatment,  $\pm SE$ ), over the course of the experiment for positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu g l^{-1}$ , △), T2 (TCS:  $20 \mu g l^{-1}$ , ●), T3 (TCS:  $50 \mu g l^{-1}$ , ◇), T4 (TCS:  $100 \mu g l^{-1}$ , ▲), T5 (TCS:  $150 \mu g l^{-1}$ , □). (B)-Changes in photosynthetic activity of microalgae in relation to the first day (100%), represented as a dashed line, mean values ( $n=6$ ,  $\pm SE$ ).

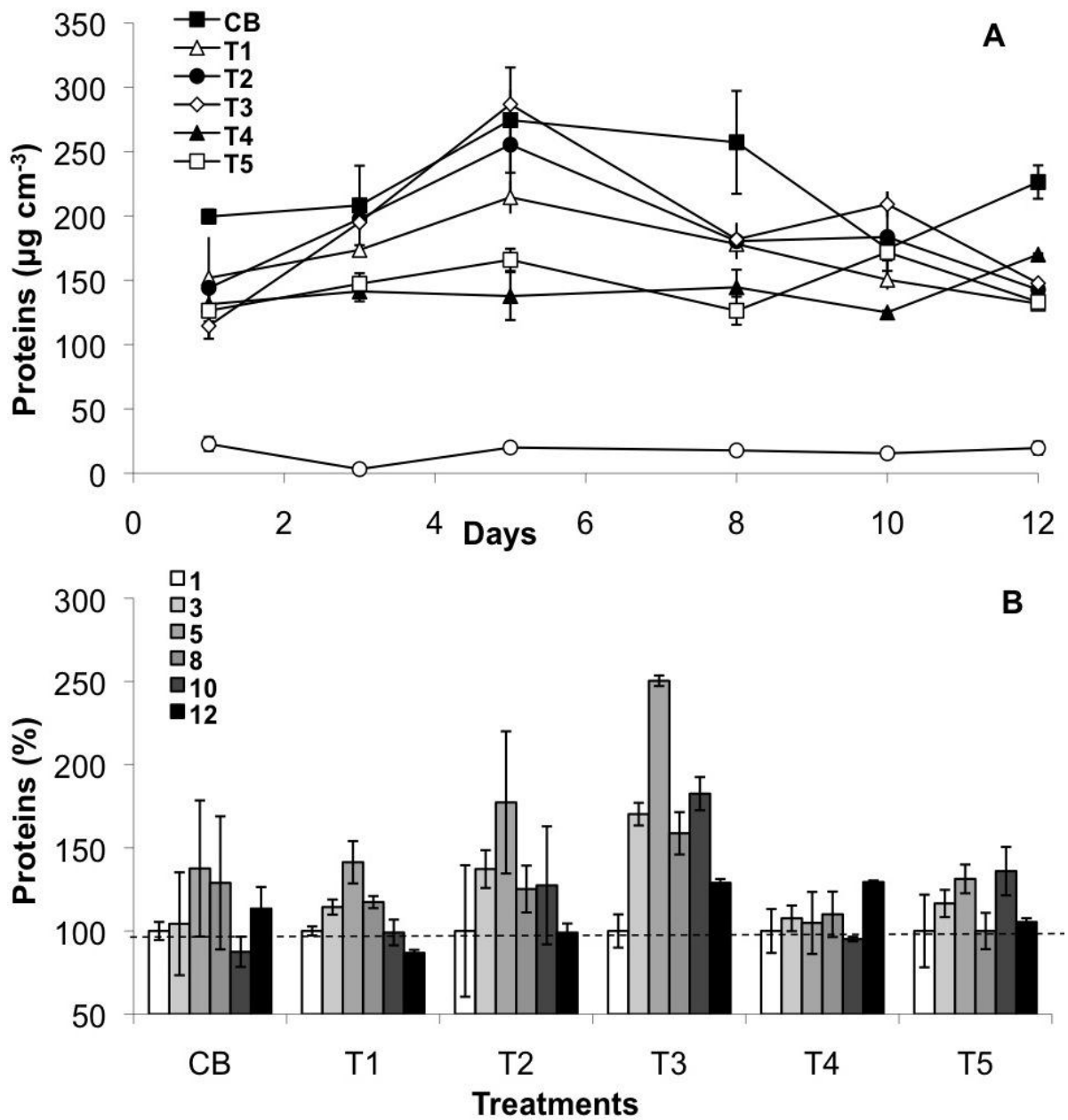
#### 7.3.4. Changes in colloidal EPS components

The colloidal carbohydrate concentrations increased over time in all treatments; however the negative controls CT showed negligible concentrations of colloidal carbohydrates over the entire period of the experiment (Figure 7.6 A). The increase was more pronounced for the treatments T3 (up to 175%), T4 (164%) and T5 (up to 88%) (Figure 7.6 B). The colloidal carbohydrate concentration in these treatments reached a maximum in day 5 of experiment, and ranged between 83.3-229  $\mu\text{g cm}^{-3}$ , 87.9-232  $\mu\text{g cm}^{-3}$  and 92.5-201.2  $\mu\text{g cm}^{-3}$  respectively and were significantly higher than CB, T1 and T2 (KW,  $\chi^2=18.3$ ,  $df=6$ ,  $p<0.01$ , followed by post-hoc SNK test). The positive control CB and treatments T1 and T2 reached a maximum at day 10 of experiment and ranged between 137.4-169.2  $\mu\text{g cm}^{-3}$ , 131.5-154.4  $\mu\text{g cm}^{-3}$  and 122.8-177.8  $\mu\text{g cm}^{-3}$  respectively. At the end of the experiment (day 12) the treatments T3 and T4 still significantly higher than positive control and treatment T1 and T2 (KW,  $\chi^2=14.3$ ,  $df=6$ ,  $p<0.05$ , followed by post-hoc SNK test), however no significant difference was found between treatments CB, T1, T2 and T5.

The water-extractable protein concentrations increased up to the middle of experiment and gradually decreased thereafter in all treatments with low and medium TCS concentration and the positive control (Figure 7.7 A). The increase was more pronounced for treatment T3 (150%), followed by >T2 (77%)>T1 (41%)>CB (37%) (Figure 7.7 B) with ranges between 114.5-286.8  $\mu\text{g cm}^{-3}$  for T3, 144-255.4  $\mu\text{g cm}^{-3}$  for T2, 151.8-214.5  $\mu\text{g cm}^{-3}$  for T1 and 199.5-274.5  $\mu\text{g cm}^{-3}$  for CB. In contrast, the treatments T4 and T5 with the highest TCS concentration showed a much reduced increase over the first week that continued until the end of the experiment (Figure 7.7 A). At the beginning of experiment there was no significant difference between treatments, except the negative control. However after TCS spiking (day 5), the treatments CB, T1, T2 and T3 were significantly higher than T4 and T5 (KW,  $\chi^2=17.9$ ,  $df=6$ ,  $p<0.01$ , followed by post-hoc SNK test) and at the end of the experiment positive control CB was significantly higher than another treatment with TCS (KW,  $\chi^2=19.1$ ,  $df=6$ ,  $p<0.01$ , followed by post-hoc SNK test). The negative controls without biofilms showed negligible concentrations of colloidal proteins concentration over experimental period.



**Figure 7.6:** Colloidal carbohydrate concentrations, over the course of the experiment. Mean values ( $n=3$  per treatment based on  $n=3$  replicates per box,  $\pm$ SE) is shown for positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu\text{g l}^{-1}$ , △), T2 (TCS:  $20 \mu\text{g l}^{-1}$ , ●), T3 (TCS:  $50 \mu\text{g l}^{-1}$ , ◇), T4 (TCS:  $100 \mu\text{g l}^{-1}$ , ▲), T5 (TCS:  $150 \mu\text{g l}^{-1}$ , □). (B)-Changes in carbohydrates concentration in relation to the first day (100%), represented as a dashed line, mean values ( $n=6$ ,  $\pm$ SE).



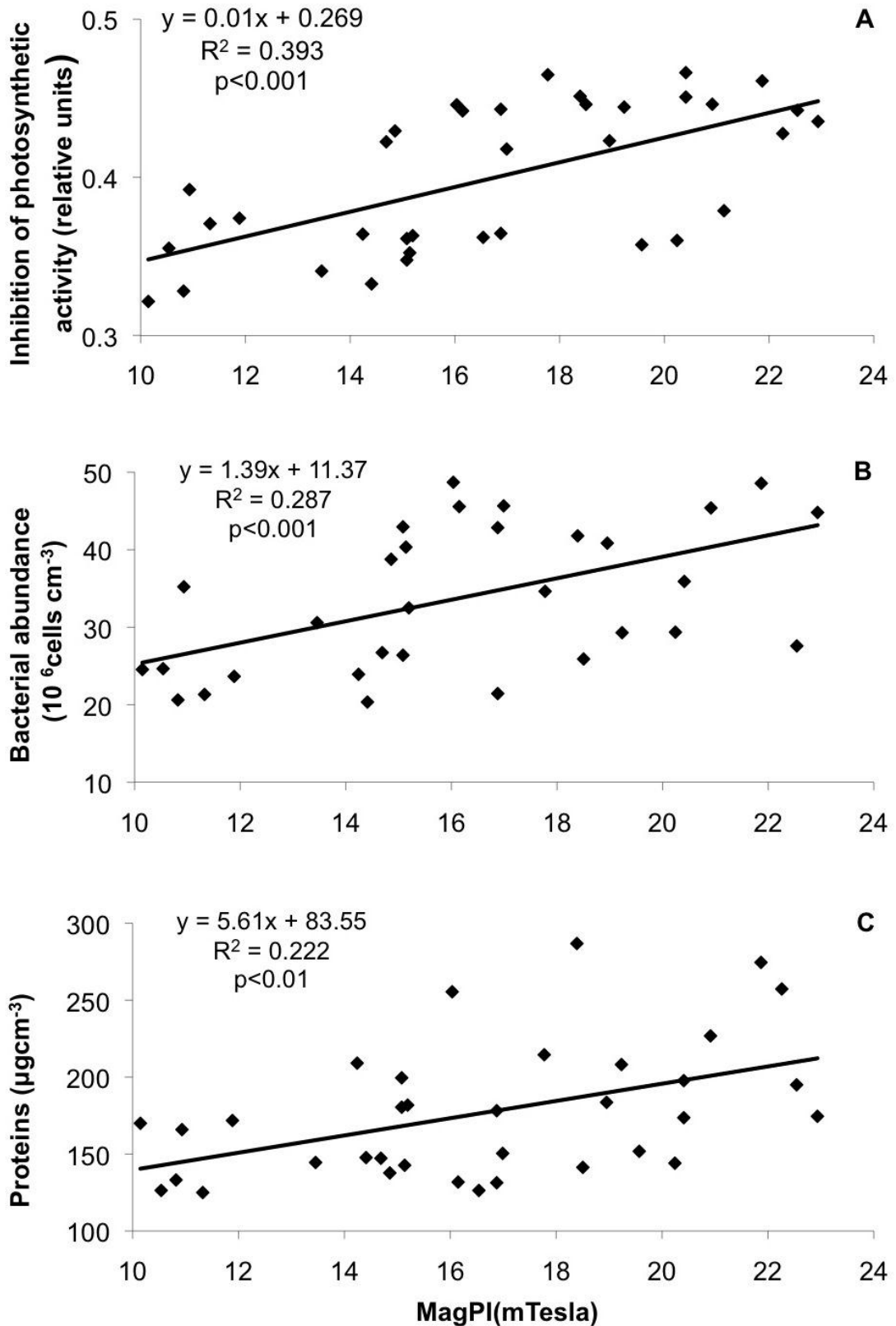
**Figure 7.7:** Colloidal protein concentrations, over the course of the experiment. Mean values ( $n=3$  per treatment based on  $n=3$  replicates per box,  $\pm SE$ ) is shown for positive control (CB, ■), negative control (CT, ○), T1 (TCS:  $2 \mu\text{g l}^{-1}$ , △), T2 (TCS:  $20 \mu\text{g l}^{-1}$ , ●), T3 (TCS:  $50 \mu\text{g l}^{-1}$ , ◇), T4 (TCS:  $100 \mu\text{g l}^{-1}$ , ▲), T5 (TCS:  $150 \mu\text{g l}^{-1}$ , □). (B)-Changes in proteins concentration in relation to the first day (100%) represented as a dashed line, mean values ( $n=6$ ,  $\pm SE$ ).

### 7.3.5. Relation between biological variables, surface adhesion/stability and triclosan exposure

A strong linear relationship was determined between sediment stability measurements and the effective quantum yield of photosystem PSII ( $r=0.627$ ,  $n=35$ ,  $p<0.001$ , Figure 7.8 A) as well as bacterial cell numbers ( $r=0.536$ ,  $n=35$ ,  $p<0.001$ , Figure 7.8 B) and EPS proteins ( $r=0.471$ ,  $n=35$ ,  $p<0.01$ , Figure 7.8 C); however, the relationship to the colloidal carbohydrate was not significant. Analysis suggests that there was no significant correlation between the sediment stability measurement by MagPI and all other variables at the beginning of the experiment (day 1 and day 3) (Table 7.3). However, during the rest of the experiment, stability was closely related to colloidal proteins, bacterial cell number and the effective quantum yield of photosystem PSII and to a lesser extent, to colloidal carbohydrates (Table 7.3). On the last day of the experiment there was no significant correlation between MagPI measurement and colloidal carbohydrates, and strong negative correlation was determined between MagPI measurement and bacterial abundance.

**Table 7.3** Pearson's correlation coefficients between surface adhesion (MagPI) and colloidal carbohydrates and proteins bacterial cell number and the effective quantum yield of photosystem PSII, per each day of the experiment. The significance levels are the following: \*\*\*  $p<0.001$ . \*\*  $p<0.01$ . \*  $p<0.05$

<b>Treatment</b>	<b>Carbohydrates</b>			<b>Proteins</b>			<b>Bacterial cell</b>			<b>PS II</b>		
<b>Day 1</b>	-0.205	21		-0.372	21		-0.207	21		0.173	21	
<b>Day 3</b>	-0.214	21		0.163	21		0.312	21		0.519	21	*
<b>Day 5</b>	0.639	21	**	0.778	21	**	0.790	21	**	0.774	21	**
<b>Day 8</b>	0.524	21	*	0.774	21	**	0.740	21	**	0.669	21	**
<b>Day 10</b>	0.546	21	*	0.678	21	**	0.796	21	**	0.719	21	**
<b>Day 12</b>	0.362	21		0.719	21	**	-0.634	21	**	0.754	21	**



**Figure 7.8:** The relationship ( $n=35$ ) between microbial biofilm adhesion expressed by MagPI (mTesla) versus the effective quantum efficiency PS II (A), bacterial cell numbers (B) and colloidal protein concentrations (C).



#### 7.4. Discussion

##### ***The effect of triclosan on substratum stabilisation potential of a natural biofilm***

This is a pioneering study that investigates the effect of triclosan (TCS) on the stabilization potential of natural freshwater biofilms. A biofilm was developed in flow-through glass channels with TCS concentrations relevant to environmental occurrence. As was explained in section 7.2, selected experimental set-up may create problems with pseudoreplicates, so some caution needs to be exerted in the interpretation of the results. However, on every day of the experiment, the monitoring of experimental conditions in each channels was performed (this data is not presented in this work). Results of monitoring parameters, such as temperature, pH-value, conductivity, O<sub>2</sub>-content and O<sub>2</sub>-saturation allow to conclude that experimental conditions in all channels were similar, thus differences between treatments may be addressed to the effect of TCS to microbial community.

In previous experiment (Chapter 6), the negative effect of triclosan on bacteria stabilization potential was observed. The microalgae in former experiments were found to be more sensitive to TCS and this fact alongside the highly species specific interaction between bacteria and microalgae, make the prediction of the effect of toxins on biostabilisation potential of natural biofilm complex. On the first day of measurements, after 3 weeks of growth, the sediment stability value as measured by MagPI was ~20mTesla. This value is comparable with results obtained in previous experiments described in Chapter 6. In previous experiment TCS was inhibitory, but did not prevent the settlement of a bacterial biofilm and hence, the stability increased in all treatments. In this experiment, however, the rapid decrease in stability of natural biofilm was observed in all treatments after TCS spiking, and over time it was more pronounced with increasing TCS concentration. In contrast to that, sediment stability in positive control CB was increased continuously until the end of the experiment. Despite the relatively low initial value, stability of the biofilm in treatment CB at the end of the experiment was 1.4 times higher than stability in treatments with low and medium TCS concentration and two times higher than treatments with higher TCS concentrations. These results suggest a clear negative effect of TCS on stability of natural biofilms. This effect cannot be explained by bacterial-microalgae interaction such as the production of poly-saturated aldehydes from microalgae (Wichard et al. 2005, Ribalet et al. 2008) or algicidal compounds

from bacteria (Fukami et al. 1997, Jung et al. 2008) or “batch culture” effects, because such a decrease in sediment stability was observed only in TCS treated biofilm and not in the positive control. The work also confirmed previous results (Reiss et al. 2002, Tatarazako et al. 2004, Neumegen et al. 2005) that microalgae are more sensitive to TCS than the directly targeted bacteria. On the other hands, additional nutrient produced by microalgae may allow bacterial culture to be are more sensitive to TCS than nutrient depleted cultures (Johnson et al. 2009). After one week of experiment treatments with low TCS concentration, the cultures showed slightly increases or remained stable in terms of adhesive capacity until the end of experiment, which may indicate some adaptations or resistance developed by the microorganism to the presence of low concentrations of the TCS. In the present experiment, a “batch culture” effect was not observed. This may be due to additional nutrient supply from microalgae, via the additional fresh water added after one week of experiment or perhaps the effect become stronger after more than 2 weeks of experiment. In all treatments the stability was significantly higher than the negative control with triclosan. This fact suggests that TCS does not affect the cohesion of the sediment particles and the stability originated from development of the biofilm. Sediment stability values in the negative control CT do not show variations over the course of the experiment, confirming that there was no contribution of gravity forces to overall stabilisation.

#### ***EPS responsible for biostabilisation?***

In recent studies (Underwood and Paterson 2003, Gerbersdorf et al. 2009), natural biofilm biostabilisation was directly linked to quantities of EPS components, carbohydrates and proteins, and showed increasing EPS production may result in an increase of sediment stability. In the present experiment, the higher carbohydrate concentration was determined in treatments with higher TCS concentrations. This is opposite to sediment stability data, where higher stabilisation effect was observed in treatments with no or low TCS concentration. The data suggest that TCS may elevate EPS carbohydrate production along the TCS gradient. These results support previous studies, that the presence of toxins could elevate EPS productions from microorganisms (Schmitt et al. 1995, White and Gadd 1998, Fang et al. 2002). However there were no significant differences in quantity of EPS carbohydrate in

treatments CB, T1 and T2. This may be explained if only medium and higher TCS concentrations cause microorganisms to produce higher EPS levels. Also at higher levels triclosan appears to act as bactericide which may cause cell lysis and subsequent fatal loss of intracellular material (Villalain et al. 2001). Thus some of the recorded EPS compounds may related to intracellular pools (Priester et al. 2006) which may not be effective for sediment stabilisation. The bactericidal effect in treatment T3, T4 and T5 was confirmed by microbial biomass data. At the end of the experiment bacterial cell number in treatment T3, T4 and T5 were significantly lower than all other treatments. Inhibition of microalgal photosynthetic activity was also observed in these treatments as well as in treatment T2. This may be explained by higher sensitivity of microalgae when low concentrations of triclosan may have already inhibited the photosynthetic activity. Similar to the previous experiments (Chapter 3, Chapter 4), after one week of experiment significant decreases in EPS carbohydrate production in all of the treatments was observed, which may be due to nutrient-depleted conditions in all treatments. However, after additional fresh water was supplied (day 10) a slight increase in EPS carbohydrate production was determined in all treatments. In contrast to EPS carbohydrate, the EPS protein production was more pronounced for positive control and treatments with low and medium TCS concentration (T1, T2 and T3). This may be due to different origins of EPS compounds. It was previously recognized that microalgae secrete mainly polysaccharides (Staats et al. 1999) and bacteria mostly contributed in proteins pools (up to 60%, Flemming and Wingender 2001b). Bacteria may be more tolerant to the presence of low concentrations of TCS than microalgae and therefore produce higher amounts of EPS protein to protect cells as response to the toxin.

Similar to EPS carbohydrate, after one week of experiment, a decrease in EPS protein concentration was observed in all of the treatments. However the decrease was more pronounced for treatment under TCS exposure as compared to the positive control CB, suggesting that under nutrient-depleted conditions, TCS may affect microorganism secretion of EPS protein, with direct implication on the adhesion capacity and sediment stability. The sediment stability is probably a net result of coexisting of EPS compound, such as carbohydrates and proteins (Pennisi 2002, Gerbersdorf et al. 2009), in terms of stabilizations the reduction of one compound may be compensated for by enhancement another and vice versa.

***Sediment stability and microbial biomass.***

Stability has been directly linked to EPS quality and quantity together with microbial biomass in the past (Yallop et al. 2000, Perkins et al. 2004, Gerbersdorf et al. 2008). In the present experiment microbial cells were obviously stressed by the presence of TCS; however bacteria demonstrated a delayed response in terms of cell number. After TCS spiking bacterial cell numbers increased in all treatments, however the increase was more pronounced for treatments with low and medium TCS concentration. This fact may be explained by hormesis, a well-known dose-response phenomenon characterized by a stimulatory response of various growth parameters at low toxin doses, followed by an inhibitory response at higher doses (Calabrese 2001b) and can occur after an initial disruption in homeostasis (Calabrese 2001a). After one week, bacterial cell numbers decreased in all TCS treatments except treatment T1 (Figure 7.4). After this time the bacterial cell numbers slightly increased (T1) or remained stable (T2) and at the end of experiment the bacterial cell number in treatments CB, T1 and T2 were not significantly different from each other. This may indicate that bacteria developed a resistance (T1) or adapted (T2) to the presence of TCS at the relevant concentration. In contrast, the negative effect on bacterial biomass was observed in the treatments with medium and higher TCS concentration. Bacteria biomass decreased  $T3 > T4 > T5$  along the gradient of TCS concentration after the first week of the experiment. At the end of experiment, the bacterial cell number in these treatments, were comparable with values at the start of the experiment and were not significantly different from each other. These results may suggest the bactericidal effect of TCS in concentrations in excess of  $100 \mu\text{g l}^{-1}$ .

To assess to disturbances to the photosystem of the algae, Chl *a* fluorescence quenching analysis was applied. This method has been developed to monitor the influence of a stress factor on microalgae photosynthesis and provides information of physiological responses concerning the photosynthetic status of microalgae (Schmitt-Jansen and Altenburger 2008). Similar to the bacterial cell number, after TCS spiking, the photosynthetic efficiency of microalgae in positive control CB and treatment with low TCS concentrations T1, increased slightly or remained stable and was significantly higher than other treatments. After one week of the experiment the inhibition of the photosynthetic efficiency of these treatments was observed (day 8). The inhibition of

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photosynthetic activity in these treatments may be due to the presence of TCS concentration in the treatment T1, as well as minimum concentration of TCS in positive control. However, an increase in the photosynthetic yield in these treatments with time indicated the adaptation of microorganism to these concentrations or possible shift from a sensitive to a progressively more tolerant community. In other treatments, the inhibition of the photosynthetic yield PSII was observed immediately after TCS spiking (day 5) with continuous decrease until the end of experiment and was more pronounced for treatments with higher TCS concentrations T4 and T5. These results indicate that inhibition of photosynthetic activity of microalgae may occur with the presence of minimal TCS concentrations, however inhibition of bacterial growth rate only occurred in treatments with much higher concentrations. These data suggest a different mode of TCS action ranging from suppressing metabolism to bactericidal effects depending on the TCS concentration. General relationships between biological variables and sediment stability (MagPI) indicate that in the presence of a toxin the substratum stabilization is correlated with microbial biomass as well as colloidal EPS proteins, and to a lesser extent to colloidal carbohydrates. In fact, the statistical correlations for each sampling day suggest there were no significant correlations between sediment stability and EPS compounds (carbohydrates and proteins) and bacterial cell number at the beginning (day 1, day 3) of the experiments. However after TCS spiking, the MagPI measurements were strongly correlated with all of the parameters. The described experiments were designed to simulate natural scenarios for ecological relevance and applicability. The significant impairment of stabilisation capacity of the natural freshwater biofilm was determined. The data suggest that exposure to triclosan affects on microbial biomass, may change the EPS production and has a great influence on the dynamics of sediments and associated pollutants with wider implications for the aquatic ecosystems and beyond.

**References**

- Adolfsson-Erici, M., M. Pettersson, J. Parkkonen, and J. Sturve. 2002. Triclosan, a commonly used bactericide found in human milk and in the aquatic environment in Sweden. *Chemosphere* **46**:1485-1489.
- Aguera, A., A. R. Fernandez-Alba, L. Piedra, M. Mezcua, and M. J. Gomez. 2003. Evaluation of triclosan and biphenylol in marine sediments and urban wastewaters by pressurized liquid extraction and solid phase extraction followed by gas chromatography mass spectrometry and liquid chromatography mass spectrometry. *Analytica Chimica Acta* **480**:193-205.
- Bandow, N., R. Altenburger, U. Lubcke-von Varel, A. Paschke, G. Streck, and W. Brack. 2009a. Partitioning-Based Dosing: An Approach To Include Bioavailability in the Effect-Directed Analysis of Contaminated Sediment Samples. *Environmental Science & Technology* **43**:3891-3896.
- Bandow, N., R. Altenburger, G. Streck, and W. Brack. 2009b. Effect-Directed Analysis of Contaminated Sediments with Partition-Based Dosing Using Green Algae Cell Multiplication Inhibition. *Environmental Science & Technology* **43**:7343-7349.
- Bell, W. H., J. M. Lang, and R. Mitchell. 1974. Selective stimulation of marine bacteria by algal extracellular products. *Limnology and Oceanography* **19**:833-839.
- Bhargava, H. N. and P. A. Leonard. 1996. Triclosan: Applications and safety. *American Journal of Infection Control* **24**:209-218.
- Bruckner, C. G., R. Bahulikar, M. Rahalkar, B. Schink, and P. G. Kroth. 2008. Bacteria associated with benthic diatoms from lake Constance: phylogeny and influences on diatom growth and secretion of extracellular polymeric substances. *Applied and Environmental Microbiology* **74**:7740-7749.
- Calabrese, E. J. 2001a. Overcompensation stimulation: A mechanism for hormetic effects. *Critical Reviews in Toxicology* **31**:425-470.
- Calabrese, E. J. 2001b. The role of hormesis in ecotoxicology and ecological risk assessment. *Human & Experimental Toxicology* **20**:497-497.
- Cantwell, M. G., B. A. Wilson, J. Zhu, G. T. Wallace, J. W. King, C. R. Olsen, R. M. Burgess, and J. P. Smith. 2010. Temporal trends of triclosan contamination in dated sediment cores from four urbanized estuaries: evidence of preservation and accumulation. *Chemosphere* **78**:347-352.
- Cerca, N., S. Martins, G. B. Pier, R. Oliveira, and J. Azeredo. 2005. The relationship between inhibition of bacterial adhesion to a solid surface by sub-MICs of antibiotics and subsequent development of a biofilm. *Research in Microbiology* **156**:650-655.
- Ciba. 1998. Brochure no. 2521: IRGASAN DP/IRGACARE MP: Toxicological and ecological data. Basel, Switzerland.
- Clark, G., W. J. Dougherty, and F. H. Kasten, editors. 1981. *General Methods*. Williams & Wilkins, Baltimore.
- Cole, J. J. 1982. Interactions between bacteria and algae in aquatic ecosystems. *Annual Review of Ecology and Systematics* **13**:291-314.
- Coogan, M. A., R. E. Edziyie, T. W. La Point, and B. J. Venables. 2007. Algal bioaccumulation of triclocarban, triclosan, and methyl-triclosan in a North Texas wastewater, treatment plant receiving stream. *Chemosphere* **67**:1911-1918.

- Costerton, J. W., P. S. Stewart, and E. P. Greenberg. 1999. Bacterial biofilms: A common cause of persistent infections. *Science* **284**:1318-1322.
- Cyr, H. and K. E. Morton. 2006. Distribution of biofilm exopolymeric substances in littoral sediments of Canadian Shield lakes: the effects of light and substrate. *Canadian Journal of Fisheries and Aquatic Sciences* **63**:1763-1776.
- Dade, W. B., J. D. Davis, P. D. Nichols, A. R. M. Nowell, D. Thistle, M. B. Trexler, and D. C. White. 1990. Effect of bacterial exopolymer adhesion on the entrainment of sand. *Geomicrobiology Journal* **8**:1-16.
- de Winder, B., N. Staats, L. J. Stal, and D. M. Paterson. 1999. Carbohydrate secretion by phototrophic communities in tidal sediments. *Journal of Sea Research* **42**:131-146.
- Decho, A. W. 1990. Microbial exopolymer secretions in ocean environments-their role(s) in food webs and marine processes. *Oceanography and Marine Biology* **28**:73-153.
- DeLorenzo, M. E., J. M. Keller, C. D. Arthur, M. C. Finnegan, H. E. Harper, V. L. Winder, and D. L. Zdankiewicz. 2008. Toxicity of the antimicrobial compound triclosan and formation of the metabolite methyl-triclosan in estuarine systems. *Environmental Toxicology* **23**:224-232.
- Escalada, M. G., J. L. Harwood, J. Y. Maillard, and D. Ochs. 2005. Triclosan inhibition of fatty acid synthesis and its effect on growth of *Escherichia coli* and *Pseudomonas aeruginosa*. *Journal of Antimicrobial Chemotherapy* **55**:879-882.
- Fang, H. H. P., L. C. Xu, and K. Y. Chan. 2002. Effects of toxic metals and chemicals on biofilm and biocorrosion. *Water Research* **36**:4709-4716.
- Flemming, H. C. and J. Wingender. 2001a. Relevance of microbial extracellular polymeric substances (EPSs) - Part I: Structural and ecological aspects. *Water Science and Technology* **43**:1-8.
- Flemming, H. C. and J. Wingender. 2001b. Relevance of microbial extracellular polymeric substances (EPSs) - Part II: Technical aspects. *Water Science and Technology* **43**:9-16.
- Franz, S., R. Altenburger, H. Heilmeier, and M. Schmitt-Jansen. 2008. What contributes to the sensitivity of microalgae to triclosan? *Aquatic Toxicology* **90**:102-108.
- Fukami, K., T. Nishijima, and Y. Ishida. 1997. Stimulative and inhibitory effects of bacteria on the growth of microalgae. *Hydrobiologia* **358**:185-191.
- Furneri, P. M., A. Garozzo, M. P. Musumarra, A. C. Scuderi, A. Russo, and G. Bonfiglio. 2003. Effects on adhesiveness and hydrophobicity of sub-inhibitory concentrations of netilmicin. *International Journal of Antimicrobial Agents* **22**:164-167.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Gerbersdorf, S. U., W. Manz, and D. M. Paterson. 2008. The engineering potential of natural benthic bacterial assemblages in terms of the erosion resistance of sediments. *Fems Microbiology Ecology* **66**:282-294.
- Glaser, A. 2004. The ubiquitous triclosan: A common antibacterial agent exposed. *Pesticides and You* **24**:12-17.
- Goto, N., O. Mitamura, and H. Terai. 2001. Biodegradation of photosynthetically produced extracellular organic carbon from intertidal benthic algae. *Journal of Experimental Marine Biology and Ecology* **257**:73-86.

- Grossart, H. P. 1999. Interaction between marine bacteria and axenic diatoms (*Cylindrotheca fusiformis*, *Nitzschia laevis*, and *Thalassiosira weissflogii*) incubated under various conditions in the lab. *Aquatic Microbial Ecology* **19**:1-11.
- Halden, R. U. and D. H. Paull. 2005. Co-occurrence of triclocarban and triclosan in US water resources. *Environmental Science & Technology* **39**:1420-1426.
- Haynes, K., T. A. Hofmann, C. J. Smith, A. S. Ball, G. J. C. Underwood, and A. M. Osborn. 2007. Diatom-derived carbohydrates as factors affecting bacterial community composition in estuarine sediments. *Applied and Environmental Microbiology* **73**:6112-6124.
- Imai, I., Y. Ishida, and Y. Hata. 1993. Killing of marine -phytoplankton by a gliding bacterium *Cytophaga* sp, isolated from the coastal sea of Japan *Marine Biology* **116**:527-532.
- Ishibashi, H., N. Matsumura, M. Hirano, M. Matsuoka, H. Shiratsuchi, Y. Ishibashi, Y. Takao, and K. Arizono. 2004. Effects of triclosan on the early life stages and reproduction of medaka *Oryzias latipes* and induction of hepatic vitellogenin. *Aquatic Toxicology* **67**:167-179.
- Iyer, A., K. Mody, and B. Jha. 2004. Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloacae*. *Marine Pollution Bulletin* **49**:974-977.
- Jabusch, T. W. and D. L. Swackhamer. 2004. Subcellular accumulation of polychlorinated biphenyls in the green alga *Chlamydomonas reinhardtii*. *Environmental Toxicology and Chemistry* **23**:2823-2830.
- Johnson, D. R., K. Czechowska, N. Chevre, and J. R. van der Meer. 2009. Toxicity of triclosan, penconazole and metalaxyl on *Caulobacter crescentus* and a freshwater microbial community as assessed by flow cytometry. *Environmental Microbiology* **11**:1682-1691.
- Jung, S. W., B. H. Kim, T. Katano, D. S. Kong, and M. S. Han. 2008. *Pseudomonas fluorescens* HYK0210-SK09 offers species-specific biological control of winter algal blooms caused by freshwater diatom *Stephanodiscus hantzschii*. *Journal of Applied Microbiology* **105**:186-195.
- Kolpin, D. W., E. T. Furlong, M. T. Meyer, E. M. Thurman, S. D. Zaugg, L. B. Barber, and H. T. Buxton. 2002. Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environmental Science & Technology* **36**:1202-1211.
- Lawrence, J. R., B. Zhu, G. D. W. Swerhone, J. Roy, L. I. Wassenaar, E. Topp, and D. R. Korber. 2009. Comparative microscale analysis of the effects of triclosan and triclocarban on the structure and function of river biofilm communities. *Science of the Total Environment* **407**:3307-3316.
- Lindstrom, A., I. J. Buerge, T. Poiger, P. A. Bergqvist, M. D. Muller, and H. R. Buser. 2002. Occurrence and environmental behavior of the bactericide triclosan and its methyl derivative in surface waters and in wastewater. *Environmental Science & Technology* **36**:2322-2329.
- Lubarsky, H. V., C. Hubas, M. Chocholek, F. Larson, W. Manz, D. M. Paterson, and S. U. Gerbersdorf. 2010. The stabilisation potential of individual and mixed assemblages of natural bacteria and microalgae. *Plos One* **5**:e13794.
- McAvoy, D. C., B. Schatowitz, M. Jacob, A. Hauk, and W. S. Eckhoff. 2002. Measurement of triclosan in wastewater treatment systems. *Environmental Toxicology and Chemistry* **21**:1323-1329.
-



- McClellan, K., R. Altenburger, and M. Schmitt-Jansen. 2008. Pollution-induced community tolerance as a measure of species interaction in toxicity assessment. *Journal of Applied Ecology* **45**:1514-1522.
- McDonnell, G. and D. Pretzer. 1998. Action and targets of triclosan. *Asm News* **64**:670-671.
- McMurry, L. M., M. Oethinger, and S. B. Levy. 1998. Triclosan targets lipid synthesis. *Nature* **394**:531-532.
- Neumegen, R. A., A. R. Fernandez-Alba, and Y. Chisti. 2005. Toxicities of triclosan, phenol, and copper sulfate in activated sludge. *Environmental Toxicology* **20**:160-164.
- Okumura, T. and Y. Nishikawa. 1996. Gas chromatography mass spectrometry determination of triclosans in water, sediment and fish samples via methylation with diazomethane. *Analytica Chimica Acta* **325**:175-184.
- Orvos, D. R., D. J. Versteeg, J. Inauen, M. Capdevielle, A. Rothenstein, and V. Cunningham. 2002. Aquatic toxicity of triclosan. *Environmental Toxicology and Chemistry* **21**:1338-1349.
- Paterson, D. M. 1989. Short-term changes in the erodibility of intertidal cohesive sediments related to the migratory behaviour of epipelagic diatoms. *Limnology and Oceanography* **34**:223-234.
- Paterson, D. M., T. J. Tolhurst, J. A. Kelly, C. Honeywill, E. de Deckere, V. Huet, S. A. Shayler, K. S. Black, J. de Brouwer, and I. Davidson. 2000. Variations in sediment properties, Skeffling mudflat, Humber Estuary, UK. *Continental Shelf Research* **20**:1373-1396.
- Pennisi, E. 2002. Materials science - Biology reveals new ways to hold on tight. *Science* **296**:250-251.
- Perkins, R. G., D. M. Paterson, H. Sun, J. Watson, and M. A. Player. 2004. Extracellular polymeric substances: quantification and use in erosion experiments. *Continental Shelf Research* **24**:1623-1635.
- Priester, J. H., S. G. Olson, S. M. Webb, M. P. Neu, L. E. Hersman, and P. A. Holden. 2006. Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Applied and Environmental Microbiology* **72**:1988-1996.
- Reiss, R., N. Mackay, C. Habig, and J. Griffin. 2002. An ecological risk assessment for triclosan in lotic systems following discharge from wastewater treatment plants in the United States. *Environmental Toxicology and Chemistry* **21**:2483-2492.
- Ribalet, F., L. Intertaglia, P. Lebaron, and R. Casotti. 2008. Differential effect of three polyunsaturated aldehydes on marine bacterial isolates. *Aquatic Toxicology* **86**:249-255.
- Ricart, M., H. Guasch, D. Barcelo, R. Brix, M. H. Conceicao, A. Geiszinger, M. J. L. de Alda, J. C. Lopez-Doval, I. Munoz, C. Postigo, A. M. Romani, M. Villagrana, and S. Sabater. 2010. Primary and complex stressors in polluted mediterranean rivers: Pesticide effects on biological communities. *Journal of Hydrology* **383**:52-61.
- Roberson, E. B. and M. K. Firestone. 1992. Relationship between desiccation and exopolysaccharide production in a soil *pseudomonas* sp. *Applied and Environmental Microbiology* **58**:1284-1291.
- Russell, A. D. 2004. Whither triclosan? *Journal of Antimicrobial Chemotherapy* **53**:693-695.

- Sabater, S., H. Guasch, M. Ricart, A. Romani, G. Vidal, C. Klunder, and M. Schmitt-Jansen. 2007. Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Analytical and Bioanalytical Chemistry* **387**:1425-1434.
- Sastry, V. and G. R. K. Rao. 1994. Antibacterial substances from marine-algae-successive extraction using benzene, chloroform and methanol. *Botanica Marina* **37**:357-360.
- Schaefer, H., B. Abbas, H. Witte, and G. Muyzer. 2002. Genetic diversity of "satellite" bacteria present in cultures of marine diatoms. *Fems Microbiology Ecology* **42**:25-35.
- Schmitt-Jansen, M. and R. Altenburger. 2005a. Predicting and observing responses of algal communities to photosystem II-herbicide exposure using pollution-induced community tolerance and species-sensitivity distributions. *Environmental Toxicology and Chemistry* **24**:304-312.
- Schmitt-Jansen, M. and R. Altenburger. 2005b. Toxic effects of isoproturon on periphyton communities - a microcosm study. *Estuarine Coastal and Shelf Science* **62**:539-545.
- Schmitt-Jansen, M. and R. Altenburger. 2008. Community-level microalgal toxicity assessment by multiwavelength-excitation PAM fluorometry. *Aquatic Toxicology* **86**:49-58.
- Schmitt, J., D. Nivens, D. C. White, and H. C. Flemming. 1995. Changes of biofilm properties in response to sorbed substances - An FTIR-ATR study. *Water Science and Technology* **32**:149-155.
- Schreiber, F. and U. Szewzyk. 2008. Environmentally relevant concentrations of pharmaceuticals influence the initial adhesion of bacteria. *Aquatic Toxicology* **87**:227-233.
- Schweizer, H. P. 2001. Triclosan: a widely used biocide and its link to antibiotics. *Fems Microbiology Letters* **202**:1-7.
- Singer, H., S. Muller, C. Tixier, and L. Pillonel. 2002. Triclosan: Occurrence and fate of a widely used biocide in the aquatic environment: Field measurements in wastewater treatment plants, surface waters, and lake sediments. *Environmental Science & Technology* **36**:4998-5004.
- Staats, N., B. De Winder, L. J. Stal, and L. R. Mur. 1999. Isolation and characterization of extracellular polysaccharides from the epipellic diatoms *Cylindrotheca closterium* and *Navicula salinarum*. *European Journal of Phycology* **34**:161-169.
- Stal, L. J. 2003. Microphytobenthos, their extracellular polymeric substances, and the morphogenesis of intertidal sediments. *Geomicrobiology Journal* **20**:463-478.
- Stal, L. J. and A. E. Walsby. 2000. Photosynthesis and nitrogen fixation in a cyanobacterial bloom in the Baltic sea. *European Journal of Phycology* **35**:97-108.
- Suller, M. T. E. and A. D. Russell. 1999. Antibiotic and biocide resistance in methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococcus. *Journal of Hospital Infection* **43**:281-291.
- Sutherland, I. W. 2001a. The biofilm matrix - an immobilized but dynamic microbial environment. *Trends in Microbiology* **9**:222-227.
- Sutherland, I. W. 2001b. Microbial polysaccharides from Gram-negative bacteria. *International Dairy Journal* **11**:663-674.

- Tabak, M., K. Scher, E. Hartog, U. Romling, K. R. Matthews, M. L. Chikindas, and S. Yaron. 2007. Effect of triclosan on *Salmonella typhimurium* at different growth stages and in biofilms. *Fems Microbiology Letters* **267**:200-206.
- Tatarazako, N., H. Ishibashi, K. Teshima, K. Kishi, and K. Arizono. 2004. Effect of triclosan on various aquatic organisms. *Environmental Science* **11**:133-140.
- Underwood, G. J. C. and D. M. Paterson. 2003. The importance of extracellular carbohydrate production by marine epipelagic diatoms. *Advances in Botanical Research* **40**:183-240.
- Villalain, J., C. R. Mateo, F. J. Aranda, S. Shapiro, and V. Micol. 2001. Membranotropic effects of the antibacterial agent triclosan. *Archives of Biochemistry and Biophysics* **390**:128-136.
- Wang, L. Z. and J. C. Priscu. 1994. Stimulation of aquatic bacterial-activity by cyanobacteria. *Hydrobiologia* **27**:145-158.
- White, C. and G. M. Gadd. 1998. Accumulation and effects of cadmium on sulphate-reducing bacterial biofilms. *Microbiology-UK* **144**:1407-1415.
- Wichard, T., S. A. Poulet, and G. Pohnert. 2005. Determination and quantification of alpha, beta, gamma, delta-unsaturated aldehydes as pentafluorobenzyl-oxime derivatives in diatom cultures and natural phytoplankton populations: application in marine field studies. *Journal of Chromatography B-Analytical Technologies in the Biomedical and Life Sciences* **814**:155-161.
- Wicke, D., U. Bockelmann, and T. Reemtsma. 2007. Experimental and modeling approach to study sorption of dissolved hydrophobic organic contaminants to microbial biofilms. *Water Research* **41**:2202-2210.
- Wilson, B. A., V. H. Smith, F. Denoyelles, and C. K. Larive. 2003. Effects of three pharmaceutical and personal care products on natural freshwater algal assemblages. *Environmental Science & Technology* **37**:1713-1719.
- Wotton, R. S. 2004. The ubiquity and many roles of exopolymers (EPS) in aquatic systems. *Scientia Marina* **68**:13-21.
- Yallop, M. L., D. M. Paterson, and P. Wellsbury. 2000. Interrelationships between rates of microbial production, exopolymer production, microbial biomass, and sediment stability in biofilms of intertidal sediments. *Microbial Ecology* **39**:116-127.
- Zaranko, D. T., R. W. Griffiths, and N. K. Kaushik. 1997. Biomagnification of polychlorinated biphenyls through a riverine food web. *Environmental Toxicology and Chemistry* **16**:1463-1471.

## Chapter 8

### General Conclusion and Future Work

To summarise this study I would like to emphasise the advantages and limitations of described methods and to propose some ideas to future work.

#### **Methods:**

A great advantage of MagPI, described in Chapter 3, is the ability to measure biofilm adhesion in a non-destructive manner, a variable that has rarely been considered, but is at the same time of great significance for binding pollutants, trapping nutrients, enhancing sediment stability, and capturing newly deposited particles. For instance, the ecosystem service (Paterson et al. 2008) of particle capture and retention is of great importance to sediment systems in balancing the replacement of material lost by tidal erosion (Verney et al. 2006) or wave action (Andersen et al. 2007), enhancing the nutrient status (Freeman and Lock 1995) and offering binding sites for pollutants (Ghosh et al. 2003). This biofilm adhesion can be measured with high sensitivity, and small changes in developing biofilms can be demonstrated that would be unnoticed using established erosion devices. MagPI comes at comparatively low cost, and with basic practical skills and technical understanding it is comparatively easy to build and use.

Although the permanent magnet is valuable for the use in the field, MagPI cannot easily be used if a wet biofilm is not submerged, such as during tidal emersion period.

The measurements have to be performed underwater by the help of a water-filled chamber, otherwise the magnetic particles interact with the surface tension of the water film and these forces confound the measurement of adhesion.

This method can be used for any subtidal or intertidal sediment, including complex biofilm-based systems such as stromatolites (Paterson et al. 2008), but the measurements of moist surface should be made underwater because of surface tension effect. In addition, dry exposed surface, where adhesion is important, might also be examined. To-date, during this experiment a very few substrata were tested, but stonework, tree surfaces, leaves, etc., remain possible candidates for investigation. The MagPI represents an economically viable, easily constructed, easy-to-use tool to determine surface adhesion, a proxy for the retentive capacity of the substratum. The knowledge of surface adhesion can provide useful insights for particulate pollutant capture, nutrient trapping, enhancing sediment stability, and capturing particles in various depositional systems such as intertidal flats, shallow submerged sediment systems, and stromatolites, to name but a few. In contrast to established erosion devices, MagPI can determine small changes in surface properties below the point of incipient erosion with high sensitivity, high accuracy, and high repeatability. The calibration of the device by the gauss meter makes the comparison of the data between different experiments and various laboratories possible, which is an important prerequisite for future success in biofilm research. Two types of magnets have been examined here; the high-power permanent magnet for increased mobility and application in the field and the electromagnet, which is to be preferred in the laboratory due to a higher accuracy in calibration and measurement. The MagPI methods presented here are likely to have future applications in environmental, medical, and biotechnological research.

#### ***Microbial interactions and effects on stability***

Results presented in Chapter 4 suggest that bacteria may produce as copious amounts of EPS as microalgae culture, while the combination of bacteria and diatoms in the mixed assemblages might result in synergy in terms of EPS carbohydrate production but not for colloidal protein production. In-depth investigation of interaction of main biofilm components such as heterotrophic bacteria and autotrophic microalgae, and as mixtures in terms of their individual contribution to the EPS pool was done.

However, contamination of the microbial culture in this study was problematic, thus a mixture of antibiotics was used to inhibit bacterial growth, which in itself created problems. Chloramphenicol has been reported to suppress the growth of microalgae; however the actual effect of antibiotics on microalgae EPS production is still unknown, leaving an opening for future investigation into the effects of antibiotics on microbial EPS production.

Whilst the positive effect of nematodes on microbial growth and EPS production was determined, their impact on sediment stability is mostly unknown. Despite impressive enhancement of microbial growth and EPS production in the presence of nematodes, their feeding activity or bioturbation may have a negative effect on overall stabilization processes and this needs to be investigated further, using traditional or newly development technics.

The results described in Chapter 4 conclude that interaction between species is highly species-specific under varying abiotic scenarios and requires further investigation. Increased levels of biodiversity, such as combinations of bacteria and microalgae or natural microbial assemblages and nematodes may provide enhancement of EPS production. However, as this study suggests, differences in EPS composition may have significant effects on biostabilisation and need to be investigated with high resolution chemical analysis.

The microbial assemblages-isolated from estuarine sediments-significantly stabilized the non-cohesive test substratum from day 1 onwards as determined by MagPI. Thus, this new technique has been very successful in determining early and subtle changes in growing biofilm properties. By the sensitive measurement of biofilm adhesion, a proxy for sediment stability, this method provides a useful addition to the variables measurable by established erosion devices (Gerbersdorf et al. 2009). The stabilisation of the substratum as described in Chapter 5 was highly correlated with microbial biomass and was due to the secreted EPS matrix. Both EPS concentrations (quantity) and EPS components (quality) were important. In this context, the EPS protein seems to play a critical role for adhesion/cohesion of the substratum. Bacterial assemblages had a significant higher stabilisation potential as compared to the axenic microalgae cultures. The explanation is probably in the conformation of the polymeric matrix and may reflect the functional roles (attachment, movement) that the EPS provides. The

tendency in the literature to exclude the contribution of bacterial EPS to sediment stability in the field should be re-addressed and the importance of bacterial assemblages recognized. The investigation of biostabilisation in natural marine or freshwater biofilm should not be performed without assessment of bacterial impact on these processes. On the other hand, monospecific microalgae culture shows differences in stabilisation capacity, probably due to different degrees of substitution and different structures of the main EPS chains and in this regard may be characterised as strong or weak species in adhesion/cohesion. The mixed assemblages provided greater sediment stability than either community on its own. This probably due to a different origin of EPS produced by bacterial or microalgae assemblages and suggests both assemblages have an important role in substratum stabilisation and are more effective together.

#### ***Influence of toxins on microbial stabilisation capacity***

Microbial colonisation resulted in significant substratum stabilisation as was shown in previous chapters. However, in response to varying abiotic conditions (e.g. presence of pollutant) notable shifts in the populations affected the secretion of EPS and thus, biostabilisation. The possible effect of triclosan (TCS) on stabilisation potential of bacterial assemblages was described throughout this thesis. Chapter 6 focused on the effect of triclosan on bacterial assemblages due to their impressive stabilisation potential. These microbes dominate in submerged biofilms in rivers and coastal areas. The work described in Chapter 6 is a pioneering study into the impact of toxins on biostabilisation potential of aquatic microorganisms. These investigations were only possible by using the newly development and very sensitive MagPI technique. The negative effect of TCS on bacterial biomass and growth rate were previously described. It is a widely accepted fact that presence of toxins may stimulate microbial EPS production (Fang et al. 2002, Iyer et al. 2004, Priester et al. 2006) and as a result enhance bioaccumulation capacity of microbial biofilm (Schmitt et al. 1995). In this regard, prediction of biostabilisation processes in biofilms with the presence of triclosan is complex. In order to determine the substratum stabilisation process in the presence of toxins, more investigations are required. Results obtained suggest that concentrations of TCS relevant to environmental conditions do not to stop development of a bacterial biofilm. However, it has a significantly inhibitory effect on

bacterial stabilisation capacity. This effect was more pronounced along the triclosan concentration gradient. These results were mirrored by EPS carbohydrate production and less significantly by EPS proteins. TCS exposure also affected bacterial growth rate, but the most damaging effect on bacteria was observed in treatments with highest TCS concentrations. It is recognised that bacteria are very sensitive to changes in abiotic conditions. Changes in nutrients or salinity may have a huge impact on bacterial stabilisation capacity. For further extension of this study, experiments performed with different abiotic condition, such as nutrients, salinity or temperature regimes should be carried out.

Triclosan was developed as a broad-spectrum antibacterial compound. However it also generates acute and chronic toxic effects on non-target organisms, especially on microalgae. Based on the findings of Chapter 6, an investigation into the impairment of the stabilisation capacity of natural freshwater biofilms by TCS exposure to different concentrations was described (Chapter 7). Biochemical parameters and sediment stability were measured over two weeks of experimental time. The results showed significant changes in EPS quantity and quality over time along with inhibition effect of TCS on microbial biostabilisation. This negative effect was more pronounced when triclosan concentration increased. It is possible that a bacteriostatic effect was observed in treatments with low triclosan concentration. However, in treatments with high TCS concentrations bacterial growth was significantly hampered. Microalgal photosynthetic activity was also inhibited by TCS determined. To summarise, triclosan exposure affects on microbial biomass and EPS production and impairing the stabilisation capacity of microbial biofilm. The studies on environmental risk assessment of pollutants on biostabilisation processes needs to be continued on the different substrata (e.g. natural sediment), by involving of other aquatic organisms (e.g. nematodes) and testing other hazardous compounds (such as pesticides, heavy metals or surfactants). Further, studies are needed to address the environmental risk of pollutants and identify the causes of ecological deterioration as the European Water Framework Directive requires. Microbial population and biofilms are at the centre of the ecology and metabolism of many aquatic ecosystems and they may act as an early warning system for the impact of toxic chemicals on the environment and as such we need to expand our knowledge to these systems.



## References

- Andersen, T. J., J. Fredsoe, and M. Pejrup. 2007. In situ estimation of erosion and deposition thresholds by Acoustic Doppler Velocimeter (ADV). *Estuarine Coastal and Shelf Science* **75**:327-336.
- Fang, H. H. P., L. C. Xu, and K. Y. Chan. 2002. Effects of toxic metals and chemicals on biofilm and biocorrosion. *Water Research* **36**:4709-4716.
- Freeman, C. and M. A. Lock. 1995. The biofilm polysaccharide matrix-a buffer against changing organic substrate supply. *Limnology and Oceanography* **40**:273-278.
- Gerbersdorf, S. U., R. Bittner, H. Lubarsky, W. Manz, and D. M. Paterson. 2009. Microbial assemblages as ecosystem engineers of sediment stability. *Journal of Soils and Sediments* **9**:640-652.
- Ghosh, U., J. R. Zimmerman, and R. G. Luthy. 2003. PCB and PAH speciation among particle types in contaminated harbor sediments and effects on PAH bioavailability. *Environmental Science & Technology* **37**:2209-2217.
- Iyer, A., K. Mody, and B. Jha. 2004. Accumulation of hexavalent chromium by an exopolysaccharide producing marine *Enterobacter cloacae*. *Marine Pollution Bulletin* **49**:974-977.
- Paterson, D. M., R. J. Aspden, P. T. Visscher, M. Consalvey, M. S. Andres, A. W. Decho, J. Stolz, and R. P. Reid. 2008. Light-Dependant biostabilisation of sediments by stromatolite assemblages. *Plos One* **3**.
- Priester, J. H., S. G. Olson, S. M. Webb, M. P. Neu, L. E. Hersman, and P. A. Holden. 2006. Enhanced exopolymer production and chromium stabilization in *Pseudomonas putida* unsaturated biofilms. *Applied and Environmental Microbiology* **72**:1988-1996.
- Schmitt, J., D. Nivens, D. C. White, and H. C. Flemming. 1995. Changes of biofilm properties in response to sorbed substances - An FTIR-ATR study. *Water Science and Technology* **32**:149-155.
- Verney, R., J. C. Brun-Cottan, R. Lafite, J. Deloffre, and J. A. Taylor. 2006. Tidally-induced shear stress variability above intertidal mudflats in the macrotidal Seine estuary. *Estuaries and Coasts* **29**:653-664.