Microbial Diversity in

Baltic Sea Sediments

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Doctoral thesis
Swedish University of Agricultural Sciences
Uppsala 2007

Acta Universitatis Agriculturae Sueciae
2007:26

ISSN 1652-6880
ISBN 91-576-7325-1

Tryck: SLU Service/Repro, Uppsala 2007

Abstract

Edlund, A. 2007. Microbial diversity in Baltic Sea sediments.
Doctoral dissertation.
ISSN 1652-6880; ISBN 91-576-7325-1.

This thesis focuses on microbial community structures and their functions in Baltic Sea sediments. First we investigated the distribution of archaea and bacteria in Baltic Sea sediments along a eutrophication gradient. Community profile analysis of 16S rRNA genes using terminal restriction length polymorphism (T-RFLP) indicated that archaeal and bacterial communities were spatially heterogeneous. By employing statistical ordination methods we observed that archaea and bacteria were structured and impacted differently by environmental parameters that were significantly linked to eutrophication. In a separate study, we analyzed bacterial communities at a different site in the Baltic Sea that was heavily contaminated with polyaromatic hydrocarbons (PAHs) and several other pollutants. Sediment samples were collected before and after remediation by dredging in two consecutive years. A polyphasic experimental approach was used to assess growing bacteria and degradation genes in the sediments. The bacterial communities were significantly different before and after dredging of the sediment. Several isolates collected from contaminated sediments showed an intrinsic capacity for degradation of phenanthrene (a PAH model compound). Quantititative real-time PCR was used to monitor the abundance of degradation genes in sediment microcosms spiked with phenanthrene. Although both *xylE* and *phnAc* genes increased in abundance in the microcosms, the isolates only carried *phnAc* genes. Isolates with closest 16S rRNA gene sequence matches to *Exigobacterium oxidotolerans*, a *Pseudomonas* sp. and a *Gammaproteobacterium* were identified by all approaches used as growing bacteria that are capable of phenanthrene degradation. These isolates were assigned species and strain designations as follows: *Exiguobacterium oxidotolerans* AE3, *Pseudomonas fluorescens* AE1 and *Pseudomonas migulae* AE2. We also identified and studied the distribution of actively growing bacteria along red-ox profiles in Baltic Sea sediments. Community structures were found to be significantly different at different red-ox depths. Also, according to multivariate statistical ordination analysis organic carbon, nitrogen, and red-ox potential were crucial parameters for structuring the bacterial communities on a vertical scale. Novel lineages of bacteria were obtained by sequencing 16S rRNA genes from different red-ox depths and sampling stations indicating that bacterial diversity in Baltic Sea sediments is largely unexplored.

Keywords: Baltic Sea sediment, eutrophication, polyaromatic hydrocarbon (PAH), red-ox, terminal-restriction fragment length polymorphism (T-RFLP), bromodeoxyuridine (BrdU), Exioguobacterium, phenanthrene.

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In memory of my father Erland Edlund

(1946-2006)

Contents

Appendix

Papers I-IV

This thesis is based on the following publications and manuscripts, which are referred to by their Roman numerals:

I. Edlund, A., Soule, T., Sjöling, S. & Jansson, J. K. 2006. Microbial community structure in polluted Baltic Sea sediments. Environmental Microbiology, 8, 223-232.

II. Edlund, A. & Jansson, J. K. 2006. Changes in active bacterial communities before and after dredging of highly polluted Baltic Sea sediments. Applied and Environmental Microbiology, 72, 6800-6807.

III. Edlund, A. & Jansson. J. K. 2006. Identification of metabolically active phenanthrene degrading bacteria in polluted Baltic Sea sediments. Manuscript.

IV. Edlund, A., Hårdeman, F., Jansson. J. K. & Sjöling, S. Active bacterial population structures along vertical red-ox gradients in Baltic Sea sediment. Manuscript.

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My contributions to the papers included in this thesis have been as follows:

I. I planned the experiments together with my supervisors and contributed many of the ideas. I performed all of the sediment sampling and laboratory work. I was extensively involved in the writing of the manuscripts. I also contributed with ideas and data sets for developing the APLAUS computer program.

II. I planned the experiments together with my supervisors and contributed many of the ideas. I performed all the sediment sampling, laboratory work, computing and drawing of phylogenetic trees. I was extensively involved in the writing of the manuscript.

III. I planned the experiments together with my supervisors and contributed many of the ideas. I performed the sediment sampling and laboratory work. I was extensively involved in the writing of the manuscript.

IV. I planned the experiments together with my supervisors and contributed many of the ideas. Sampling of sediments was done together with Fredrik Hårdeman. Collection and analysis of chemical data, laboratory work concerning DNA extractions, immunocapture of BrdU labeled DNA, T-RFLP and statistical analyses were performed by me. I was extensively involved in the writing of the manuscript.

Additional publication:
Gorokhova, E., Edlund, A., Hajdu, S. & Zhivotova, E. Nucleic acid levels in copepods: dynamic response to the phytoplankton bloom in the northern Baltic proper. *Marine Ecology Progress Series*, in press.

"I make no apologies for putting microorganisms on a pedestal above all other living things. For if the last blue whale choked to death on the last panda, it would be disastrous but not the end of the world. But if we accidentally poisoned the last two species of ammonia oxidizers, that would be another matter, it could be happening now and we wouldn't even know…"

Tom Curtis, 2006

Introduction

During the last two centuries, the delivery of nutrients and toxic pollutants, such as polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and heavy metals, have greatly impacted the Baltic Sea ecosystem (Dahlberg & Jansson, 1997; Elmgren, 1989; Elmgren, 2001; Kuparinen & Tuominen, 2001; Larsson, Elmgren &Wulff, 1985). A number of toxic pollutants have accumulated in Baltic Sea sediments since many synthesized and foreign, "xenobiotic", compounds are extremely recalcitrant to biodegradation. This recalcitrance is due to the chemical and physical properties of the pollutants, their sub-optimal concentrations and diffusion rates and their low bioavailability to microorganisms. However, a large number of microorganisms in sediments have the capacity to degrade some pollutants by a diverse array of enzymatic processes (Anderson & Lovley, 1997; Lovely, 2003; Vogel, 1996; Wackett & Hershberger, 2001). The complexity of bacterial diversity and identification of important key species involved in pollutant degradation in benthic sediments of the Baltic Sea have not yet been elucidated. The link between bacterial diversity and bacterial community function in Baltic sediments is also lacking experimental data. The present thesis aims to fill some of these gaps and also to provide new insights on the benthic bacterial flora in both polluted and less polluted areas of the Baltic.

Anthropogenic influences on the Baltic Sea

Coastal eutrophication

Eutrophication is recognized as one of the foremost threats facing the Baltic aquatic ecosystem today. Eutrophication can be defined as the increased accumulation of organic matter in a system (Nixon, 1995). This primarily results from an excess of nitrogen and phosphorous being delivered to water bodies. The major sink for this extra delivery of nutrients is the Baltic benthic sediment (Carman & Wulff, 1989; Wulff & Rahm, 1988; Wulff, Stigebrandt & Rahm, 1990).

Productivity in coastal ecosystems is normally nitrogen (N) limited (Graneli *et al.*, 1990; Howarth *et al.*, 1988). Therefore increased N availability can

stimulate algal growth, degrade water quality, and affect ecosystem functioning. Eutrophication causes elevated algal production and biomass, and breakdown of this increased organic matter causes hypoxia (i.e. oxygen depletion) that, in turn, affects benthic microbial community structures (Lake *et al.*, 2000). The increased productivity also decreases light penetration and alters benthic production rates (Kelly, 2001; Rabalais *et al.*, 2002). In the Baltic Sea, excessive nutrient loading has resulted in significant eutrophication of coastal areas. This is characterized by oxygen depletion followed by dramatic changes of biotic communities (Cerderwall & Elmgren, 1990; Dahlberg & Jansson, 1997; Elmgren, 1989; Hansson & Rudstam, 1990; Laine *et al.*, 1997; Powilleit & Kube, 1999).

Pollutant compounds

The input and accumulation of pollutant compounds is also a major threat for the Baltic Sea ecosystem. Many pollutants, for example polychlorinated biphenyls (PCBs), polycyclic aromatic hydrocarbons (PAHs) and heavy metals, accumulate in marine sediments due to their recalcitrance to biodegradation. Depending on the chemical structure and environmental factors, some compounds can be adsorbed onto organic matter or be trapped in micropores and form strong bonds with sediment particles. These factors help to contribute to low bioavailability. PCBs and PAHs are also directly carcinogenic (Cerniglia, 1984; Mastrangelo, Fadda & Marzia, 1996) and can also cause genotoxic effects (Cerniglia, 1992; Mueller, Cerniglia & Pritchard, 1996). Mercury, another toxic compound found in the Baltic, has genotoxic effects (Betti, Davini & Barale, 1992) and can also act as a neuro- and immuno toxin (Dieter *et al.*, 1983; Ilback, Sundberg & Oskarsson, 1991; O'Connor & Nielsen, 1981). In general, the above-mentioned pollutants derive from industrial activities and combustion of fossil fuels. However, the releases of PAHs are to some extent natural since they are also formed during forest- and brush fires and the decaying of organic matter. Removal of these pollutants can be performed by dredging of the sediments (i.e. physical removal) or by employing indigenous bacterial metabolic capacity (i.e. bioremediation).

Characteristics of marine sediment

Aerobic and anaerobic worlds and boundary layers

Marine sediments are complex environments that are affected by both physiological and biological factors, for example, water movements and burrowing animals. To explain this environment from a microbiological point of view it is reasonable to start with describing which metabolic energy resources are present. Thermodynamic considerations suggest that the energetically most favorable process should occur first. This process involves the reduction of oxygen. When oxygen resources are consumed other compounds are preferred for cell respiration and the sediments become anoxic. By considering the rate of microbial oxygen uptake it can be predicted that a small sediment particle (1-2 mm) may maintain an anoxic centre even when the particle is surrounded by air or oxygenated water (Fenchel & Finlay, 1995). Therefore, in benthic sediments anoxic environments do not only exist in isolation from their oxic surroundings. Some of the most anaerobic active habitats in surface sediments occur as islands in

a microaerobic matrix or they are only temporarily anaerobic (Fenchel & Finlay, 1995). Thus, it is important to consider the boundaries between aerobic and anaerobic habitats.

The basis of chemical gradients

Much of the organic material that accumulates in marine sediments is mineralized by microorganisms (bacteria, archaea and fungi). However, there are certain restrictions for where these processes can occur, such as the availability of electrons for cellular respiration. In benthic sediments (ignoring burrowing animals and their ventilatory water currents) transport of dissolved oxygen takes place through molecular diffusion. The consumption of oxygen leads to a diffusional flux from the water into the sediment, where it is consumed (Gundersen & Jørgensen, 1990). Heterotropic organisms inhabiting these sediments catalyze the restoration of chemical equilibrium through the oxidation of reduced carbon produced by photosynthetic organisms (Fig. 1). In benthic sediments oxygen is depleted close to the surface (1-2 mm) and only after it is depleted will nitrate (NO_3^-) serve as an electron acceptor. These are then followed by manganese (Mn^{4+}), iron (Fe^{3+}), sulfate (SO_4^{2-}) and carbon dioxide (CO_2) (Fig. 1). This biogeochemical sequence has been extensively studied in sediments (Canfield *et al.*, 1993; Thamdrup, Fossing & Jørgensen, 1994), whereas only limited data are available on the subsequent stratification of microorganisms and how it is correlated to these processes (Hunter, Mills & Kostka, 2006; Pett-Ridge & Firestone, 2005; Urakawa *et al.*, 2000).

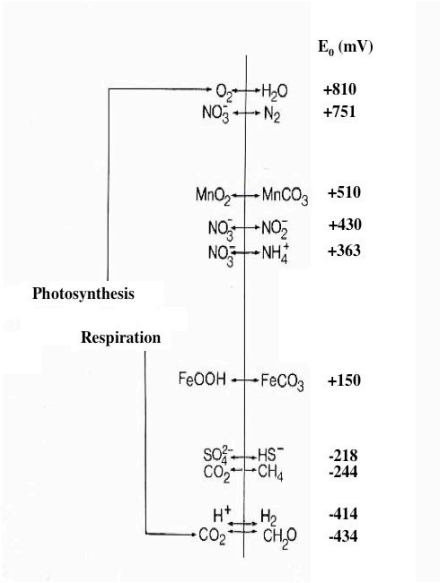

Figure 1. Photosynthesis by autotrophic bacteria creates a chemical disequilibrium while respiration by heterotrophic bacteria and methanogens catalyze the return to equilibrium. In conclusion, a number of external electron acceptors are available but the gain in energy differs according to the different available redox-couples. E_0 represents free energy in mV. Adapted from Fenchel & Finlay (1995).

Living cells that catalyze these processes use part of the free energy they gain for growth and for cell division. However, as long as oxygen is present, aerobic respirers will outcompete bacteria using any other electron acceptors. Below the aerobic zone there is a "suboxic" zone, which is characterized by the biologically mediated reduction of nitrate, manganese and iron. Below the "suboxic" zone, sulfate reduction dominates (the sulfidic zone) and beneath this, sulfate is depleted and archaeal methanogenesis dominates.

Cycling of major elements; C, N, S and P

Bacteria dominate both the production and catabolic processes involving organic carbon in the marine environment. However, only little information is available about the role of archaea in organic carbon cycling in the marine environment (Biddle *et al.*, 2006). Thus, it is well established that methanogenic archaea are ecologically important in the biodegradation of organic matter in nature since methanogenesis is the final step in the decay of organic matter. Therefore, without methanogenesis, a great deal of carbon would accumulate in anaerobic environments. Some archaea are also capable of autotrophic CO_2 fixation and represent an unexpected source of primary productivity in the Sea (Herndl *et al.*, 2005). The dominance of bacteria and archaea in nutrient cycling processes can be explained by their high numbers, large surface-to-volume ratio, and transport systems efficient at low substrate concentrations (Moran & Hodson, 1990).

Cycling of organic carbon in the Baltic Sea sediment was previously shown to be strongly influenced by seasonal changes (Meyer-Reil, 1983; Meyer-Reil, 1987). Meyer-Reil found that the highest bacterial activity in sediments was in November. This increase was shown to be related to the accumulation of organic material deriving from autumn blooms of phytoplankton (Meyer-Reil 1987).

Nitrogen and sulfur are cycled in a complex oxidoreductive fashion. Their reduced form supports chemolithotrophic metabolism. The oxidative forms are used as electron sinks in anaerobic environments. The nitrogen cycle consists of several steps, each mediated by different microorganisms and each having different environmental constraints. All of the critical steps in the nitrogen cycle are exclusively carried out by microorganisms. In the nitrogen fixation step, bacteria convert molecular nitrogen to ammonia. In the Baltic Sea, eutrophication has led to an increase of algal blooms (Elmgren, 1989; Rönngren & Bonsdorff, 2004). This has lead to proliferation of nitrogen-fixing cyanobacteria which results in elevated nitrogen levels in the marine ecosystem (Arrigo, 2005). Conversely, loss of nitrogen via denitrification (the reduction of nitrate, NO_3, to dinitrogen gas, N_2) occurs during microbial decomposition of organic matter in anoxic and near-anoxic environments. It is well documented that reserves of total nitrogen in the Baltic Sea have been rising (Elmgren, 1989; Kuparinen & Tuominen, 2001). The removal of nitrogen by denitrification is therefore a key process in balancing the nitrogen budget. In the Baltic Sea water column, denitrification was previously observed to be responsible for 30% of the removal of the total N input (Stockenberg, 1998). In Baltic Sea sediments, denitrification was strongly regulated by organic carbon availability and quantity (Stockenberg, 1998). Previous studies suggest that nitrogen burial and denitrification together contribute

to 60% of the total nitrogen removal from the Baltic ecosystem (Stockenberg, 1998). Nitrogen is also eliminated from the marine ecosystem by a process called anaerobic ammonia oxidation ('anammox'). This is a process wherein ammonium (NH_4^+) is anaerobically oxidized by bacteria, using nitrite (NO_2^-) as oxidant (Dalsgaard, Thamdrup & Canfield, 2006; Dalsgaard *et al.*, 2003; Kuypers *et al.*, 2006). However, this process has not yet been investigated in Baltic Sea sediments.

Sulfur cycling involves sulfate reduction to hydrogen sulfide, and sulfide oxidation to sulfate. In the presence of oxygen, reduced sulfur compounds are capable of supporting chemolithotrophic microbial metabolism. Some bacteria are capable of photoreduction of carbon dioxide (CO_2) while oxidizing hydrogen sulfide (H_2S) to elemental sulfur (S^0). These bacteria grow at the mud-water interfaces of aquatic habitats. Sulfate reducing bacteria are key community members of marine sediments where they are responsible for 50% of the total organic carbon (Canfield *et al*., 1993; Jørgensen, 1982; Llobet-Brossa *et al*., 2002). This division of bacteria includes species that are not only bound to reduced environments but may also live under oxidized conditions where they may respire with nitrate or even with oxygen (Dilling & Cypionka, 1990; Sahm *et al.*, 1999). There is also evidence that sulfate reduction may proceed under highly oxic conditions (Cohen, 1989). In a previous study it was shown that methane producing archaea coexist with sulfate reducing bacteria in shallow sediments (0-20 cm) indicating their importance. Thus, further investigations are needed to evaluate their role in sulfur cycling (Koizumi *et al.*, 2003).

The phosphorous cycle does not involve oxidation-reduction reactions. Most phosphorous transformations that are mediated by microorganisms can be viewed as transfers of inorganic to organic phosphate or transfers of phosphate from insoluble, immobilized forms to soluble mobile compounds. Phosphate often limits the growth and productivity of microorganisms. Excessive addition of phosphate can result in toxic algal blooms and eutrophication, which are frequently observed in the Baltic Sea (Bianchi *et al.*, 2000). Phosphorus loading has increased about eight-fold in the Baltic Sea during this century. This can be attributed to human activities with the highest concentrations of phosphorus in shallow Baltic Sea sediments (Carman & Wulff, 1989).

Degradation of organic pollutants in marine sediments

Definitions and concepts

A useful strategy for cleaning up both terrestrial and aquatic environments from pollutants is to use the enzymatic activity of microorganisms (Liu & Suflita, 1993; Madsen, 1998; Vogel, 1996). The process of cleaning up polluted environments using microbial degradation capabilities is referred to as bioremediation. Ideally bioremediation strategies should be designed based on knowledge of the microorganisms that are present in the contaminated environments. One must also take into account their metabolic capacities, and how they respond to changes in environmental conditions (Lovely, 2003). Unfortunately, in practice much of the required information is not readily available and the use of microorganisms in bioremediation is more empirical than knowledge based. In the following sections

bioremediation approaches will be discussed in three categories: natural attenuation, biostimulation and bioaugmentation.

Natural attenuation

Natural attenuation occurs constantly and is usually a very slow process where indigenous bacteria develop their natural pollutant degrading ability (Leahy & Colwell, 1990; Pritchard, 1992). When a community has been exposed to a pollutant for a sufficient period, they may evolve appropriate degradation enzymes. Or alternatively, they may acquire degradation genes from other organisms. Although possible, it is not certain that the indigenous microorganisms will develop abilities to degrade pollutants in a reasonable time period, especially if the pollutant is recalcitrant or present in high concentrations (Walker, Colwell & Petrakis, 1975).

Biostimulation

Biostimulation includes supplementing the indigenous microorganisms with nutrients (such as phosphorous or nitrogen) and/or oxygen, thereby stimulating their degradative abilities (Atlas & Unterman, 1981; Atlas & Untermann, 1999). This clean up strategy can in many cases be enough to stimulate degrading populations and decrease levels of toxic pollutants. However, since this method relies on the presence of indigenous microorganisms with adequate degrading abilities it is not always suitable (Atlas & Unterman, 1981; Atlas & Untermann, 1999). Thus, biostimulation strategies that are successful in one environment may not work in another.

Bioaugmentation

Bioaugmentation involves inoculation with laboratory-grown microorganisms carrying the degradation capacity required to clean up the contaminated site (DeFlaun & Steffan, 2002; Vogel, 1996). The inoculum can consist of one to several degrading microorganisms, which may be pre-adapted to the contaminant in the laboratory (DeFlaun & Steffan, 2002). It is possible to initiate a selective enrichment process for increased degradative capacity by adapting the organisms to increasing concentrations of the pollutant.

Archaeal and bacterial biodiversity

The domains, *Archaea* and *Bacteria,* are currently divided into several lineages, which constitute heterogeneous groups of species. Almost any consequential microbial community will have 10^{10} to 10^{17} bacteria that could comprise more than 10^{7} differing taxonomic groups and countless functional groups (Curtis & Sloan, 2005). For archaea, these number are not well known, however in several marine environments it has previously been shown that archaea contribute up to 20-30% of total microbial biomass indicating their significant importance (DeLong, 2003). Consequently, when considering these high numbers and also the complex evolution of archaeal and bacterial entities, classification is not a simple task.

There are several reasons why archaea and bacteria need to be taxonomically classified. For example, when studying natural environments, bacterial classification in combination with statistical ordination tools can be used

as a chronometer to monitor responses to environmental variables (DeLong *et al.*, 2006; Edlund & Jansson, 2006; Edlund *et al.*, 2006). Also, classification can be used to understand which metabolic processes are carried out in a specific environment (DeLong *et al.*, 2006). This knowledge is important since bacteria and archaea are the key components in the cycling of inorganic- and organic matters in all ecosystems.

The earliest attempts to classify bacteria were largely based on morphological properties in analogy with the classification of animals and plants. It was soon recognized that bacterial morphology is in most cases too simple and crude to serve as a basis for classification or identification. A variety of other phenotypic traits were therefore used, and among them, properties of metabolism were prominent. Archaea were not recognized as a major domain of life until relatively recently (Woese & Fox, 1977). They were originally recognized as abundant in environments that are normally hostile to other life forms, such as hot sulfur springs (Marteinsson *et al*., 2001). However, archaea are now known to not be restricted to extreme environments; for example, studies have shown that they are also abundant members of the phytoplankton of the open sea (DeLong, 2003) and soil communities (Bintrim *et al*., 1997). Much is still to be learned about the function of archaea since the majority have not been cultivated to date, but based on molecular studies it is clear that archaea comprise a remarkably diverse and successful domain of organisms.

Currently, two archaea or bacteria are classified as the same species if they exhibit a 70% or greater DNA-DNA reassociation value (Hagström, Pinhassi & Zweifel, 2000; Stackebrandt & Göbel, 1994). However, the species concepts continue to develop and recently it has been proposed that molecular sequence data can be used to define natural units of bacterial diversity that possess the fundamental properties of species (Cohan, 2002). These units can be recognized as clusters of sequences that share greater similarity to each other than to related sequences and are believed to delineate ecologically distinct populations or ecotypes (Cohan, 2002). Ecotypes may arise through various processes including geographical isolation or natural selection and can be difficult to resolve using highly conserved loci such as the 16S rRNA gene (Gevers *et al*., 2005; Fox, Wisotzkey & Jurtshuk, 1992; Palys, Nakamura & Cohan, 1997; Staley & Gosink, 1999). This has led to an increased reliance on protein coding genes and, more recently, multilocus sequence analysis for the resolution of intrageneric relationships (Gevers *et al.*, 2005). In several cases, it has been demonstrated that named species are comprised of multiple ecotypes (Palys *et al.*, 2000). This would lead one to believe that bacterial species generally recognized today are, in fact composites of multiple ecotypes each possessing the dynamic properties of individual species (Cohan, 2002). The bacterial species concept is also applied to archaea, however the major discussion regarding the species concept derives from studies of bacteria.

The present study

Objectives of the thesis

The main purpose of this thesis was to investigate microbial community structures in relatively clean and polluted Baltic Sea sediments and to explore links between bacterial community structures and function. The particular objectives were to:

- Determine the distribution and composition of archaeal (Paper I) and bacterial (Papers I, II and IV) communities along horizontal and vertical gradients in Baltic Sea sediments.
- Test which environmental variables impact horizontal and vertical community structures of archaea (Paper I) and bacteria (Papers I, II and IV).
- Develop an algorithm and software (APLAUS), which enables us to link bacterial community structures defined by T-RFLP with putative bacterial identities (Paper I).
- Link actively growing bacterial communities with functions in Baltic Sea sediments using a polyphasic approach (Papers II, III, IV).
- Isolate bacteria from polluted Baltic Sea sediments that are promising phenanthrene degraders (Papers II, III).

Methods

It is now well established that combinations of molecular tools facilitate the characterization of complex microbial communities. Different suits of tools should be used, and/or adapted depending on the hypothesis to be tested. In this thesis the following approaches were applied.

Bromodeoxyuridine immunocapture

Bromodeoxyuridine (BrdU) immunocapture was previously developed to identify growing bacteria independently of their ability to be cultured (Borneman, 1999; Urbach, Vergin & Giovannoni, 1999; Yin *et al.*, 2000). This method permits identification of specific populations that grow in specific environmental conditions or that grow in response to specific stimuli. The BrdU immunocapture approach relies on the incorporation of BrdU, as a thymidine analogue (Fig. 2), into growing cells during DNA replication. The BrdU labeling is followed by an immunocapture procedure where the newly synthesized DNA is isolated by using antibodies against BrdU (Borneman, 1999; Urbach, Vergin & Giovannoni, 1999; Yin *et al.*, 2000; papers II, III, IV).

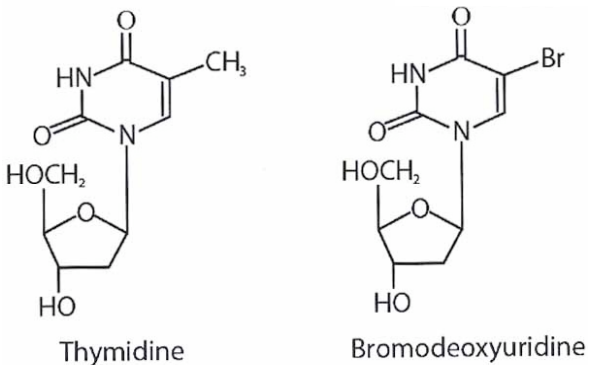

Figure 2. The DNA nucleoside thymidine and its structural analogue bromodeoxyuridine.

The next step in this process is to PCR amplify specific genes of interest from the DNA extract that are subsequently analyzed by cloning and sequencing (papers II, III, IV). Alternatively one could use a molecular fingerprinting method for determination of the active bacterial community composition (Fig. 3; papers II, III and IV). Genes encoding functions of interest representing the actively growing community members can also be amplified and quantified from BrdU incorporated DNA by quantitative real time PCR (qPCR); see below for a description of this method (paper III). By using combinations of these approaches, species identities and relevant metabolic processes can be identified within the actively growing community. The communities can be phylogenetically classified by sequencing of 16S rRNA encoding genes (Fig. 3; papers II, III, IV). The major concern regarding this approach is that it is currently not known which bacterial taxa or species are unable to incorporate BrdU into their DNA. It has been suggested that the majority of bacteria take up and incorporate radiolabeled thymidine, and therefore, it is likely that BrdU can be similarly taken up and incorporated in most organisms (Borneman, 1999). To test whether BrdU was either stimulating or inhibiting bacterial growth at higher concentrations we repeatedly added BrdU to one of the sediment microcosms series (paper III). This resulted in a decrease in community richness (based on the number of TRFs), which probably reflected that BrdU was inhibiting growth for some community members, or alternatively stimulating growth of specific populations. Because of the uncertainty of universal microbial uptake of BrdU, results should be interpreted with caution. On the other hand, this method is rapid and simple to perform and highly suitable for proving that specific populations of bacteria are actively growing; or at least actively synthesizing DNA. However, BrdU immunocapture cannot unambiguously prove that a population is not growing, unless a particular species has previously been shown to be capable of incorporating BrdU under the same environmental conditions. Currently, this approach is one of the most useful tools for studying and identification of specific microbial populations that are growing under defined conditions.

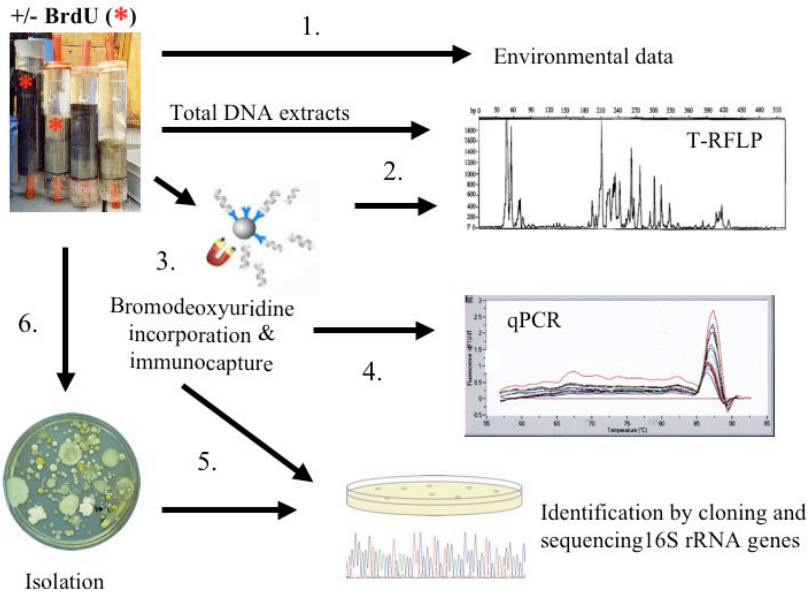

Figure 3. Schematic drawing of a polyphasic experimental approach, involving; 1) collection of environmental data, 2) T-RFLP community fingerprinting, 3) BrdU immunocapture, 4) qPCR, 5) clone libraries and sequencing, 6) isolation on selective media.

Reverse transcriptase (rt) PCR

As a complement to the BrdU immunocapture approach we also used reverse transcription (rt) PCR. The idea behind this method is that after extracting total RNA, reverse transcriptase is added to synthesize complementary DNA (cDNA). Since cellular RNA content is linearly correlated with transcription, i.e. growth rate, this method can be employed to quantify and identify growing bacterial populations (Bremer & Dennis, 1987; DeLong, Wickman & Pace, 1989; Schaechter, Maaløe & Kjeldgaard, 1958). Here we amplified 16S rRNA genes from the reverse transcribed cDNA and performed T-RFLP analysis to monitor the bacterial community structures (paper IV). In paper IV we compared the rt-PCR approach and the BrdU approach to address which groups of bacteria each method could detect. It was clear that a wider range of bacterial divisions were detected with the BrdU approach than when using rt-PCR. The reason for this discrepancy could be that when using rt-PCR, the community members that are active at the specific sampling moment are analyzed, while the BrdU immunocapture method requires a certain incubation time enabling a larger variety of different metabolically active bacteria to be assessed. Furthermore, the two different approaches reflect different intracellular levels of activity (i.e. DNA replication and transcription), which also may contribute to the observed differences. Also, rt PCR includes an additional PCR step which may be disadvantageous since amplicons in PCR increase exponentially. Furthermore, an additional PCR step may increase the chance for introducing PCR errors in the analysis. However, by using rt-PCR as a complement to BrdU immunocapture we could detect more unique bacterial sequences in sediments from different red-ox depths (paper IV).

Terminal restriction fragment length polymorphism (T-RFLP)

Terminal restriction fragment length polymorphism (T-RFLP) is a PCR based method that provides fingerprints of dominant members of complex microbial communities (Fig. 3). This approach enables one to compare microbial community profiles obtained from different environmental samples or to do treatment or temporal comparisons of the same samples. In brief, DNA extracted from a sample is amplified by PCR using primers homologous to a conserved region in a target gene, most commonly the 16S rRNA gene. One of the primers, usually the forward primer, has a fluorescent tag attached to it. After PCR cycling using these primers, DNA fragments (amplicons), which are of equal length, are digested with restriction endonucleases. Consequently, amplified DNA from different organisms containing different restriction sites will yield terminally labeled fragments of different sizes due to polymorphisms in their 16S rRNA gene sequences. The digested amplicons are then separated by electrophoresis on either a polyacrylamide gel or by capillary gel electrophoresis. Usually a DNA sequencer with a fluorescence detector is used so that only fluorescently labeled terminal restriction fragments (TRFs) are visualized. An automated fragment analysis program then calculates the lengths of the TRFs (basepairs) by comparing TRF peak retention time to a DNA size standard. These programs integrate the electropherograms and return TRF peak height and area. The patterns of TRF peaks can then be numerically compared between samples using a variety of multivariate statistical methods (Kitts, 2001) papers I, II, III and IV). Sequence databases based on the input of the lengths of the TRFs and their individual relative abundances (Marsh *et al.*, 2000) paper I) can be used to phylogenetically separate the identified organisms from each other. The analysis can also predict the contribution of various taxa to a specific community. Consequently, individual TRFs in an electropherogram can be identified by comparisons to clone libraries or by predictions from existing databases of sequences such as "A plausible microbial community analysis 3" (APLAUS+; http://mica.ibest.uidaho.edu/trflp.php; paper I), see below, and Fragsort 4.0 (http://www.oardc.ohio-state.edu/trflpfragsort/). T-RFLP is most commonly used to provide a fingerprint that is characteristic of the community from which the DNA was originally extracted.

In general, T-RFLP analysis of dominant microbial communities has gained increased usage in the scientific community because it is fast and has a high resolution (Marsh, *et al.*, 2000). It is, however, subject to all of the caveats routinely applied to molecular approaches that are dependent on efficient extraction of community DNA, such as PCR amplification and restriction digestion with an endonuclease of a target gene (Osborn, Moore & Timmis, 2000; van Elsas, Mäntynene & Wolters, 1997). These problems consequently include concerns regarding preferential extraction of genomic DNA (e.g. the extraction procedure is biased towards those organisms having DNA more easily extracted). Other concerns include amplification bias during PCR cycling and incomplete restriction digestion with endonucleases (Marsh, *et al.*, 2000; Osborn, Moore & Timmis, 2000). Nevertheless, this technique provides useful information about community structures and shifts in dominant populations in microbial communities (papers I, II, III and IV). In this thesis it is demonstrated that this technique has a great potential as a fingerprinting technique combined with

additional molecular approaches, including the above described BrdU immunocapture- and rt-PCR approaches (Fig 3; papers II, III and IV).

A Plausible Community Analysis (APLAUS)

In paper I we developed the "easy access"-computer software APLAUS, for the purpose of identifying dominant microbial community members in T-RFLP community profiles. APLAUS was developed based on the Ribosomal Data Project II (Cole *et al*., 2003) and is available at http://mica.ibest.uidaho.edu/trflp.php. When working with APLAUS it is important to keep in mind that microbial species may have the same TRF length. Therefore, when considering TRFs, it is possible for more than one population in the community to be represented within the same peak. In addition, the T-RFLP resolution may not be capable of distinguishing between TRFs that are within 1-3 bp in length. However, when digesting the gene of interest with multiple restriction enzymes these populations can normally be distinguished. In paper I we putatively identified the most dominant bacterial species in surface sediments along a eutrophication gradient in the Baltic Sea by using three different restriction enzymes. In total, nine different bacterial divisions were detected with APLAUS. Their contribution to the total community abundance varied along the eutrophication gradient. Also, several community members were unclassified, indicating that they were novel with no representatives in existing databases. The archaeal T-RFLP community profiles from the eutrophication gradient contained several TRFs but since only few archaeal 16S rRNA sequences were deposited in the RDPII database at the time of analysis we were limited in our ability to identify most of the community members. APLAUS is a well-suited approach for assigning putative identities to microbial populations represented in T-RFLP profiles. However, it is important to keep in mind that the obtained identities using APLAUS are putative and that other methods may be needed as a complement for more confident identification.

Quantitative real-time PCR (qPCR)

Quantitative gene expression assays are either based on absolute quantification or relative quantification of DNA (Bustin, 2000). Quantitative real-time PCR (qPRC) enables an estimation of the abundance of a specific target DNA sequence in a sample. An important drawback that limits absolute quantification is the variation of material loading for different samples (e.g. uncontrolled biases in DNA extraction).

With the use of fluorescent probes one can monitor the amplification of a target sequence. The two most common ways for detection are DNA binding fluorescent molecules, such as SYBR green, or use of a reporter-quencher system, as represented by Taq-man probes. The Taq-man technology uses a probe that contains a reporter fluorophore and a quencher fluorophore. Before PCR amplification no fluorescence is detected due to the quencher absorbing the light emitted by the reporter fluorophore. However, during PCR amplification the *Taq* polymerase cleaves the probe and fluorescence is emitted.

To obtain absolute quantification the changes in abundance of a specific gene are compared to a standard control DNA sequence with known copy numbers. Gene copy numbers can then be calculated from an external standard curve (Fig. 4). In paper III the absolute changes in abundances of the dioxygenase-

encoding genes, *xylE* and *phnAc,* were determined using the SYBR green approach (paper III). After completion of the PCR cycling a melting curve analysis can be done as a measure of the quality of the amplicon (Fig. 4). The sharper the melting curve, the fewer specific DNA sequences are generated during amplification. When working with DNA extracted from environments harboring a vast genetic diversity, amplification specificity has to be verified by sequencing of the amplified product.

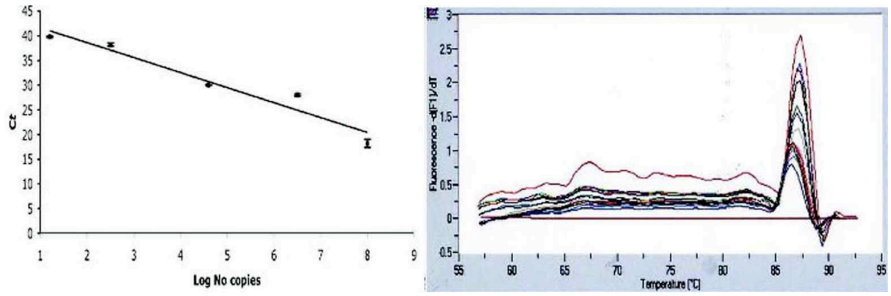

Figure 4. Standard curve (left panel) and melting curve (right panel) of amplicons generated from BrdU labeled DNA extracted from polluted Baltic Sea sediments with primers encompassing the *phnAc* gene during qPCR. Left panel x-axis: the logarithm number of plasmid copies; y-axis: threshold cycle (C_t); right panel x-axis: temperature; y-axis: fluorescence intensity.

Isolation of PAH degraders

Conventional cultivation of microorganisms from environmental samples can be difficult and time consuming. Often the growth conditions and nutritional requirements for growth are unknown. In addition, cultivation conditions normally used are selective and biased for the growth of specific microorganisms (Eilers *et al.*, 2000; Ferguson, Buckley & Palumbo, 1984). This reflects the artificial conditions inherent in most culture media (for example, extremely high substrate concentrations or the lack of specific nutrients required for growth) (Zengler *et al.*, 2002). Recently, it was shown that previously uncultured organisms could be grown in culture if provided with the chemical components of their natural environment (Connon & Giovannoni, 2002; Kaeberlein, Lewis & Epstein, 2002; Rappé *et al.*, 2002). Here, we aimed to isolate polycyclic aromatic hydrocarbon (PAH)-degrading bacteria that were actively growing *in situ* in PAH polluted Baltic Sea sediments (papers II and III). Phenanthrene (Fig. 5) served as a model low molecular weight PAH compound that is commonly found in water and sediments in contaminated regions of the Baltic Sea.

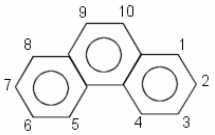

Figure 5. Phenanthrene: a low molecular polycyclic aromatic hydrocarbon comprised of three fused benzene rings.

To activate the growing community members in the polluted sediments, we spiked sediment samples with phenanthrene and incubated at the *in situ* sediment temperature (5°C) in the dark (paper II). Samples were withdrawn and plated on agar plates after one week of incubation (paper II). After plating we adopted the previously developed sublimation technique, which allows incubation with water-insoluble substrates, such as phenanthrene (Alley & Brown, 2000) (Fig. 6).

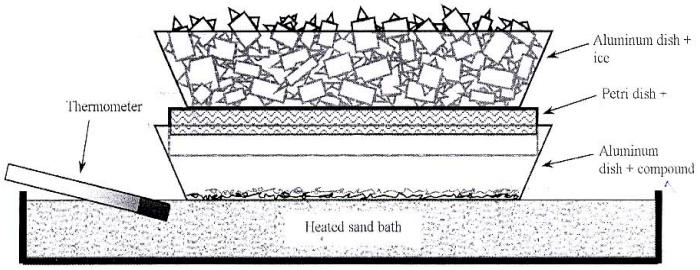

Figure 6. A schematic drawing of the sublimation system. The compound (here phenanthrene) to be sublimed and an inverted petri plate containing inoculated 10% tryptic soy agar rest in a heated aluminum dish. While resting on the petri plate, the second aluminum dish containing ice serves to cool the agar during sublimation. The sand bath was placed on a thermostatically controlled hot plate and the temperature was monitored with a thermometer placed below the surface of the sand. Adapted from Alley & Brown (2000). Copyright © 2007, the American Society for Microbiology, and printed here with permission.

When growth appeared on agar plates, colonies and agar were assessed for color changes and clearing zones under UV light illumination indicative of phenanthrene transformation. Potential positive colonies were tested for potential catechol dioxygenase activity by spraying 0.5 M catechol solution over plates and assessing eventual color change of colonies or/and agar medium (Ingram *et al.*, 1989). The bacterial strains that were identified by both molecular (T-RFLP and sequencing of clones) and cultivation techniques were also tested for their genetic capacity for phenanthrene degradation. This was done by conventional PCR amplification of dioxygenase genes that were previously found to increase in abundance in phenanthrene spiked microcosms according to qPCR (paper III). Furthermore, to test the intrinsic phenanthrene degradation capacity the isolates were cultivated in phenanthrene spiked and filtered sediment water from the Baltic Sea (paper III). Phenanthrene degradation during the incubation time was monitored by gas chromatography-mass spectrometry (GC-MS) (paper III).

Key findings

Metabolically active bacteria

To specifically study the community structures of metabolically active and growing bacteria in marine sediments we employed the BrdU immunocapture approach (papers II, III and IV) and reverse transcription (rt) of RNA followed by PCR amplification of 16S rRNA cDNA (paper IV). The results from these methods were evaluated by T-RFLP and sequencing of cloned 16S rRNA genes (paper IV).

In papers II and IV we demonstrated that bacterial 16S rRNA genes amplified from metabolically active and growing bacteria were significantly different than 16S rRNA genes amplified from total DNA extracts. These results imply that the metabolically active and growing fraction of bacterial communities in natural marine sediments is very small. These results strengthen findings from earlier studies suggesting that approximately 85% of the total bacterial community is comprised of either dead or dormant bacteria (Dell'Anno & Corinaldesi, 2004; Luna, Manini & Danovaro, 2002). By contrast, when we studied phenanthrene-spiked sediments in a microcosm study (paper III) several of the dominant bacteria identified in community DNA extracts were also actively growing, suggesting that the incubation conditions had selected for bacteria that grew and dominated the community. However, some of the growing populations, although not dominant in the community extracts, were only found in the phenanthrene-spiked sediments, suggesting that they were specifically favored to grow in the presence of phenanthrene.

In Paper IV, we compared the BrdU immunocapture- and rt-PCR approaches. Both of these techniques, in combination with molecular fingerprinting approaches, were suitable methods for identification of metabolically active and growing bacteria in sediments. An advantage is that both of these approaches are less time consuming and less technically demanding than stable isotope probing (SIP) which can also be used for similar purposes (Radajewski *et al*., 2003). Although, there was good similarity in phylotypes identified by each of these methods, there were also some differences in the number and types detected. These differences could be due to intrinsic biases in the respective methods; i.e. BrdU immunocapture requires uptake of BrdU into the cells. By contrast, rt-PCR has a bias during the reverse transcription step and the PCR amplification of cDNA can cause a further bias. Another difference in the two approaches is that the rt-PCR technique results in a snapshot of the populations that are active at the exact moment of RNA extraction, whereas the BrdU immunocapture technique allows for a lengthier incubation time and may capture a wider phylogenetic range of microbes. Further investigations are needed to support these hypotheses and to more thoroughly test limitations of these methods.

Spatial distribution of microorganisms in sediment

We found that in Baltic Sea sediments dominant archaeal and bacterial communities clustered separately along a eutrophication gradient according to T-RFLP results analyzed with statistical ordination methods (paper I; Fig. 2). The

structures of the bacterial communities were most strongly correlated to water depth, followed by organic carbon, oxygen, salinity and silicate levels. In contrast, archaeal communities were most strongly correlated to oxygen, salinity, organic carbon, silicate and nitrate levels (Fig. 7). These results suggest that the microbial communities were spatially structured by environmental variables directly linked to eutrophication (i.e. organic, carbon, oxygen and silicate and nitrate) along the eutrophication gradient. In addition, these results suggest that archaeal and bacterial communities are spatially structured by different environmental factors. Therefore, anthropogenic impacts, such as eutrophication play a role in the structure of the resulting microbial communities in sediments and presumably have impacts on microbial function. Although we did not study functional genes in this study, it would be interesting to see how their levels are correlated to the environmental factors and to the identities of the dominant populations identified in the sediments.

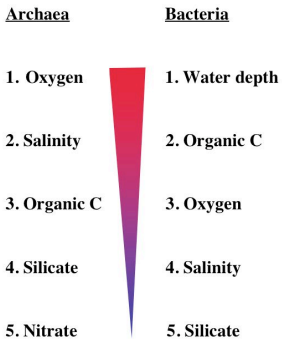

Figure 7. Environmental parameters that have an impact on archaeal and bacterial 16S rRNA gene distributed along a eutrophication gradient in Baltic Sea sediments. The parameters are presented in descending rank order (1-5; red- blue) according to correlation analysis of CA coordinates and chemical data.

Vertical distribution of bacteria in sediment

In paper IV, our principle aim was to test if the distribution of metabolically active and growing bacterial communities was influenced by red-ox parameters (i.e. available electron acceptors) present along vertical sediment profiles in the Baltic Sea. Based on results from T-RFLP analysis of 16S rRNA gene fragments amplified from total DNA extracts and BrdU labeled DNA, bacterial communities were significantly different at the different red-ox depths (paper IV; Fig. 2). The bacterial community profiles were also significantly impacted by organic carbon-, nitrogen content and red-ox potential (paper IV; Table 2). Interestingly, a large fraction of the sequenced 16S rRNA genes from the different red-ox depths showed low sequence similarities (approximately 93%) to previously deposited sequences in the greengenes database. This would indicate that Baltic Sea sediments harbor a largely unidentified microflora (paper IV). Also, 16S rRNA gene sequences belonging to ecologically important groups involved in sulfate reduction and denitrification were predominant in the reduced layers of the sediments. In addition, it should be noted that several community members

belonged to lesser-known candidate divisions, for example OP3, WS3, SBR1093, etc. (paper IV; Fig. 3).

Isolation of key community members

Although estimates exist that more than 90- 99% of bacteria from environmental samples have not been cultivated (Amann, Ludwig & Schleifer, 1995; Fuhrman, McCallum & Davis, 1993; Pace, 1997; Zengler *et al.*, 2002), we were able to successfully cultivate bacteria of interest from Baltic Sea sediments in this study. In papers II and III we show that inventive cultivation approaches including adding selection pressure and cultivation in environmental media allows isolation of metabolically active community members in polluted Baltic Sea sediments (Fig. 8).

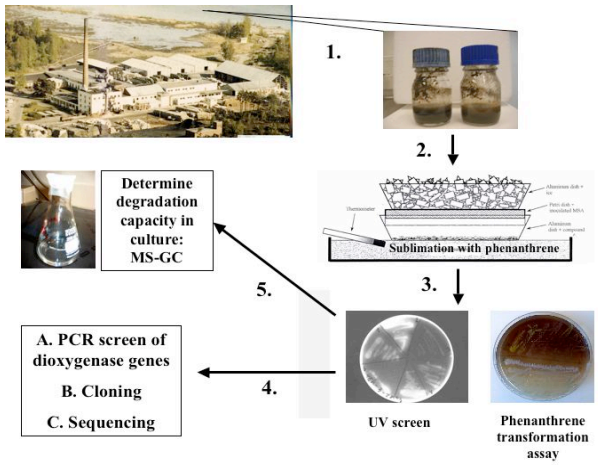

Figure 8. Cultivation approach to select for actively growing community members involved the following steps: 1) spiking of polluted sediments with phenanthrene and incubation at *in situ* sediment temperature (5°C) in microcosms, 2) inoculation of agar plates with spiked sediments and addition of water insoluble phenanthrene by employing a sublimation technique, 3) growing bacteria at 5°C and screening for dioxygenase activity by visualizing clearing zones under UV light illumination and by observing color changes of agar and bacterial colonies, 4) screening for genetic capacity of phenanthrene degradation by PCR (A) cloning (B) and sequencing (C) of PCR products to verify gene sequences, 5) cultivating dioxygenase positive isolates in sediment water extracts spiked with phenanthrene and screening for phenanthrene degradation using gas chromatography-mass spectrometry (GC-MS).

Three bacteria that were identified as growing by BrdU immunocapture followed by T-RFLP and sequencing of 16S rRNA genes were also isolated using the approach outlined above (Fig. 8). These isolates, *Exiguobacterium oxidotolerans* AE3, *Pseudomonas fluorescens* AE1 and *Pseudomonas migulae* AE2 showed both genotypic and phenotypic characteristics of phenanthrene degradation (i.e. they contained the dioxygenase gene, *phnAc*, and they were capable of removal of phenanthrene from liquid medium). When taking these data into account we propose that these bacteria were responsible or at least involved in phenanthrene

degradation in the polluted sediments *in situ* (papers II and III). We found differences in the rates of phenanthrene removal by the three bacteria: *E. oxidotolerans* AE3 removed phenanthrene more rapidly than *P. fluorescens* AE1 and *P. migulae* AE2. We propose that *E. oxidotolerans* is a potential candidate for future bioremediation applications in marine sediments with low temperatures. To date, not much is known about the *Exiguobacterium* genus in general. Therefore, these results add a clue as to their function in the environment, with respect to pollutant degradation.

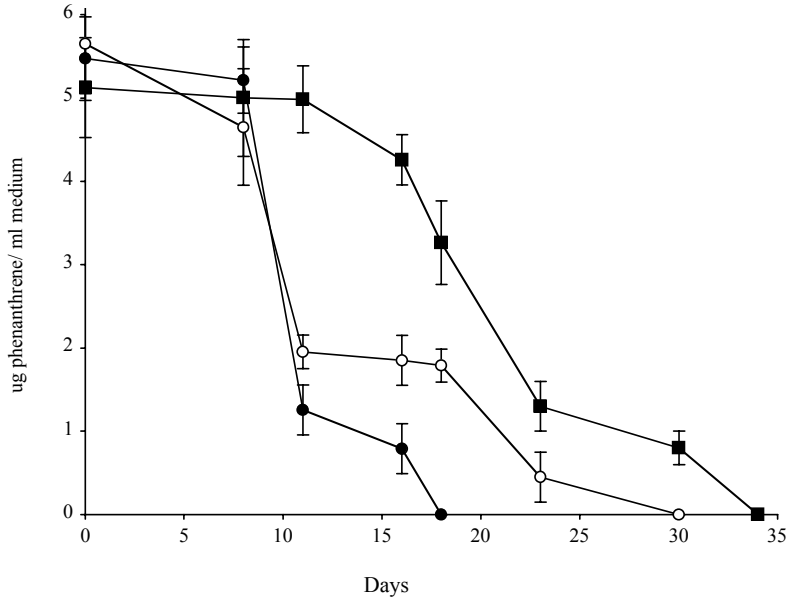

Figure 9. Phenanthrene degradation in cultures containing phenanthrene spiked sediment medium and *Exiguobacterium oxidotolerans* AE3 (filled circles), *Pseudomonas fluorescens* AE1 (open circles), and *Pseudomonas migulae* AE2 (filled boxes).

Concluding remarks and future perspectives

The major findings of this work can be summarized as follows:

- Archaeal and bacterial community structures are spatially heterogeneous and correlate significantly to different environmental parameters that are directly linked to eutrophication in top surface sediments along a pollution gradient in the Baltic Sea (paper I).
- In Baltic Sea sediments, metabolically active and growing bacterial community members are not necessarily the most dominant community members as inferred from molecular analysis of DNA (Papers II, III and IV) and RNA (paper IV).
- Novel lineages of bacteria were obtained by sequencing 16S rRNA genes from clones and by cultivating bacteria from different sediment depths and sampling locations respectively, indicating that bacterial diversity in Baltic Sea sediments is still largely unexplored (papers I and IV).
- By applying a polyphasic approach we were able to detect and isolate three bacterial strains possibly involved in *in situ* phenanthrene degradation. We suggest that these isolates are representative candidates for phenanthrene bioremediation of polluted Baltic Sea sediments (papers II and III).
- Bacterial communities are significantly different along vertical red-ox profiles in Baltic Sea sediments and the community structures are mainly impacted by organic carbon, nitrogen and red-ox potentials (paper IV).

These studies have elucidated several areas of research, which have previously been unexplored in Baltic Sea sediment. However, several new questions have also been raised from this work. For instance, what is the functional role of bacterial lineages belonging to the species *Exiguobacterium oxidotolerans* in marine sediments? Representatives of this genus were present at all sampling locations and the *E. oxidotolerans* AE3 isolate could degrade phenanthrene rapidly in culture. In addition, representatives of *Schewanella* and *Methylomonas* were identified as the most dominant members of the sediment community in the presence of phenanthrene. It would be interesting to isolate strains representative of these genera in order to study their functional roles in the sediments more thoroughly and to combine them in co-cultures to determine whether degradation rates are enhanced.

The mechanisms behind the spatial heterogeneity of archaea and bacterial urge further investigation. This would require individual testing of a group of environmental variables *in situ* in a dose dependent fashion to determine their respective impacts on bacterial and archaeal communities. To achieve a holistic view about ecological functions and interactions in sediments, organisms from all major phyla (i.e. viruses, archaea, bacteria, and eukaryotes; fungi, protozoa and macrofauna) should be sampled and studied in parallel. This would enable responses to environmental variables at different tropic levels to be analyzed in conjunction.

An important insight from these studies is that in order to detect the functionally active fraction of sediment microbial communities, it is necessary to specifically target metabolically active and growing bacteria, as many of the

fingerprinting approaches used to date can also detect extracellular DNA as well as dead and dormant bacteria that might be dominant in sediments, but have a minor functional role.

Clearly, the Baltic Sea sediment harbors an extensive bacterial flora with a unique biodiversity, which has to date been relatively unexplored. We believe it is highly relevant to continue our work with identifying metabolically active community members and functional genes that are expressed in Baltic Sea sediment. These studies would aid in the understanding of the fundamental driving forces behind nutrient cycling and pollutant degradation in the Baltic Sea ecosystem.

Here we developed a promising polyphasic suite of methods involving traditional cultivation in combination with molecular tools. We propose that these tools can be applicable for future studies aiming to link bacterial community diversity with ecological functions. In addition, the molecular data can be used to determine which key community members are of interest to isolate for potential applications, such as for bioremediation purposes.

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Acknowledgements

First I would like to acknowledge **Södertörn University College** and the **Baltic Sea foundation** for supporting this work and for paying my salary all these years!

Then I like to thank you all for your fantastic support through this last year's emotional disaster as I unexpectedly lost my precious father. Without your support this thesis would not be. In this section, I hope I have not forgotten to mention anyone and if I did, I am sure I will suffer somehow.

Janet Jansson, my supervisor, thanks for being the Super Nova shining on me! You have kept me under your wings and supported me anytime I needed it. Thanks for your continues care and for being one of the most encouraging persons I have ever met. You have inspired me tremendously to discover the universe of microbes. Thanks for sending me all around the world to involve me in the microbial society and to meet the most fantastic microbiologists! Thanks for supporting the private me- I really needed you to be there in ups and downs. You mean so much to me!

Sara Sjöling, my supervisor, thanks for showing me the way into the marine ecology field and for keeping me on the right track! Thanks for all fruitful discussions, for challenging and teaching me so many things about science and ethics. You are the smartest cookie and I will always look up to you. Without your help I would never have found our first sediment samples. Thanks for caring so much for me and for sharing everything.

To my group at Södertörn: Thanks for all the fun and your super strong support. **Fredrik,** it has been great sharing all these years from undergraduate students to PhD. Thanks for all your help, all the laughter, your warm support and for putting up with the grouchy working machine I know I can be. **Karolina,** you have been my role model scientist and a very good and inspiring friend. Thank you very much Mrs Maniatis! **Agneta,** thanks for being a very good friend and a fun colleague to spend time with. **Cia,** thanks for all your help, fruitful T-RFLP discussions and for inspiring conversations about everything! **Ninwe**, thanks for your pleasant company and interesting parties. I look forward to become your neighbour in California. **Johan N, Lu, Åsa, Elisabet, Irene, Maria** and **Michal** thanks for your lovely company and interesting chats. **Annika** and **Annelie,** Janet's oldest, thanks for very inspiring talks and for treating me so well.

All the people at Södertörn: Sam, the Macintosh Mogul, thanks for solving all my computer problems, for your tremendous patience, for teaching me about computers and your pleasant company; **Fergal** (and **Jill** of course) for your very nice company and support and for introducing your very nice friend Ryan. **Tord Eriksson**, thanks for being so nice and for your excellent help with MS-GC analysis. **Inger PH,** thanks for all the nice horse chats and for being a very good friend. Many thanks to **Mats G, Emma, Petra, Oskar, Lars F, Mike L, Anna-Carin and Magnus** for adopting me into your group and for a fantastic time together. I will miss you so much! Thanks to everybody at floor 4, **Håkan L,**

Tomas B, Rose-Marie, Gunnar, Hossein, Ivo, Galina, Odd, Elinor, Jan-Erik, Mikael H, Lisbet, Gaby, Kalle, Andreas and **Andrea** for creating a nice environment and for sharing everything from internal standards to lunch boxes. Thanks to **Shawn** for being a very good friend and for help with bioinformatics issues – Good luck in Florida! Thanks to **Per K** for your nice company and for inspiring me to continue to study. Thanks to **Mikael G and Clair** for an interesting collaboration, inspiring discussions and very nice company. **Lotta, Karin, Helena Marie B, Anders B, Johan T, Marie G, Andres, Patrick D, Stefan, Peter S, Maria T, Maria T, Einar and Håkan O** - thanks for all your help and for being so nice to me.

To my group at SLU: Magnus, thanks for teaching me about phylogeny, alignments and for letting me occupy your office, snatching you candies and for many laughs. **Karin** and **Claes** for very nice company and inspiring chats. **Veronika,** thanks for being a very good colleague and helping me with BrdU. **Åsa, Maria H, Lotta, Sara H, Karin E, Maria U, Johan D** and **Joakim,** it has been a pleasure to know you and be a part of your group. Also, **Ingvar, Harald** and **Anna-Ida**, thanks for very inspiring conversations. A special thank to **Johan Schnürer** for being an excellent supporter whenever I needed and for the tremendously funny dancing at the castle in Ljubljana! A heavy "thank you" has to go to **Susanne Broqvist** for being so nice and for answering all my e-mails within a second. **Elisabet** and **Leticia,** your help and knowledge have been invaluable to me. Thank you for everything!

Thanks to **Sonja Löfgren** and **Benjamin Edvinsson** at the Karolinska Institute for all your help and patience with me and my qPCR and for very nice chats.

Thanks to **Elena Gorokhova, Ulf Larsson and Sture Hansson** at the Department of Systems Ecology, Stockholm University for being excellent inspiration sources. Thank you Ulf for help with sampling in Himmerfjärden and for sharing environmental data. Elena, thanks for being a very good friend and a colleague and for always giving me the best advices.

Anne Stockenberg, thanks for being so nice to me and for your support and for sharing your knowledge about Baltic sediments.

To Professor **Forest Rohwer and colleagues** at the San Diego State University- thanks for paying interest in my research and for creating the most incredibly inspiring future plans!

…

Dad, your contribution to this thesis is vast. Knowing the remarkable person you were means everything to me in work and private. There are no words that can explain how much we miss you every moment.

Birgitta, thanks for saving me! Your support means everything to me. Thanks for bringing the best into our lives and for being the warmest and most adorable person we all love so much.

To rest of my grand family; **Rickard, Tove, Amanda and Linus; Anna, Patrick, William and Charlie; Anna-Maria, Jimmy, Sanna, Wilma and Alexander; Lena, Johan, Erik and Felix**; **Karin, Gunnar, Robin and Inez; Sigrid and Ingvar; Staffan and Jo-Anna and Ester** I just can't imagine what I would do without you?? Lena and Rickard, you shall have an extra award for taking care of dad's business so well which made it possible for me to finish my PhD. Also, to my family in Australia, Canada and USA: **Arne, Robert, Helen, Geoffrey, Kevin, Gunilla and family, Gord and Laura, Rick and the girls, Darcy, Carol and the boys,** thanks for your continuous support!

Many humble thanks to our old racehorse and World Champion **Hallsta Lotus** for fulfilling my father's dreams and for supporting me and my family economically through a trying study time. May you eat fresh green grass, drink water from a golden bucket for the rest of your horsy life!

Antti, thanks for your support and for trying so hard to make everything work out!

Kajsa Ohlbjörns (another Goos Hill chick), Stephanie Plouda, Petra Wiik-Öjemark, Kiki Buchta, Brita Zilg, Helena Bister thanks for your support, inspiration and for staying by my side all these years!

Emma Ehn, Johan Lindh and Alva thanks for always being very close to me in work and private and for all the incredible fun times we have had together during our study time Emma. Thanks for your tremendous support! With you life is sooo much easier!

Petra, David, Kajsa and Aiden Souter, thanks for all the fun times together. Petra, thanks for being an excellent colleague, for your comfort and for becoming one of my closest friends.

Thank you **Marco and Alessia**, for all the laughter, your fantastic support and for taking care of us in Stockholm and in San Diego. I look forward to spend lots of quality time with you in a close future.

The last and heaviest acknowledgement goes to the little group of people who is my syntrophic family: **Ryan, Juni and Madison**. **Ryan**, my Super man, thanks for putting up with the microbiologist in me who always relates to bacteria and goes out sampling even in the sleep! Thanks for putting all the pieces together and straighten me out. You are the most helpful and caring person and I really look forward sharing an exciting and loving future with you. **Juni**, the most patient and understanding girl I have ever known. Thanks for your fantastic support and for forcing me to get my act together as a young mother. I hope you are not too traumatized by late night sampling occasions, stinky sediments, playing computer games in the office, pipetting in the lab or taking care of Madison… This thesis would not be without you. You are something extraordinary and I owe you everything! **Madsion**, thanks for coming to this world and making it complete! Thanks for your patience as well and for all the laughter. You are the best vitamin pill and the most wonderful kiddo.