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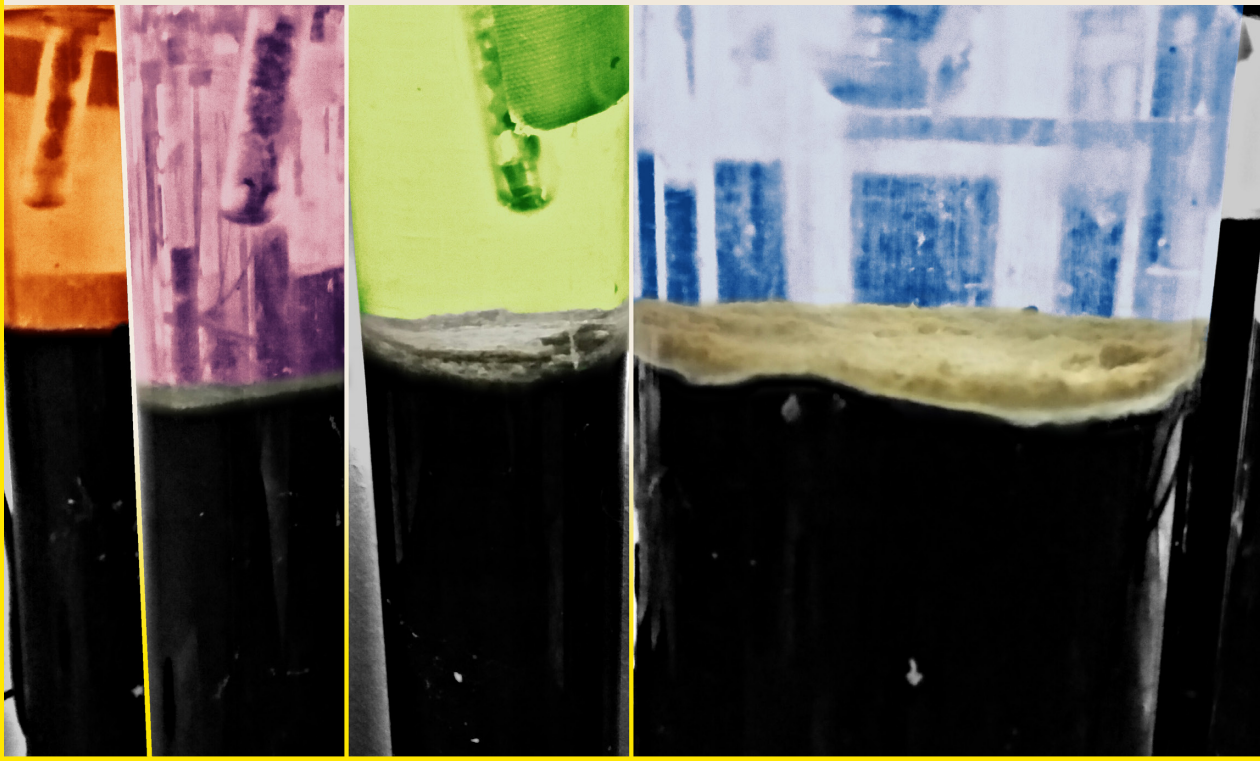
Elias Broman

Ecology and evolution of coastal Baltic Sea 'dead zone' sediments



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Ecology and evolution of coastal Baltic Sea 'dead zone' sediments:
Doctoral Dissertation, Department of Biology and Environmental Science,
Linnaeus University, Kalmar, 2018

ISBN: 978-91-88761-00-2 (print), 978-91-88761-01-9 (pdf)
Published by: Linnaeus University Press, 351 95 Växjö
Printed by: DanagårdLiTHO, 2018

Abstract

Broman, Elias (2018). *Ecology and evolution of coastal Baltic Sea 'dead zone' sediments,* Linnaeus University Dissertations No 302/2018, ISBN: 978-91-88761-00-2 (print), 978-91-88761-01-9 (pdf). Written in English.

Since industrialization and the release of agricultural fertilizers began, coastal and open waters of the Baltic Sea have been loaded with nutrients. This has increased the growth of algal blooms and because a portion of the algal organic matter sinks to the sea floor, hypoxia has increased. In conjunction to this, natural stratification of the water column makes the bottom zones especially prone to oxygen depletion due to microbes using oxygen and organic matter to grow. Hypoxia (<2 mg/L O_2) and anoxia (no oxygen) are deadly for many organisms and only specialists (typically some microorganisms) are able to survive. Due to the harsh conditions these bottom zones are commonly referred to as 'dead zones'. The focus of this thesis was to look closer at the microbial community changes upon degradation of algal organic matter and the effect of oxygenating coastal Baltic Sea 'dead zone' sediments on chemistry fluxes, phyto- and zooplankton, the microbial community structure, and microbial metabolic responses. Results from field sampling and incubation experiments showed that degradation of algal biomass in nutrient rich oxic sediment was partly related to the growth of archaea; that oxygenation of anoxic sediments decreased stored organic matter plus triggered hatching of zooplankton eggs increasing the benthic-pelagic coupling; and resting diatoms buried in hypoxic/anoxic sediment were alive and triggered to germinate by light rather than oxygen. Changes in the microbial community structures to oxygen shifts were dependent on the historical exposure to oxygen and that microbial generalists adapted to episodic oxygenation were favored during oxygen shifts. Facultative anaerobic sulfur/sulfide oxidizing bacterial genera were favored upon oxygenation of hypoxic/anoxic sediment plus sulfur cycling and nitrogen fixation genes were abundant. Finally, it was discovered that oxygenation regulates metabolic processes involved in the sulfur and methane cycles, especially by metabolic processes that results in a decrease of toxic hydrogen sulfide as well as the potent greenhouse gas methane. This thesis has explored how 'dead zones' change and develop during oxygen shifts and that re-oxygenation of 'dead zones' could bring favorable conditions in the sediment surface for re-establishment of new micro- and macroorganism communities.

Keywords: Baltic Sea, sediment, oxygen, metatranscriptomics, metagenomics, 16S rRNA gene, RNA-seq, dead zone, re-oxygenation

Sammanfattning

Arealerna av 'döda bottnar' i Östersjön har ökat som en följd av industrialiseringen och användandet av gödningsmedel. Föroreningen av Östersjöns kust och öppna vatten med näringsämnen leder till en ökad tillväxt av algblomningar. En del av dessa alger sjunker till havsbotten och orsakar att så kallad hypoxia utvecklas. Den naturliga stratifieringen av vattenkolummen avgränsar yt- och bottenvattnet vilket leder till att bottenzonen är speciellt utsatt för syrebrist. Detta eftersom mikroorganismer i bottensedimentet använder syre och organiskt material för att leva. Hypoxia (<2 mg/L O_2) och anoxia (inget syre) är dödligt för de flesta organismer och endast specialiserade organismer (vanligtvis vissa mikroorganismer) kan överleva. Det är av denna anledning dessa bottenzoner ofta kallas för 'döda bottnar'. Målet med denna avhandling var att undersöka förändringar i de mikrobiologiska samhällena vid nedbrytning av organiskt algmaterial, och undersöka vilken effekt syresättning har på ekologin i döda bottensediment i Östersjöns kust. I mer detalj studerades kemiska flöden, växt- och djurplankton, samt mikrobiologiska samhällen och deras metaboliska processer. Resultaten från fältprovtagningar och inkubationer i laboratoriet visade att nedbrytning av algmaterial i syrerikt sediment till viss del gynnade arkéer; syretillsättning av anoxiska sediment minskade det lagrade organiska materialet och ledde till ökad kläckning av djurplanktonägg; vilande kiselalger begravda i hypoxisk/anoxisk sediment var levande och vaknade vid tillförsel av ljus snarare än syre. Förändringar i mikrobiologiska samhällen vid syreförändringar var beroende av historisk exponering av syre i sedimentytan. Det observerades också att mikroorganismer anpassade till episodiska förändringar i syre gynnades. Fakultativt anaerobiska svavel/sulfidoxiderande bakteriesläkten gynnades efter syresättning av hypoxisk/anoxiskt sediment och gener involverade i omvandling av svavelämnen och kvävefixering var vanliga. Slutligen visade resultaten att syresättning reglerar metaboliska processer involverade i kretsloppen för svavel och metan. Speciellt genom processer som leder till en minskning av den gifta gasen svavelväte och växthusgasen metan. Denna avhandling har undersökt hur döda bottensediment förändras och utvecklas vid skiftande syreförhållanden och visar att syresättning av 'döda bottnar' kan skapa gynnsamma förhållanden i sedimentytan för återetablering av mikro- och makroorganismsamhällen.

Nyckelord: Östersjön, sediment, syre, metatranskriptomik, metagenomik, 16S rRNA gen, RNA-sekvensering, döda bottnar, syretillsättning

"The greatest scientific discovery was the discovery of ignorance. Once humans realised how little they knew about the world, they suddenly had a very good reason to seek new knowledge, which opened up the scientific road to progress."
— Yuval Noah Harari, Homo Deus: A Brief History of Tomorrow

"Staying alive is not enough to guarantee survival. Development is the best way to ensure survival."
— Liu Cixin, The Dark Forest

The covor photo depicts incubation experiments with the water digitally colored

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List of publications

I. **Broman E.**, Sjöstedt J., Pinhassi J., Dopson M. (2017) Shifts in coastal sediment oxygenation cause pronounced changes in microbial community composition and associated metabolism. *Microbiome* 5(96). doi: 10.1186/s40168-017-0311-5

II. **Broman E.**, Brüsin M., Dopson M., Hylander S. (2015) Oxygenation of anoxic sediments triggers hatching of zooplankton eggs. *Proceedings of the Royal Society of London B: Biological sciences* 282(1817), 20152025. doi: 10.1098/rspb.2015.2025

III. **Broman E.**, Li L., Fridlund J., Svensson F., Legrand C., Dopson M. (2018) Eutrophication induced early stage hypoxic 'dead zone' sediment releases nitrate and stimulates growth of archaea. *Submitted*

IV. **Broman E.**, Sachpazidou V., Pinhassi, J., Dopson M. (2017) Oxygenation of hypoxic coastal Baltic Sea sediments impacts on chemistry, microbial community composition, and metabolism. *Frontiers in Microbiology,* in press

V. **Broman E.**, Sachpazidou V, Dopson M., Hylander S. (2017). Diatoms dominate the eukaryotic metatranscriptome during spring in coastal 'dead zone' sediments. *Proceedings of the Royal Society of London B: Biological sciences* 284(1864), 20171617. doi: 10.1098/rspb.2017.1617

Authors contribution to the papers

Paper I
Concept and design: Broman E, Dopson M, Pinhassi J
Sampling: Broman E
Laboratory work: Broman E, Sjöstedt J
Data analysis: Broman E, Sjöstedt J
Drafting manuscript: Broman E
Proofreading and edit: Broman E, Sjöstedt J, Pinhassi J, Dopson M

Paper II
Concept and design: Hylander S, Broman E and Dopson M
Sampling: Broman E
Laboratory work: Broman E, Hylander S, Brüsin M
Data analysis: Broman E, Hylander S
Drafting manuscript: Hylander S, Broman E
Proofreading and edit: Broman E, Brüsin M, Dopson M, Hylander S

Paper III
Concept and design: Broman E, Dopson M, Legrand C
Sampling: Broman E, Fridlund J, Li L
Laboratory work: Broman E, Li L, Fridlund J, Svensson F
Data analysis: Broman E
Drafting manuscript: Broman E
Proofreading and edit: Broman E, Fridlund J, Li L, Svensson F, Legrand C, Dopson M

Paper IV
Concept and design: Broman E, Dopson M, Pinhassi J
Sampling: Broman E, Sachpazidou V
Laboratory work: Sachpazidou V, Broman E
Data analysis: Broman E
Drafting manuscript: Broman E
Proofreading and edit: Broman E, Sachpazidou V, Pinhassi J, Dopson M

Paper V
Concept and design: Hylander S, Broman E, Dopson M
Sampling: Broman E, Sachpazidou V
Laboratory work: Sachpazidou V, Hylander S
Data analysis: Broman E, Hylander S
Drafting manuscript: Broman E, Hylander S
Proofreading and edit: Broman E, Sachpazidou V, Dopson M, Hylander S

Other publications not included in this thesis

Broman E., Jawad A., Wu X., Christel S., Ni G., Lopez-Fernandez M., Sundkvist J.E., Dopson M. (2017) Low temperature, autotrophic microbial denitrification using thiosulfate or thiocyanate as electron donor. *Biodegradation* 28(4), 287-301. doi: 10.1007/s10532-017-9796-7

Roman P, Klok JBM, Sousa JAB, **Broman E.**, Dopson M., Van Zessen E., Bijmans M.F.M., Sorokin D.Y., Janssen A.J.H. (2016) Selection and application of sulfide oxidizing microorganisms able to withstand thiols in gas biodesulfurization systems. *Environmental Science & Technology* 50(23), 12808-12815. doi: 10.1021/acs.est.6b04222

Koehler B., **Broman E.**, Tranvik L.J. (2016) Apparent quantum yield of photochemical dissolved organic carbon mineralization in lakes. *Limnology and Oceanography* 61(6), 2207-2221. doi: 10.1002/lno.10366

Introduction

During the past five decades the dissolved oxygen has decreased in the oceans in conjunction with the expansion of vertical oxygen-minimum-zones. This decrease of oxygen is accelerated by warmer waters and is suggested to further decrease due to climate change (Schmidtko et al., 2017). Oxygen is respired by micro- and macroorganisms in the waters and its decline has been found to cause damage to e.g. fisheries via increased fish mortality (Diaz and Rosenberg, 2008). In addition, water systems heavily polluted with nutrients leads to a subsequent increase in organic biomass that can cause further oxygen depletion by microbial mediated biomass degradation coupled to the reduction of oxygen (Middelburg and Meysman, 2007). Since industrialization and the use of agricultural fertilizers this has been especially prominent in the Baltic Sea (Conley, 2012).

1.1 The Baltic Sea

The Baltic Sea is a brackish water body with an area of 370,000 km^2 (Kullenberg and Jacobsen, 1981). It is located in northern Europe and is surrounded by nine countries: Russia, Sweden, Finland, Germany, Poland, Lithuania, Latvia, Estonia, and Denmark (Voss et al., 2011). The Baltic Sea consists of a series of basins comprising 21,000 km^3 of water with a mean and maximum depth of 56 and 459 m, respectively (Kullenberg and Jacobsen, 1981). The main drainage areas of the water body include the northern Gulf of Bothnia and the southern Baltic Proper (Figure 1). The Gulf of Bothnia is surrounded by 50% forest and 20% shrubland while the Baltic Proper is surrounded by 20% forest and 50% agricultural land (Voss et al., 2011).

The salinity in the Baltic Sea is less than marine water that has typically 30-35‰ salinity and is therefore classified as a brackish system. Average salinity in the Baltic Sea is lowest in the 0-60 m water depth (salinity of ~7 ‰) and increases gradually to 60-125 m ~9‰ and 125-240 m ~12 ‰ (Hansson and

Gustafsson, 2011). This difference in vertical water column salinity forms a stratification (halocline) separating the upper and lower waters (Matthäus and Schinke, 1999). There is also a difference in salinity horizontally which forms a gradient in the Baltic Sea with as low as 1‰ in the surface waters of the northern Gulf of Bothnia and 9‰ in the southern Baltic Proper (Kullenberg and Jacobsen, 1981). Water retention time (complete water exchange) in the Baltic Sea has been estimated to be up to 35 years (Kullenberg and Jacobsen, 1981; Matthäus and Schinke, 1999) and ~9 years in the Baltic Proper (Savchuk, 2005). This is in contrast to Swedish coastal waters that have been estimated to have an average retention time of ~9 days (Dimberg and Bryhn, 2014).

Figure 1 Overview of the major subdivisions in the Baltic Sea. © OpenSeaMap contributors.

Long water retention time in conjunction with the halocline prevents oxygen in the surface water to ventilate the deeper, bottom water (Zillén et al., 2008). Therefore, throughout history the Baltic Sea has had naturally occurring bottom zones devoid of oxygen (Per et al., 1990; Zillén et al., 2008). Oxygen fluctuations below the halocline can also occur naturally during inflow events of high saline, oxygen-rich water. Due to the higher salinity of the inflow marine water compared to the brackish water, the marine water sinks and

oxygenates bottom areas in the Baltic Sea. However, the occurrence of these events has become more rare compared to three decades ago (Kabel et al., 2012). During the 1960s it was brought to public attention that the area of oxygen deficiency had increased more rapidly than previously observed (Elmgren, 2001). From 1960 to 1980 nutrient input of nitrogen and phosphorous into the Baltic Sea was estimated to have increased up to 4 and 8 times, respectively (Larsson et al., 1985). Thereafter it became evident that the increase of nutrients fueled algal biomass growth and upon death, a portion sank to the sediment and was aerobically degraded by microorganisms (Elmgren, 2001).

1.2 Eutrophication of the Baltic Sea

During the last 60 years, an increase in agriculture and industry has indirectly fed the Baltic Sea with nutrients (Gustafsson et al., 2012). Fertilizers enrich the leached water from agricultural land with nutrients (nitrogen and phosphorous). In addition, industrial and sewage waste going into the sea also contain high levels of nutrients (Conley, 2012). These nutrients increase the growth of algae and cyanobacteria (formerly blue-green algae) in the water, resulting in phytoplankton blooms. This increase is further enhanced by climate warming due to the increased temperature accelerating and prolonging the blooms (Emeis et al., 2000). Larger blooms occur throughout the year in the Baltic Sea with a spring bloom mainly consisting of silica rich algae diatoms and brown algae dinoflagellates whereas, the summer bloom is dominated by cyanobacteria and dinoflagellates (Figure 2) (Håkanson and Bryhn, 2008). The spring bloom occurs in mid-April and May and lasts from one week up to two months (Fleming and Kaitala, 2006) while the summer bloom lasts a few weeks up to two months during July and August (Stal et al., 2003). Common diatoms during the spring bloom are *Chaetoceros wighami*, *Chaetoceros dholsaticus*, *Thalassiosira baltica*, *Thalassiosira levanderi*, and *Skeletonema costatum* (Tamelander and Heiskanen, 2004) with the summer bloom being rich in cyanobacteria *Anabaena* spp., *Nodularia spumigena* and *Aphanizomenon* spp. (Stal et al., 2003).

During and after the blooms decaying phytoplankton sink and are partially degraded in the water column. Eventually a large portion lands on the sediment surface (Peinert et al., 1982; Conley and Johnstone, 1995; Tallberg and Heiskanen, 1998; Emeis et al., 2000; Conley, 2012). Aerobic microbes in the sediment degrade the organic carbon derived from the water column and thereby reduce the available oxygen. Eventually the oxygen concentration drops below the minimum for survival of aerobic benthic organisms (Middelburg and Meysman, 2007). Oxygen concentrations below 2 mg/L are termed hypoxic, areas devoid of oxygen are defined as anoxic, and together

these zones are referred to as ‘dead zones’ (Conley, 2012). Dead zones are widespread in the offshore of the Baltic Sea and have increased from ~40 000 km^2 to ~60,000 km^2 between the years 1961- 2010 (Meier et al., 2011). In conjunction to offshore hypoxia, oxygen deficient coastal areas have also increased substantially during the last 60 years (Conley et al., 2011).

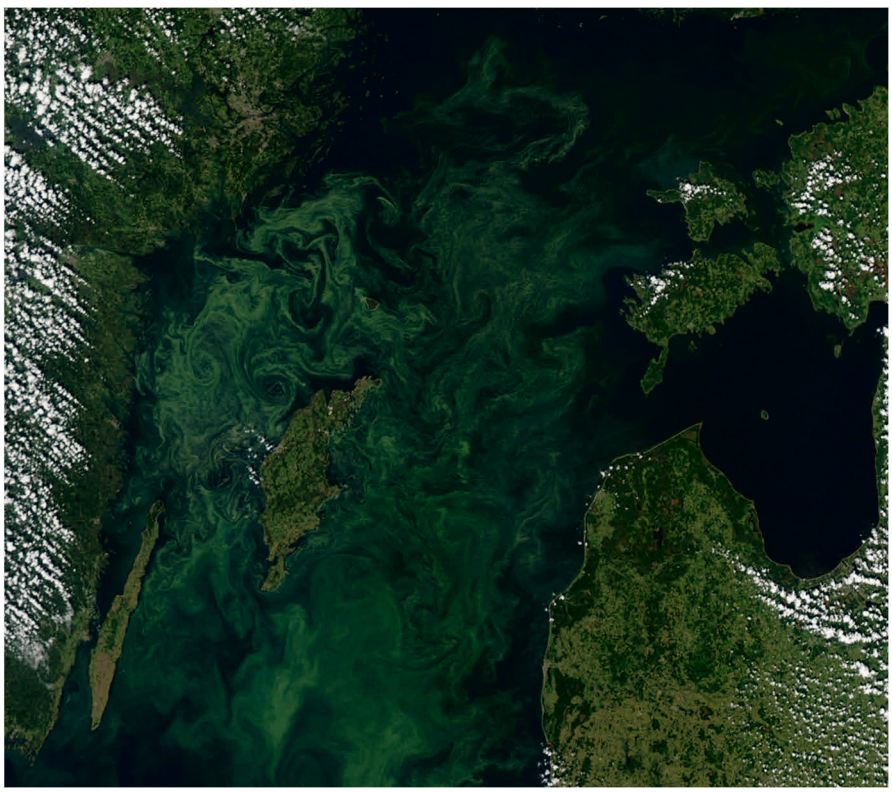

Figure 2 Satellite image of the summer phytoplankton bloom in the Baltic Proper. Credit: Jeff Schmaltz. Visible Earth, NASA.

1.3 Water-sediment exchange

Sinking particles, commonly referred to as ‘marine snow’, are rich in organic carbon and have attached microorganisms (Alldredge and Silver, 1988). Certain organisms have both pelagic and benthic life stages that transfer carbon between the water column and the sediment surface. Some examples of these organisms are different taxa of phytoplankton that have dormant stages (e.g. diatoms) in the sediment (Lampert, 1995; Rengefors et al., 1998; McQuoid et al., 2002; Geelhoed et al., 2009; Orlova and Morozova, 2009) as well as various zooplankton such as rotifers, cladocerans, and copepods

(Gyllstrom and Hansson, 2004). For example, diatoms sinking to the sediment are known to either produce spores or enter a resting stage (Smetacek, 1985) and have been found to survive in both darkness and anoxia (Kamp et al., 2013). A second organism that couples the water column and sediment surface is zooplankton that lays eggs in the water column that sink and, if not hatched in the water column, are buried in the sediment. These eggs can hatch when environmental conditions later become favorable, allowing these organisms to survive harsh periods (De Stasio, 1989). There are different types of eggs such as subitaneous quiescent eggs and diapausing eggs. Compared to diapausing eggs, subitaneous quiescent eggs have a short development pause to withstand limiting factors such as food availability and/or low temperature (Dahms, 1995). Diapausing eggs have an obligatory pause during extreme harsh environmental conditions, e.g. low/high temperature and oxygen deficiency (Brendonck and De Meester, 2003; Gyllstrom and Hansson, 2004). In environments undergoing fluctuations in temperature and oxygen, the organisms increase the hatching frequency by laying many and different types of eggs (Hairston et al., 1996; Brendonck and De Meester, 2003; Gyllstrom and Hansson, 2004). In addition to organic and other material being transferred from the water to the sediment surface, chemical compounds and gases are released from the sediment to the water column by various biotic and abiotic redox processes.

1.4 Sediments and 'dead zones'

In the sediment surface organic matter is degraded (oxidized) in conjunction with reduction of dissolved oxygen into water. However, if no oxygen is present the organic matter is preserved (Koho et al., 2013) due to reduced degradation rates using anaerobic oxidants such as nitrate (NO_3^-) (Kristensen et al., 1995; Sun et al., 2002). Therefore, microaerophilic and anaerobic microorganisms thrive in the oxygen deficient sediment by utilizing alternative terminal electron acceptors. Available acceptors in the sediment yield different amounts of energy and consist of, in order of energy yield: O_2, NO_3^-, manganese (Mn^{3+} and Mn^{4+}) oxides, ferric iron (Fe^{3+}) oxides, and sulfate (SO_4^{2-}). Additionally, below the SO_4^{2-} reduction zone methane (CH_4) is microbially produced (i.e. methanogenesis) by the reduction of carbon dioxide (CO_2) or low molecular weight (lmw) carbon compounds such as acetate (Burdige, 2006). Due to these differences in energy yield, a vertical redox cascade is formed in the sediment surface (Burdige, 2006) that can reach just a few mm below the sediment surface (Burdige, 1993; Jørgensen, 2006; Middelburg and Meysman, 2007). See Table 1 for a description of these redox zones and common microbial processes occurring in them.

The reduction of these anaerobic oxidants result in reduced products (e.g. Mn^{2+}, ferrous iron (Fe^{2+}), and hydrogen sulfide (H_2S^{2-})) which diffuse upwards in the sediment pore-water and eventually reach the benthic water overlying the sediment surface. During diffusion or when reaching the benthic water, the reduced products can be re-oxidized either chemically or in aerobic/anaerobic microbial processes such as denitrification, Fe^{+2} oxidation, and H_2S oxidation (Straub et al., 1996; Burdige, 2006; Takai et al., 2006). See Table 2 for a description of these reductants and common chemical and microbial processes when they become oxidized. Many of the microbial aerobic processes mentioned in Table 2 can be conducted anaerobically using other oxidants than oxygen, for example the reduction of NO_3^- in conjunction with H_2S oxidation (Han and Perner, 2015). Table 3 shows some of the common microbial processes observed in sediments when oxygen is not available.

Due to the differences in energy gain using various electron acceptors, redox zones migrate upwards in the sediment during highly reduced conditions such as anoxia, e.g. turning the sediment surface into an anaerobic microbial SO_4^{2-} reduction zone producing the toxic gas H_2S (Metzger et al., 2014). In addition, Fe^{3+} is microbially reduced in anoxic zones and becomes soluble as Fe^{2+} which in conjunction with H_2S, precipitates as iron sulfides giving 'dead zones' their distinctive black color (Photo 1) (Bagarinao, 1992; Burdige, 2006). As many of the redox processes occurring in the sediment are part of microbial metabolism, the microorganisms have a key role in ecosystem chemical cycling and degradation of organic matter and the generation of 'dead zones'.

Table 1 Simplified microbial processes utilizing terminal electron acceptors (oxidants) available in the sediment. Heterotrophic microorganisms oxidize organic carbon while autotrophic microorganisms convert CO_2 to organic carbon. Due to the different energy yields of the oxidants for microorganisms the most favored oxidants are used in the sediment surface (O_2 if available) and are then depleted downwards in the sediment (Burdige, 2006).

Depth from sediment surface	Terminal electron acceptors	Microbial reduction processes
↓	O_2	Oxygen is reduced to water
	NO_3^-	Denitrification: NO_3^- is reduced to nitrite (NO_2^-), which in turn is reduced to nitrous oxide (N_2O) and eventually nitrogen gas (N_2).
	Mn^{3+} and Mn^{4+} oxides/hydroxides	The manganese in Mn^{4+} oxides (MnO) and Mn^{3+} hydroxides (MnOOH) are reduced to Mn^{2+}
	Fe^{3+} oxides/hydroxides	The iron in Fe^{3+} oxides/hydroxides (FeO/FeOOH) are reduced to Fe^{2+}
	SO_4^{2-}	SO_4^{2-} is reduced to sulfide (typically as H_2S)
	CO_2 or lmw carbon compounds	Methanogenesis: CH_4 is produced from the reduction of CO_2 or lmw carbon compounds

Table 2 Simplification of typical chemical and microbial oxidation processes of reduced compounds (terminal electron donors) coupled to reduction of O_2 to water (Burdige, 2006, unless otherwise stated). Chemical processes are denoted as C, and microbial as M.

Terminal electron donor	Aerobic oxidation of reduced compounds	Chemical or/and Microbial
Organic carbon	Organic carbon is oxidized to CO_2	M
NH_3 or NH_4^+	Nitrification: Ammonia (NH_3) or ammonium (NH_4^+) is oxidized to NO_2^-/NO_3^-	M
Mn^{2+}	Mn^{2+} is oxidized to MnOOH or Mn^{4+} oxides[1]	C, M
Fe^{2+}	Fe^{2+} is oxidized into FeO/FeOOH	C, M
H_2S	The sulfide (S^{2-}) in H_2S is oxidized to SO_4^{2-}	C, M
CH_4	Methanotrophy: CH_4 is oxidized to CO_2	M

[1] (Konhauser, 2009)

Table 3 Some anaerobic microbial processes used to oxidize reductants using other terminal electron acceptors than oxygen. Processes have been simplified with intermediate metabolic steps not shown.

Terminal electron donors	Terminal electron acceptors	Microbial anaerobic processes of reduced compounds
NH_4^+	NO_2^-	ANAMMOX: Anaerobic ammonium oxidation involving the oxidation of NH_4^+ coupled to reduction of NO_2^-, producing N_2-gas[1]
Mn^{2+}	NO_3^-	Mn^{2+} is oxidized with the reduction of nitrate[2]
Fe^{2+}	NO_3^-	Fe^{2+} is oxidized with the reduction of nitrate[3]
H_2S	NO_3^-	The sulfide (S^{2-}) in H_2S is oxidized to SO_4^{2-} with the reduction of nitrate[4]
CH_4	SO_4^{2-} or Fe^{3+}	Anaerobic oxidation of methane (AOM) involves a symbiotic microbial process oxidizing CH_4 coupled to sulfate [5] or Fe^{3+} reduction.[6]

[1] (Devol, 1978), [2] (Burdige, 2006), [3] (Straub et al., 1996), [4] (Han and Perner, 2015), [5] (Knittel and Boetius, 2009), [6] (Beal et al., 2009; Konhauser, 2009)

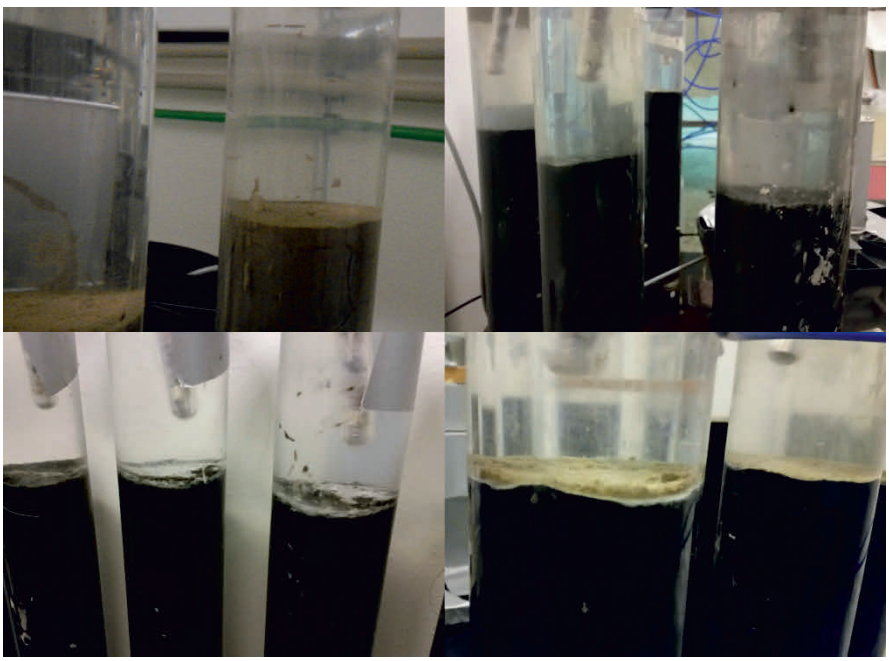

Photo 1 Varying oxygen concentrations in sediment changes its physical characteristics. *Upper left* shows oxygen-rich water and a sediment surface with oxygen penetrating a few mm below the surface. However, due to burrows constructed by macroinvertebrates oxygen is able to reach further down into the sediment. *Upper right* shows anoxic water and sediment in which iron sulfides give the sediment a black color. *Lower left* shows white precipitate (likely solid sulfur) on top of the anoxic sediment surface. *Lower right* shows how oxygen penetration of the anoxic sediment surface causes iron to oxidize and induce a change of color, while causing a white film, likely microbial oxidation of sulfide into solid sulfur, between the oxic and anoxic layer.

1.5 Sediment microbiology

Compared to the number of microbial cells in marine water (10^2 to 10^6 mL^{-1}), sediments harbor a higher cell count and can reach up to 10^{10} mL^{-1} (Solan and Wigham, 2005). It has been estimated that 76% of all bacteria on the planet thrive in marine sediment (Turley, 2000). The microbes residing in the sediment are able to couple both organic carbon and inorganic electron donors to a range of electron acceptors and are therefore key players in nutrient and carbon cycling both in the sediment and the sediment-water interface (Schallenberg and Kalff, 1993). For example, microbes can have a chemoorganotrophic lifestyle oxidizing organic carbon and/or chemolithoautotrophic converting CO_2 to organic carbon (Nealson, 1997; Burdige, 2006). The diversity of many prokaryotes conducting redox processes at various sediment depths has been previously studied (Torsvik et

al., 1996; Parkes et al., 2014). If oxygen is available in the bottom waters and the sediment surface, organotrophic prokaryotes couple organic carbon to the reduction of oxygen (Canfield, 1993). In addition, ammonia/ammonium is also used as an energy source by nitrifying bacteria when oxygen is present, such as the family *Nitrobacteraceae* (Table 2) (Belser, 1979). Another example is photosynthetic bacteria, such as green and purple sulfur bacteria capable of sulfide oxidation, that can thrive in the sediment surface if photosynthetically active radiation light penetrates the water column down to the benthic zone (de Wit and van Gemerden, 1990; Pringault et al., 1998).

In the layer between the oxygen rich sediment surface and the anoxic sediment (i.e. oxic-anoxic interface), specialist microbes oxidize compounds (e.g. sulfide and zero-valent sulfur) that are either produced *in situ* or diffuse upwards from deeper, anoxic zones (Geelhoed et al., 2009). Typical examples of microbes able to oxidize sulfide are the genus *Beggiatoaceae* belonging to *Gammaproteobacteria*, and the *Epsilonproteobacteria* genera *Arcobacter*, *Sulfurimonas*, and *Sulfurovum* (Wasmund et al., 2017), and the so-called cable bacteria (belonging to the *Deltaproteobacteria* family *Desulfobulbaceae*) able to couple sulfide oxidation in the deeper anoxic sediment with oxygen reduction in the oxygenated sediment surface (Nielsen and Risgaard-Petersen, 2015).

In the anoxic sediment below the oxic-anoxic interface, anaerobic fermenting bacteria reduce and oxidize organic carbon that yield low molecular carbon compounds that can be used in other processes such as sulfate reduction and methanogenesis (Burdige, 2006). In conjunction to this, microbes utilize alternative terminal electron acceptors such as NO_3^- to oxidize reduced products (i.e. denitrification, as described in Tables 1 and 3). Some anaerobic microbes are obligate or facultative chemolitoautotrophs and therefore do not need an organic carbon source (Boschker et al., 2014). Ammonium still available in the anoxic sediment can be anaerobically oxidized by anammox bacteria belonging to the phylum *Planctomycetes* (van Niftrik and Jetten, 2012). In addition, manganese and iron reducing bacteria also thrive, for example the *Deltaproteobacteria* family *Geobacteraceae* known to contain iron reducing bacteria (Thamdrup, 2000). Cycling of sulfur compounds and sulfate reduction in the sediment is a prominent feature when oxygen is not available (Burdige, 2006), typically conducted by e.g. anaerobic sulfate reducers such as the *Deltaproteobacteria* family *Desulfobacteraceae* (Wasmund et al., 2017). However, prokaryotes that both reduce and oxidize compounds thrive in the sediment simultaneously, such as anaerobic iron reducers and sulfide oxidizers (e.g. Sørensen, 1982; Han and Perner, 2015). Furthermore, in the anoxic sediment below the sulfate reduction zone a prominent metabolic feature is methanogenesis by anaerobic archaea such as

the taxonomical orders *Methanopyrales* and *Methanococcales* belonging to the phylum *Euryarchaeota*. This process produces methane gas from CO_2 or low molecular weight organic carbon substrates (Thauer et al., 2008). The methane diffuses upwards in the sediment and might eventually be oxidized by aerobic methanotrophic bacteria such as the *Gammaproteobacteria* genus *Methylococcus* (Hanson and Hanson, 1996) or anaerobic methanotrophic archaea belonging to the phylum *Euryarchaeota* (Knittel and Boetius, 2009). Finally, microbes are able to form symbiotic lifestyles such as bacterial sulfate reduction in combination with anaerobic methane oxidation by archaea (Knittel and Boetius, 2009).

Changes in microbial communities in relation to sediment oxygen concentration has been studied worldwide, including in the Baltic Sea (Edlund et al., 2006; Swan et al., 2010; e.g. Divya et al., 2011; Kochling et al., 2011). The importance of bacterial sulfur cycling has been highlighted, for example in a marine seasonal hypoxic coastal basin *Epsilonproteobacteria* typical for sulfide oxidation were abundant during spring when the sediment surface was oxic, while *Deltaproteobacteria* typical for sulfate reduction were favored in anoxic conditions during autumn (Lipsewers et al., 2017). In the Baltic Sea, *Planctomycetes* and *Betaproteobacteria* were found to be more abundant in the sediment when oxygen availability was high (Edlund et al., 2006). Modern molecular studies in the Baltic Sea have mainly been conducted on the water phase with few studies focused on sediment. In the permanent anoxic sediment in the deepest part of the Baltic Sea (Landsort deep, 466 m below water surface) studies looking at microbial community structure and metabolic processes have highlighted active functional traits related to degradation of organic matter, reduction of sulfate, and methanogenesis (Thureborn et al., 2016). The microbial community structure was identified as having a high abundance of sulfate reducing *Desulfobacteraceae*, as well as *Mycobacteriaceae*, *Conexibacteraceae*, *Planctomycetaceae,* and unidentified bacteria belonging to the class *Deltaproteobacteria* (Thureborn et al., 2013).

1.6 Remediation strategies of ‘dead zones’

When it became apparent during the 1970s that eutrophication is a man-made pollution, the countries surrounding the Baltic Sea formed the Helsinki Commission (HELCOM) and signed a convention to protect the Baltic Sea. Since then HELCOM has conducted routine surveys and successfully removed many direct nutrient input sources located on the coast (Voss et al., 2011). Even though a decrease in nutrients is considered to be the most beneficial remediation process (Carstensen et al., 2014a), the slow progress has caused an interest in rapid restoration strategies of the hypoxic zones (Conley et al., 2009). Such strategies consist of 1) biomanipulation by adding predators that

feed on phytoplankton; 2) lowering the growth of phytoplankton by precipitation of phosphorous e.g. with the use of aluminum; and 3) artificial re-oxygenation by pumping oxygen rich surface water down to the bottom (Conley et al., 2009). Biomanipulation has showed promising results by limiting the growth of cyanobacteria in lake studies using large-sized zooplankton (Urrutia-Cordero et al., 2016) and fish as predators (Lu et al., 2006). Trapping phosphorous in the sediment can occur naturally by the export of aluminum from soils (Kopáček et al., 2007) and has also been shown experimentally with addition of aluminum granules in Baltic Sea coastal bays (Rydin, 2014; Rydin et al., 2017). Artificial re-oxygenation has been proposed to significantly lower phosphorous in the water column after a few years of remediation and would carry a cost of ~200 million Euros for 100 pumping stations (Stigebrandt and Gustafsson, 2007). Pilot tests in an anoxic fjord on the west coast of Sweden resulted in a five-fold increased retention of phosphate trapped in the sediment (Stigebrandt et al., 2015a). In conjunction with phosphate retention, the bacterial population in the bottom water showed strong shifts and became more similar to the oxygen-rich surface water population (Forth et al., 2015). Similar findings regarding phosphate were also observed during a natural inflow of oxygen rich deep water in the Baltic Sea (Rosenberg et al., 2016).

1.7 Sequencing technology and new discoveries

The identification of prokaryotes is commonly identified by sequencing their ~1500 bp long 16S rRNA that is a part of the small ribosomal subunit. The 16S rRNA sequence can be derived either from RNA or DNA (i.e. the 16S rRNA gene coding for 16S rRNA) (Petti et al., 2005; Janda and Abbott, 2007). The small ribosomal subunit is utilized during mRNA translation to produce amino acids (Cooper, 2000). Therefore, the 16S rRNA gene is ubiquitous and conserved in prokaryotic species over long time periods (Janda and Abbott, 2007). Common laboratory practice is to extract DNA and to follow up with polymerase chain reaction (PCR) amplification of the whole or a portion of the 16S rRNA gene. The resulting amplicons (i.e. amplified 16S rRNA genes) are sequenced and the data consisting of millions of sequences (commonly referred to as 'reads') are then clustered based on similarity into OTUs (operational taxonomic units). OTUs can then be compared in terms of diversity and counted in terms of relative abundance to determine the percentage of OTUs in the population (Maciel et al., 2009; Sanschagrin and Yergeau, 2014). Today there are well-known databases consisting of sequenced 16S rRNA genes from cultured microorganisms (e.g. SILVA and NCBI GenBank) and these make it possible to taxonomically label OTUs.

With the advancement of sequencing technology whole-genome studies (WGS) have become possible. In contrast to amplicon studies, WGS offers the opportunity to potentially derive sequences from all genes available in the genome of the targeted species (Land et al., 2015). Studies involving sequencing of whole communities (i.e. more than one species in the sample) are termed metagenomic studies. With metagenome data it is still possible to screen the DNA sequences for the 16S rRNA genes and construct a taxonomic overview of the sample (Yuan et al., 2015). However, the real advantage by WGS is that functional (protein coding) genes are sequenced and an overview of the metabolic functions can be estimated in the sample (Chistoserdovai, 2010). The DNA data only shows what functions are available and it is therefore not possible to conclude if these functions are actively used during the time of sampling. Therefore, transcriptomic studies (sequencing of DNA synthesized from extracted RNA) have become a common application (Sorek and Cossart, 2010; Wolf, 2013), especially with the possibility to deplete rRNA (Sorek and Cossart, 2010). With this methodology it is possible to mainly sequence mRNA which encodes for functions being actively used by the microbes at the point of sampling (referred to as 'gene expression'; i.e. a gene transcribed into mRNA which might eventually be translated into amino acids) (Cooper, 2000). Similar to the 16S rRNA gene databases, there are today many well established protein reference databases (e.g. UniProtKB/Swiss-Prot and MetaCyc) that can be utilized to annotate (i.e. identify) sequences derived from genomic or transcriptomic studies.

These modern sequencing technologies have made new discoveries possible, such as the finding of ubiquitous ultra-small bacteria belonging to the Candidate Phyla Radiation (Brown et al., 2015) constituting a substantial portion of the current tree of life (Hug et al., 2016). In relation to sediments, the use of molecular tools have shown differences between the microbial communities in freshwater and marine sediment, with the phylum *Proteobacteria* being able to constitute up to 60% of both communities. Freshwater sediment is rich in the classes *Alpha-* and *Betaproteobacteria* while marine sediment harbors a high abundance of *Delta-* and *Gammaproteobacteria* (Bolhuis and Stal, 2011; Wang et al., 2012). Furthermore, *Epsilonproteobacteria* in the coastal zone are more abundant compared to freshwater and marine sediment (Wang et al., 2012). In the Baltic Sea, metagenomic studies have increased during the last ten years (Ininbergs et al., 2015) and have been used to e.g. identify taxonomy and obtain species specific genomes along a salinity gradient (Hugerth et al., 2015). Regarding oxygenation of coastal 'dead zones', the 16S rRNA gene microbial community structure in the bottom water after re-oxygenation of an anoxic fjord on the west cost of Sweden showed that the initial anoxic community became more similar to those residing in oxic water (Forth et al., 2015).

Aims

This thesis explores the populations of microorganisms and their functions in coastal Baltic Sea 'dead zones' and how these organisms respond and adapt to strong shifts in oxygen concentrations. Furthermore, the impact of oxygenation on buried zooplankton eggs and diatoms in hypoxic/anoxic sediment was also studied. In addition to this, the thesis further builds upon previous artificial re-oxygenation studies by incorporating the role of microorganisms and modern molecular tools. Certain chemical compounds in the sediment utilized and produced by microbes are toxic or strong greenhouse gases (e.g. H_2S and CH_4, respectively). It is therefore essential to know if microbes facilitate or hamper the outcomes of a costly restoration process such as artificial re-oxygenation.

The overall aim of the thesis was to investigate the ecology and evolution of coastal Baltic Sea 'dead zone' sediments. In more detail the aims were:

1) With the use of modern molecular tools, to investigate the microbial community structure, their metabolic functions, and chemistry fluxes in relation to shifting oxygen concentrations in oxic and anoxic sediment (**Papers I** & **IV**).

2) To investigate the impact of oxygenation on buried zooplankton eggs and diatoms in hypoxic/anoxic sediment (**Papers II** & **V**, respectively).

3) To explore the influence of phytoplankton biomass on chemistry fluxes and the microbial community structure in oxic sediment (**Paper III**).

Methods

3.1 Field sampling

Sediment was collected in a coastal Baltic Sea bay close to the locality of Loftahammar, Sweden. Three sites were chosen in the bay, 1) a 'deep site' with a water depth of 31 m, 2) an 'intermediate' site at 21 m, 3), and a 'shallow site' with a depth of 6.5 m (Figure 3). In addition to their different depths the sites had varying oxygen concentrations. The 'deep site' had a hypoxic/near-hypoxic bottom zone with anoxic black sediment, the 'intermediate site' had a hypoxic bottom zone with black anoxic sediment, and the 'shallow site' was oxic with brown sediment. Sampling of sediment was conducted several times and an overview of the sampling occasions and experiments is given in Table 4.

Figure 3 Sampling was conducted in a Baltic Sea bay (encircled) nearby the municipality of Loftahammar. The sampling sites in the bay were: **1)** a deep site with a water depth of 31 m; **2)** an intermediate site, 21 m; and **3)** a shallow site, 6.5 m. Map data is available under the Open Database License, © OpenStreetMap contributors.

Sediment was collected with the use of a Kajak-type gravity corer and accompanying tubes (60 cm polymethylmethacrylate tubes). Upon retrieval of the cores in the field they were closed at the bottom and top until incubation back in the laboratory (27 to 41 cm sediment height). In addition to collecting

sediment cores for incubation, some cores were sacrificed (i.e. sliced) in the field and the overlying benthic water and sediment were collected (Photo 2).

Table 4 Overview of the sampling campaigns, measured dissolved O_2 concentrations in the benthic water overlying the sediment surface (WTW Multiline sensors), and experiments conducted.

Date	Site	O_2 mg/L	Paper/ Experiment
Nov. 13 2013	Deep Intermediate Shallow	0.85 0.80 11.3	**I**, the effect of oxygen shifts on the sediment surface chemistry, microbial community structure, and metabolic processes (21 days study)
May 14 2014	Deep	0.20	**II**, the impact of oxygenation on buried dormant zooplankton eggs
Oct. 9 2014	Shallow	10.1	**III**, changes in the microbial community structure due to addition of cyanobacterial biomass
April 16 2015	Shallow	10.6	**III**, changes in the microbial community structure due to addition of diatomic biomass
April 15 2016	Deep	3.83	**IV**, the effect of oxygen shifts on the sediment surface chemistry, microbial community structure and metabolism (5, 9, and 15 days study)
March 22 2017	Deep	11.7	**V**, the impact of oxygenation on buried diatoms (also based on eukaryotic RNA-seq data from **Paper I** & **IV** incubations)

The overlying water was sampled and divided into tubes for further chemical measurements and DNA extraction. Afterwards, the sediment in the core was sliced at different depths and divided into tubes for further analyses (chemistry plus DNA and RNA extraction). Collected water/sediment chemistry and DNA samples were kept in a cool box filled with ice packs. Sediment RNA samples were fixed with a mix consisting of 5% water-saturated phenol in absolute ethanol (Feike et al., 2012) and then flash frozen and stored in liquid nitrogen. Once back at the laboratory, chemistry samples were stored at -20°C and water samples for DNA were filtered through a 0.2 µm filter (PALL Corporation) and the filters and sediment RNA samples were stored at -80°C.

Photo 2 The sampling campaigns generally included sampling of sediment cores and collecting the cores for incubation back in the laboratory (upper left and right). Additionally, some cores were sacrificed in the field by slicing various sediment depths (lower left) and collecting the sediment in different tubes for further chemistry and molecular determinations (lower right). Photos are used with permission and were taken by the Linnaeus University Coastal Group.

3.2 Incubation setup

Sediment cores were incubated in darkness for 15 to 26 days with intact sediment and the overlying benthic water (Photo 3). The water phase inside the cores was gently mixed with the use of submersed magnets (inside a tube) that were swirled around by the use of rotating external magnets. Incubations were conducted at 8°C (**Papers I**, **II, IV**, and **V**) except in 2014 and 2015 for the oxic 'shallow site' which was conducted at *in situ* field temperatures (11-13°C; **Paper III**). Sediment cores for incubation were divided and the water phase was either continuously bubbled with air throughout the experiments or sporadically bubbled with N_2-gas at the start, after sub-sampling, and at end of the incubation experiments. Using this methodology it was possible to study the effect of variations in oxygen by turning cores oxic/anoxic and controls maintained oxic/anoxic (**Papers I**, **II**, **IV**, and **V**). Additionally, some cores in the phytoplankton experiment (**Paper III**) were incubated without bubbling of air to study the bubbling effect on the sediment.

Photo 3 Several incubations with different aims were setup, they were *upper left*: **Paper I**, the effect of oxygen shifts on the sediment surface chemistry, microbial community structure, and microbial metabolic processes (21 days study); *upper right*: **Paper II**, the impact of oxygenation on buried dormant zooplankton eggs; *lower left*: **Paper III**, changes in the microbial community structure due to addition of phytoplankton biomass; and *lower right*: **Paper IV**, the effect of early stage oxygen shifts on the sediment surface chemistry, microbial community structure, and metabolism (5, 9, and 15 days study). In addition to these experiments, **Paper V** was based on eukaryotic sequence data collected from **Papers I** & **IV**.

3.3 Preparation of phytoplankton biomass

For the phytoplankton experiment, cyanobacteria and diatoms were grown in laboratory cultures during October 2014 and April 2015, respectively. The cyanobacteria consisted of six strains from the Kalmar Algal Collection (KAC): one *Anabaena* (KAC 16), two *Aphanizomenon* (KAC 15 and KAC 69), and three *Nodularia* species (KAC 11, KAC 13, and KAC 71) that were grown with silica free 7‰ saline f/2 medium (Guillard and Ryther, 1962). Cultures were kept at 20°C for 31 days with a diurnal cycle of 16 hours light. The diatoms consisted of five strains from the Finnish Environment Institute SYKE: *Chaetoceros wighami* (CWTW C1), *Thalassiosira baltica*, *Skeletonema marinoi* (SMTV 1), *Melosira artica* (MATV 1), and *Diatoma tenuis* (DTTV B5) that were grown with 7‰ saline f/2 medium at 16°C for 80

days with a diurnal cycle of 18 hours light. After culturing the strains they were mixed to form a cyanobacteria community, and a diatom community. Chlorophyll a was measured according to Jespersen and Christoffersen (1987) in conjunction with microscopy counts to determine the percentage of representation for each strain in the mixed cyanobacteria community. For the diatom community the percentage representation of each strain was determined by microscopy counts. At the start of the incubation experiment (**Paper III**), phytoplankton biomass was added into the water phase of the sediment cores to let the biomass sink to the sediment surface. The cyanobacteria community was added wet and the carbon weight was determined by drying and literature carbon factors (found in Goel et al., 2014), while diatoms were added dry and the carbon weight was determined by CN analysis (PerkinElmer, Series II Analyser 2400).

3.4 Chemistry measurements

The water phase of the incubated cores was sub-sampled for chemistry measurements in the field and at the end of each incubation experiment (~16 mL). The water phase was also sporadically sub-sampled throughout the experiment (typically every 4^{th} to 5^{th} day) to follow chemistry fluxes. Sediment pore-water chemistry was measured in the top 1 cm sediment surface sliced in the field, during incubations (**Papers III** to **V**), and after each experiment. Measured chemistry parameters included SO_4^{2-}, PO_4^{3-}, Fe^{2+}, Fe^{3+}, NO_2^- in combination with NO_3^-, NH_4^+, pH, redox potential, organic matter (% wt), tetrathionate, thiosulfate, and dissolved oxygen. Water samples were filtered through a 0.7 µm glass-fiber filter (Whatman GF/F or 30-SF-07 GMF syringe filter, Chromacol) prior to measurements, while sediment slices were centrifuged (2200 g for 15 min) and the supernatant (i.e. pore-water) was filtered through a similar 0.7 µm glass-fiber filter.

PO_4^{3-} and NO_2^-+NO_3^- were measured spectrophotometrically using the molybdenum blue and naphthylethylenediamine methods according to Valderrama (1995), respectively. The Hach-Lange LCK 304 and 353 kits were used to measure NH_4^+ and SO_4^{2-}, respectively (chemistry was measured on two DR 5000 Hach-Lange spectrophotometers). Iron was also measured spectrophotometrically using a SmartSpec 3000 Bio-Rad spectrophotometer following the ferrozine protocol (Dawson and Lyle, 1990). Tetrathionate and thiosulfate were measured by cyanolysis (Sörbo, 1957) according to Kelly et al. (1969). In **Paper I** both Fe^{2+} and Fe^{3+} were determined while in **Papers II** to **IV** a combination of both were reported (Fe_{tot}). pH and redox potential were measured using commercial electrodes (pHenomenal, VWR pH electrode; and Ag/AgCl SI Analytics electrode, Mettler Toledo; respectively). Sediment wet and dry weights (drying at 80°C for three days; dw) were measured. Organic

matter content (% wt) in the sediment was determined by loss on ignition of dry pre-weighed sediment at 550°C for 30 minutes in a muffle furnace (OWF 1200, Carbolite). Finally, oxygen in the water phase was measured in **Papers I, II,** & **V** using an oxygen electrode (Innovative Instruments). For **Papers III** & **IV** (and part of the **Paper V** data) oxygen was measured in the water phase and the sediment surface with the combination of a motorized micromanipulator and an optical oxygen meter (Micromanipulator MU1 and FireStingO_2 OXR50 oxygen sensor, respectively; PyroScience). Statistical testing of chemical and biological data, over incubation time and between treatments, was conducted with e.g. Pearson's correlations, one-way ANOVA, and Kruskal-Wallis tests.

3.5 Counting of zooplankton and diatoms

Throughout the incubation experiment for **Paper II** approximately two thirds (250 to 300 mL) of the water phase in the sediment cores was sporadically sub-sampled. The goal was to see if newly hatched zooplankton had emerged upon oxygenating anoxic sediment (i.e. from the 'deep site'). The sub-sampled water was filtered through a 15 µm nylon net and trapped zooplankton were transferred into 0.2 µm filtered sea water (polypropylene filter cartridge, Roki Techno). The zooplankton-free water that passed through the filter was returned to the sediment cores. The caught zooplankton were divided into two parts: one part was preserved in Lugol's solution to be quantified with the use of microscopy while the second part was cultivated in 0.2 µm filtered seawater (fed algae *Rhodomonas salina*). Later when the cultivated copepod nauplii reached adulthood they were identified. Additionally, sediments were sliced in the field at varying depths (0-3, 3-6, and 6-9 cm) and slices were incubated separately for 26 days in filtered seawater which was oxygenated by bubbling air. The water phase had *Rhodomonas salina* added to feed potential newly hatched copepod nauplii. After 26 days the water phase was filtered and preserved in Lugol's solution and the copepods were later identified and quantified. With this methodology it was possible to determine at which sediment depths copepod nauplii were able to hatch. For **Paper V** the sediment surface was sampled from the 'deep site' during 2017 and a sub-sample was preserved in Lugol's solution and diatoms were counted and identified using microscopy.

3.6 Determination of microbial communities and metabolic responses

From the sediment cores the water phase (50 mL) was sampled for DNA extraction in the field, plus at the start, during sub-sampling, and at the end of the incubations. Sampled water was filtered through a 0.2 µm filter and stored in -80°C until DNA extraction. A portion of the sediment sliced, when cores were sacrificed, and at the end of incubations was transferred into tubes and stored in -80°C until nucleic acids extraction. Frozen water filters and sediment were extracted with the PowerWater and PowerSoil DNA kits (MO BIO Laboratories), respectively. In **Paper IV**, additional replicate sediment and water samples were treated with propidium monoazide (PMA) which inhibits amplification of non-viable cells during PCR (Nocker et al., 2006). Library preparation for next generation sequencing of the 16S rRNA gene from extracted water and sediment DNA was conducted according to Lindh et al. (2015). Briefly, the V3-V4 region of the 16S rRNA gene (position 341-805) was amplified with primers 341F and 805R (Herlemann et al., 2011) according to PCR programs by Hugerth et al. (2014). Amplicons were multiplexed with the use of N5xx and N7xx Nextera indexes (Illumina). The final pooled libraries from each experiment was sequenced at SciLife Laboratories (Stockholm, Sweden) on the Illumina MiSeq platform with a 2 × 201 (half of the 16S rRNA gene samples in **Paper I**) or 2 × 301 bp pair-end setup (**Papers I** to **IV**). Besides 16S rRNA gene amplicons, metagenomic DNA was also sequenced for **Paper I** using the same sequencing technology.

RNA was extracted from sediment with the use of the PowerSoil RNA kit (MO BIO Laboratories) and DNA was removed from extracted RNA with the Turbo DNA-free kit (Ambion). Purified extracted RNA was then followed by rRNA depletion using the Ribominus Transcriptome Isolation Kit (Bacteria version; Invitrogen life technologies). In **Papers I** & **V** (2013 data), cDNA was synthesized from RNA at the sequencing facility while for **Papers IV** & **V** (2016 data) it was synthesized and purified in the laboratory using the kits Ovation RNA-Seq System V2 kit (NuGEN) and MinElute Reaction Cleanup kit (QIAGEN), respectively. RNA samples were sequenced at SciLife Laboratories (Stockholm, Sweden) on the Illumina HiSeq platform with a 1 × 126 bp pair-end setup.

Sequences were de-multiplexed and converted to .fastq files at the sequencing facility. 16S rRNA gene DNA sequences were quality trimmed, clustered for OTUs, and mapped for abundance estimation using the UPARSE pipeline (Edgar, 2013). OTUs were taxonomically identified by annotating the sequences against the SILVA database (Quast et al., 2013) and the final OTU taxonomy and abundance table was analyzed with the software Explicet

(Robertson et al., 2013). Rarefaction curves of OTUs and read counts were constructed with use of the package 'vegan' in R. Alpha diversity was calculated with Shannon's H index on OTU counts sub-sampled to the lowest sample size. To compare habitat specialization among sampling sites Levins' niche width (B) index was calculated according to Pandit et al. (2009). Correlation networks were construed in sparCC version 2016-10-17 (Friedman and Alm, 2012) and visualized in Cytoscape 3.5.1 (Shannon et al., 2003). **Paper I** also included metagenomic DNA sequences which were screened for Illumina universal adapters and removed with cutadapt 1.8.0 (Martin, 2011) and then quality trimmed using sickle 1.210. To analyze the taxonomy in the metagenomes, 16S and 18S rRNA gene sequences were extracted from the reads using SortMeRNA 2.1 (Kopylova et al., 2012) and the SILVA SSU REF database. The sequences were then clustered for OTUs, mapped, and analyzed in the same manner as the 16S rRNA gene amplicon data. To analyze the functional genes, the reads were assembled with Ray 2.31 (Boisvert et al., 2010) and annotated against the UniprotKB/Swiss-Prot database following the PROKKA pipeline (Seemann, 2014). Abundance estimation of functional genes was conducted by mapping reads onto the assembly using Picard tools 1.77 (Broad Institute) in conjunction with samtools 1.1 (Li et al., 2009) and Bowtie2 2.2.3 (Langmead et al., 2009). Finally, counts per functional gene was calculated with the use of BEDTools 2.23 (Quinlan and Hall, 2010).

Removal of Illumina universal adapters and quality trimming of RNA reads for **Papers I**, **IV,** & **V** were conducted with SeqPrep and Trimmomatic 0.32 (Bolger et al., 2014), respectively. RNA sequences were then *de novo* assembled for **Papers I** & **IV** using Trinity version 2014-07-17 (Haas et al., 2013). The assembly was then further analyzed according to the Trinotate pipeline. Briefly, the assembly was annotated for enzymes and other proteins against the UniProtKB/Swiss-Prot and PFAM databases (Punta et al., 2012). Abundance estimation of RNA transcripts were conducted by mapping reads back onto the *de novo* assembly using RSEM (Li and Dewey, 2011) in conjunction with samtools 1.1 and Bowtie2 2.2.3. Read counts were normalized among samples as Trimmed Mean of M (TMM) Fragments per Kilobase Million (FPKM) values. UniProtKB IDs linked to a taxonomic affiliation (i.e. isolated reference organism) derived from the annotation were then used to infer the active microbial community composition. The R package edgeR (Robinson et al., 2010) was used on the raw read counts (after adapter and quality trimming) to calculate differential gene expression and a statistically significant cluster of expressed RNA transcripts were extracted from the TMM FPKM dataset. UniProtKB/Swiss-Prot ID tags for significantly expressed (**Paper I**) or abundant (**Paper IV**) RNA transcripts were linked to

the KEGG Orthology (KO), GO Biological processes, and MetaCyc databases to infer functional categories.

For **Paper V** quality trimmed RNA reads collected from the 2013 and 2016 sampling in the 'deep site' were annotated against the NCBI nr database (with an e-value <0.001, and 97% identity threshold), using BLASTX 2.6.0+ (Altschul et al., 1990) in conjunction with the Diamond 0.7.9 aligner (Buchfink et al., 2015). The output files were imported into MEGAN 6 (Huson and Mitra, 2012) with InterPro protein and NCBI taxonomy database files. RNA reads affiliated with eukaryotes and diatoms were extracted and analyzed further. To identify statistically significant ($p < 0.05$) changes in the RNA data upon oxygenation edgeR analysis was conducted on the read counts from the extracted diatom cluster.

Results and discussion

4.3 Paper I: Oxygen shifts in the sediment surface

The first study in this thesis investigated changes in chemical fluxes, the microbial community structure, and their metabolic responses due to shifting oxygen levels in long-term anoxic and oxic coastal sediments. In addition, sediment from an area exposed to fluctuating oxygen concentrations during the year was also studied. To achieve this, sediment cores were sampled in the long-term anoxic 'deep site', long-term oxic 'shallow site', and the 'intermediate site' (Figure 3 in methods). The benthic water overlying the sediment surface from three cores for each site were sampled for chemistry and DNA extraction (16S rRNA gene amplification). The top 1 cm sediment surface was then sliced directly on the boat (zero time-points) for pore-water chemistry measurements and DNA/RNA extractions. A total of 15 cores were transported to the laboratory for incubation (two to three cores per site; Table 5). The collected sediment cores were incubated in darkness for 21 days at 8°C and either turned/maintained oxic (~10 mg/L dissolved O_2) by bubbling the overlying water phase with air (hereafter referred to as oxic control, anoxic-to-oxic, and intermediate-to-oxic) or turned/maintained anoxic with the use of N_2-gas (hereafter referred to as anoxic control, oxic-to-anoxic, and intermediate-to-anoxic).

Table 5 An overview of sediment cores sampled from the three different sites in the bay and their initial environmental conditions. Sediment cores were incubated for 21 days at 8°C and either turned or maintained oxic/anoxic.

	Shallow site (long-term oxic)	**Intermediate site** (fluctuating O_2)	**Deep site** (long-term anoxic)
Turned oxic	Control × 2	3	3
Turned anoxic	2	3	Control × 2

Upon oxygenation of anoxic sediment a ~0.5 cm light brown layer developed in the sediment surface which indicated that the ferrous iron was oxidized (likely derived from iron sulfides). PO_4^{3-} increased in the water phase of all cores maintained anoxic and decreased in sediment cores turned oxic while NO^{2-}+NO^{3-} decreased in all cores except the oxic control, likely due to nitrification (**Paper I - Figure 1**). Sediment pore-water PO_4^{3-} measurements showed a high variability in cores turned anoxic which was also observed in **Papers II** and **IV**. This can be explained by H_2S interference with the PO_4^{3-} analysis (Valderrama, 1995). However, there was still a trend that PO_4^{3-} decreased in anoxic-to-oxic and intermediate-to-oxic sediments while increasing in oxic-to-anoxic. Precipitated oxidized iron bound to PO_4^{3-} and reduced iron likely played a key role in regulating the PO_4^{3-} concentrations in the sediment surface. A large decrease in NO_2^-+NO_3^- concentration was observed in the oxic-to-anoxic sediment potentially because of denitrification while SO_4^{2-} was higher in the oxic control compared to the anoxic control. This increase in SO_4^{2-} was also seen in the anoxic-to-oxic sediment pore-water likely due to sulfide oxidation (**Paper I - Figure 1**). Measurement of tetrathionate remained low throughout the experiment with no significant difference among treatments and thiosulfate was only detected in the pore-water from sediment sliced in the field at the long-term anoxic 'deep site'.

The anoxic-to-oxic sediment surface organic matter (OM) content decreased significantly from 18 ± 0.5% in the field to 15 ± 1.4% wt (one-way ANOVA, $F = 28.41$, $p < 0.01$, $n = 3$, SD = 1). The anoxic-to-oxic sediment was also significantly different from the anoxic control that had 19.0 ± 0.3% OM (**Paper I - Table 1**). Surprisingly, samples from the 'intermediate site' showed no such patterns.

Explanations for these findings could be that the OM being preserved in low oxygen zones (Koho et al., 2013), that OM degradation is slower under anoxic conditions (Kristensen et al., 1995; Sun et al., 2002), and that old buried labile OM is rapidly oxidized upon re-oxygenation of anoxic sediment (Hulthe et al., 1998). Thus, it was suggested that OM in the initially anoxic sediment was more labile and that OM in the intermediate sediment was degraded *in situ* during episodic oxygen events.

Principal component analysis showed that OTUs in the initially oxic sediment were tightly clustered, while the initially anoxic and intermediate sediment was more dispersed after oxygenation. In more detail, alpha diversity (Shannon's H index) showed that diversity was significantly higher ($p < 0.01$) in the oxic samples (9.56 ± 0.11, $n = 7$) compared to intermediate (8.97 ± 0.28, $n = 8$) and anoxic (8.75 ± 0.32, $n = 7$). Furthermore, Levins' niche width (B) index was highest (and statistically significant) in the intermediate samples

and indicated that the microbial community in the intermediate sediment consisted of habitat generalists adapted to fluctuations in oxygen ($p < 0.05$, intermediate, $B = 8.11 \pm 0.27$; oxic 7.67 ± 0.28; and anoxic 7.88 ± 0.31). These results suggest that historical sediment oxygen conditions *in situ* affect the microbial community structure upon oxygen shifts.

Looking closer at the 16S rRNA gene OTUs, a wide variety of taxa were affiliated with *Bacteroidetes*, *Delta-*, *Epsilon-*, and *Gammaproteobacteria* which were dominant classes in the sediment (**Paper I - Figure 3**). Surprisingly, only 256 OTUs (of 5952) changed more than 0.1% in relative abundance between the start and end of the incubation. These changes represented 47-61% in the initially anoxic samples; 52-57% for initially intermediate; and 22-25% in the initially oxic sediment. These changes primarily belonged to the *Epsilonproteobacteria* genera *Sulfurimonas* and *Sulfurovum* along with other OTUs related to *Desulfobacula*, *Spirochaeta*, and unclassified OTUs. The genus *Sulfurimonas* consists of well-known sulfide and zero-valent sulfur oxidizing bacteria utilizing both O_2 and NO_3^- as a terminal electron acceptor (Han and Perner, 2015). *Sulfurovum* is another genus known to contain genes used in oxidation of sulfide to sulfur (Park et al., 2012; Pjevac et al., 2014) and is also able to utilize O_2 or NO^{3-} as a terminal electron acceptor (Inagaki et al., 2004).

In the oxic sediments (field, control, and oxic-to-anoxic) dominant OTUs had ≤ a two-fold change when comparing zero time-points and samples from the end of the incubation. Dominant populations in the oxic sediment were affiliated with *Desulfobulbus*, *Nitrosomonadaceae*, *Anaerolineaceae*, and *Gemmatimonadaceae*. In the initially anoxic sediment a large increase in relative abundance of *Sulfurimonas*-like OTUs was seen in the anoxic control at the end of the incubations (from 4.5% to 18.4%) and anoxic-to-oxic sediment (from 4.5% to 13.8%). In contrast, the anoxic control did not show a noteworthy change in *Sulfurovum*-like OTUs while the anoxic-to-oxic increased strongly from 0.2% to 19.0% (**Paper I - Figure 3**). *Sulfurimonas*-like species also increased in the intermediate-to-oxic and intermediate-to-anoxic sediment, from <1% to 14.4% and 8.4%, respectively. In contrast to the anoxic site, *Sulfurovum*-like OTUs only slightly increased from <1% to 4.8% in the intermediate-to-oxic and to 2.2% in the intermediate-to-anoxic sediment. *Sulfurimonas*-like OTUs in the sediment were also found to have a weak negative correlation with $NO_2^-+NO_3^-$ ($p < 0.05$, $r = -0.28$).

Extracted 16S rRNA gene sequences from the metagenomes showed similarities to the 16S amplicon microbial community. However, the relative abundance of the genera *Sulfurimonas* and *Sulfurovum* were lower but showed the same pattern (i.e. increased upon oxygenation). The metatranscriptomic

data revealed that archaea were substantially more represented in the active microbial community compared to the 16S amplicons and metagenome data with a representation up to 26% of the annotated data (proportion of FPKM); **Paper I - Figure 3**). An explanation could be due to the primers favoring the bacterial 16S rRNA gene over archaea (Hugerth et al., 2014). The genera *Sulfurmonas* and *Sulfurovum* were not abundant in the metatranscriptome data at the end of the incubation experiment (less than 1.4%) and it was suggested that sulfur cycling and growth of these genera occurred before RNA was extracted after 21 days of incubation (as also indicated by the 16S amplicon data; **Paper I - Figure 3**).

In the water phase overlying the sediment dominant microbial communities consisted of *Actinobacteria*, *Alpha-*, *Beta-*, *Epsilon-*, *Gammaproteobacteria*, *Bacteroidetes*, and unclassified OTUs (**Paper I - Figure 3**). Compared to the sediment the water phase consisted of significantly higher relative abundance of *Alphaproteobacteria* and less *Deltaproteobacteria* ($p < 0.01$). In the oxic-to-anoxic and intermediate-to-anoxic water *Actinobacteria* decreased from 22.5% to 3.9% and 13.0% to 4.0%, respectively. In contrast, *Sulfurimonas* and *Sulfurovum* increased in the intermediate-to-anoxic water while only *Sulfurovum*-like OTUs increased in the intermediate-to-anoxic water. The *Sulfurimonas*-like OTUs also decreased in the intermediate-to-oxic. In addition, *Sulfurimonas*-like OTUs were observed to increase strongly from 0.4% to 84.2% in the anoxic control water phase. Similar to the sediment data, *Sulfurimonas*-like OTUs in the water phase was also found to have a weak negative correlation with $NO_2^- + NO_3^-$ ($p < 0.05$, $r = -0.55$).

The metagenomic assemblies yielded on average 50,570 coding DNA sequences of which 6874 were found in the UniprotKB/Swiss-Prot database, resulting in 14% known and 86% hypothetical genes. Genes for methane oxidation were present in all samples. Nitrogen cycling (e.g. nitrification, dissimilatory/assimilatory nitrate reduction, and nitrogen fixation) plus sulfur cycling (e.g. dissimilatory/assimilatory sulfate reduction and thiosulfate oxidation) were present in many of the samples. A total of 204,997 RNA transcripts were *de novo* assembled from the RNA data of which 57,386 could be annotated against the UniProtKB/Swiss-Prot database. The distribution of genes varied between the different sites and treatments, with the oxic control and oxic-to-anoxic: 4938 ± 0 and 6272 ± 176 genes, respectively; the intermediate-to-oxic and intermediate-to-anoxic sediments: 7771 ± 375 and 7643 ± 303 genes, respectively; and finally anoxic control and anoxic-to-oxic sediments: 7859 ± 414 and 8274 ± 319 genes, respectively. RNA transcripts from the significant cluster of genes along with the edgeR analysis between treatments (i.e. control against treatment) were used for further interpretation.

RNA transcripts related to inorganic sulfur transformations contained several of the most abundant known genes (**Paper I - Figure 4**). This was unsurprising considering that the Baltic Sea sulfate-reducing bacteria are prevalent, including in anoxic sediment rich in organic matter (Sinkko et al., 2013). The end product of sulfate reduction is sulfide that can be oxidized back to sulfate in the presence of oxygen (Geelhoed et al., 2009). The findings indicated that oxygenation of anoxic sediment would induce sulfide oxidation by *in situ* microbes. In support of this, genes annotated as involved in sulfide to sulfur oxidation and eventually to sulfate were higher in the anoxic-to-oxic and intermediate-to-oxic sediment surface and included sulfide:quinone oxidoreductase (*sqr*), sulfide dehydrogenase (*fccB*), and dissimilatory sulfite reductase (*dsrA*) which can be used in reverse to oxidize sulfite (Harada et al., 2009). Additionally, heterodisulfide reductase (*hdrA*) was present in all cores for all treatments which is used in both methanogenesis (Hedderich et al., 1990) and zero-valent sulfur oxidation (Quatrini et al., 2009). Finally, RNA transcripts for sulfate adenylyltransferase (*sat*) were also present in all cores. This gene has been suggested to be used in reverse to oxidize sulfite to sulfate (Dopson and Johnson, 2012). In the UniprotKB/Swiss-Prot database these RNA transcripts were affiliated with *Acidothiobacillus*/*Aquifex* (*sqr*), *Betaproteobacteria* (*sat*), and *Gammaproteobacteria* (*fccB* and *dsrA*). In more detail, the sulfide oxidizer *Allochromatium vinosum* belonging to the family *Chromatiaceae* was ascribed to *fccB* and *dsrA* (with the family present in the 16S rRNA gene dataset) (**Paper I - Figure 4**).

Furthermore, RNA transcripts transcribing sulfite reductase (*dsvA*) used in anaerobic dissimilatory sulfate reduction were highest in the anoxic control sediment compared to all other treatments and ascribed to the genus *Desulfovibrio* (also present in the 16S rRNA gene dataset; **Paper I - Figure 4**). RNA transcripts encoding anaerobic reduction of tetrathionate (*ttrB*) were higher in the anoxic-to-oxic and intermediate-to-oxic sediment compared to the anoxic control and intermediate-to-anoxic. A cycle of dimethyl sulfoxide (DMSO) to dimethyl sulfide (DMS) and vice versa was suggested with anaerobic DMSO reductase (*dmsA*) and dimethyl sulfide dehydrogenase (*ddhAB*) present in the anoxic-to-oxic and intermediate-to-oxic sediment (likely below the ~0.5 cm oxic layer). Considering that both genes for reduction and oxidation of sulfur compounds occurred in the sediment, with low levels of measurable tetrathionate and thiosulfate, a cryptic cycle of sulfur was suggested. This cycling of sulfur has been reported in oxygen minimum zones that were also found to contain genes for both reduction and oxidation processes of sulfur compounds (Canfield et al., 2010).

Alongside RNA transcripts for sulfur cycling, methane cycling and nitrate reduction were significantly present in the dataset. Methanogenesis was

highest in the anoxic control and intermediate-to-anoxic sediment compared to oxygenated sediment (**Paper I - Figure 4**). In contrast, FPKM values for methane monooxygenase genes (*pmoA1A2* and *pmoB1B2*) used in methane oxidation were higher in the anoxic-to-oxic sediment compared to the anoxic control, and ascribed to *Gammaproteobacteria* species *Methylococcus capsulatus* in the UniProtKB/Swiss-Prot database (also present in the 16S rRNA gene data). This species has previously been observed to be present in the upper layers of marine sediment (Havelsrud et al., 2011) where methane oxidation mainly occurs (Hanson and Hanson, 1996). Methane oxidation was also high in the oxic control sediment. Surprisingly, methane oxidation was not notably observed in the intermediate sediment (**Paper I - Figure 4**), potentially due to oxidation during previous episodic oxygenation events. RNA transcripts encoding nitrate reductase (*narH*) used in nitrate reduction were highest in the oxic-to-anoxic sediment when compared to all other treatments, indicating enhanced denitrification due to oxygen deficiency in the sediment surface. Finally, RNA transcripts for the general stress response were present in all treatments and included e.g. heat shock proteins belonging to the HSP70 and HSP90 family (*hspA* and *hspC*, respectively) and chaperones *dnaK* and *htpG* (**Paper I - Figure 4**).

A conceptual model of a coastal bay was constructed using all major findings from **Paper I** (Figure 4), and it was estimated that it would take 73 days for the OM in the long-term anoxic field site to reach the same level as the oxic site (considering no addition of OM from the water column).

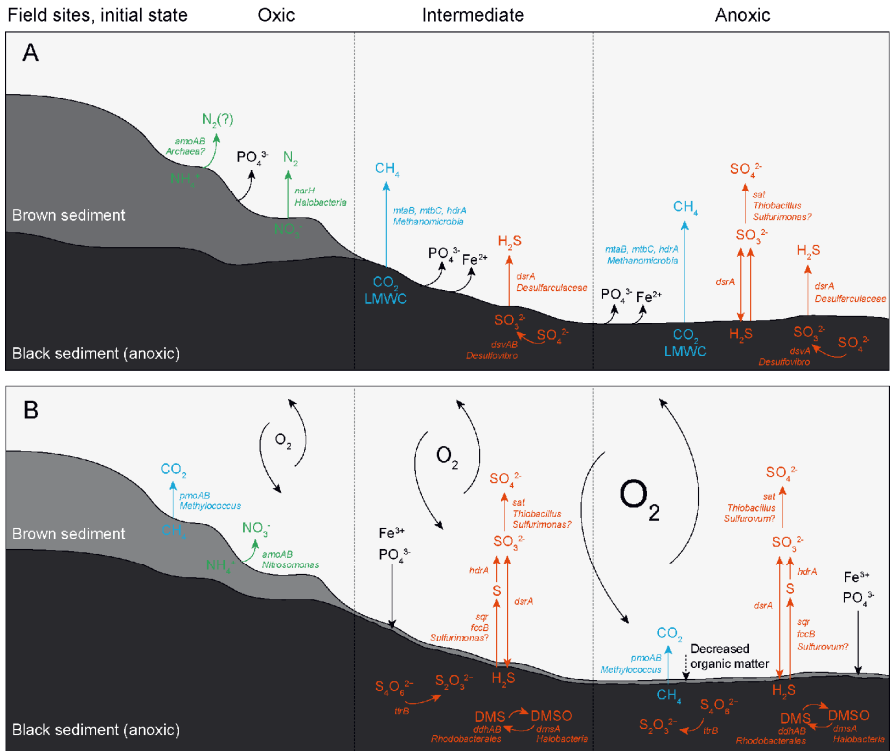

Figure 4 The major changes/findings from **Paper I** were used to construct a conceptual model of a coastal bay. The sulfur cycle is shown in orange, methane as light blue, and nitrogen as green. A) denotes initial conditions turned/maintained anoxic while B) denotes initial conditions turned/maintained oxic. LMWC = Low molecular-weight carbon substrates. Modified from Broman et al. (2017), Paper I.

To conclude, in **Paper I** it was discovered that historical sediment oxygen conditions *in situ* affected the adaptability of the microbial community and its metabolic responses. Therefore if coastal sediment sites have previously been subjected to long-term oxic, -anoxic, or fluctuating oxygen levels will impact oxygenation scenarios of 'dead zones'. Furthermore, there was a simultaneous increase between the relative abundance of sulfide oxidizing genera (e.g. *Sulfurovum*) and RNA transcripts encoding genes for sulfide oxidation in the sediment turned oxic. Additionally, genes for methanotrophy had a higher level of RNA transcripts in sediment turned oxic, with the exception of the intermediate-to-oxic.

4.2 Paper II: Hatching zooplankton eggs in anoxic sediment

The hypothesis for **Paper II** was that anoxic sediments would trap dormant zooplankton eggs and thus, stop them from hatching. To investigate if oxygenation would be a sufficient trigger for zooplankton eggs to hatch, anoxic sediment was sampled and incubated in darkness at 8°C. After 26 days of oxygenating the surface of coastal anoxic sediment sampled from the 'deep site' (Figure 3 in methods) an average of 1.5×10^5 m^{-2} nauplii copepods hatched from the top 3 cm in the sediment surface (**Paper II - Figure 2**). This amount is in the range (10^3 to 10^7 m^{-2}) of previously reported resting eggs in sediment (Viitasalo and Katajisto, 1994; Katajisto et al., 1998; Gyllstrom and Hansson, 2004; Jiang et al., 2004; Uriarte and Villate, 2006). In contrast, control cores maintained anoxic had an average of 0.02×10^5 m^{-2} hatched copepods indicating that oxygen was the hatching trigger (**Paper II - Figure 2**).

Previously established factors for hatching cues are temperature and photoperiodicity (Gyllstrom and Hansson, 2004) and even though a link between oxygen and hatching has been observed (Katajisto, 2004), it has not been considered as important as the other cues (Gyllstrom and Hansson, 2004). Copepods emerging upon oxygenation belonged to *Acartia* spp. and *Eurytemora* spp. along with rotifers belonging to *Syncheata* spp. and *Keratella* spp. As current carbon flow models tend to not include this form of benthic-pelagic coupling (Dzierzbicka-Glowacka et al., 2010; Turner, 2015) a model was constructed of the studied anoxic zone in the bay. It was estimated that during summer with water temperatures of 15°C, hatching of dormant copepod eggs reaching adulthood would constitute ~20% of the total population in the water column (Figure 5).

Considering that copepod eggs have been estimated to survive up to 13-19 years in Baltic Sea sediment (Katajisto, 1996) this hatching event would also increase genetic variation among the pelagic copepod population. Higher genetic variability in populations has been found to increase e.g. the population size stability, lower vulnerability to stress, as well as a decreased risk of extinction (Forsman and Wennersten, 2015). Additionally, sinking rates of copepod eggs have been estimated to 15-35 m^{-1} d^{-1} (Knutsen et al., 2001; Jiang et al., 2006) and a large portion of the eggs in the studied site (31 m water depth) would therefore reach the sediment. Therefore, the buried eggs in the anoxic sediment could potentially stop the recruitment of copepods to the pelagic zone, especially during spring when the abundance of adult copepods is low (Hairston et al., 2000). In addition, re-oxygenation would potentially help to initiate the annual population cycle.

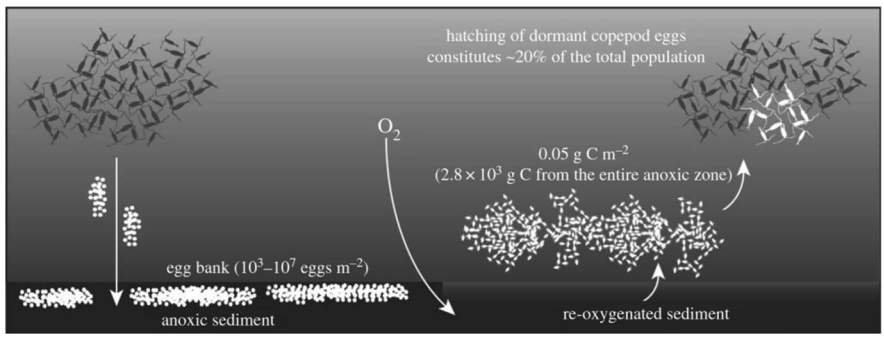

Figure 5 The studied anoxic zone in the bay was used to construct a carbon flow model using the data on hatched zooplankton. The model indicates that during summer when the water temperature is high, and if the anoxic sediment is oxygenated, hatched copepods reaching adulthood would constitute ~20% of the total population in the water. Reprinted with permission from the Royal Society

In conclusion, it was suggested that re-oxygenation strategies to remediate anoxic sediments would trigger hatching of dormant zooplankton eggs in the sediment surface. This would in turn strengthen the benthic-pelagic coupling and potentially influence trophic interactions considering that zooplankton are important prey for fish.

4.1 Paper III: Effect of phytoplankton biomass on oxic sediment

The aim of the study was to better understand changes in the sediment surface microbial community structure upon early anoxia of long-term oxic sediments. The hypothesis was that precipitation of decayed diatom and cyanobacterial biomass (i.e. spring and summer blooms, respectively) induces changes in the microbial diversity favoring degradation of the OM. Changes in the sediment surface chemistry were also expected due to these processes. As the 'shallow site' was observed to be oxic throughout the years, sediment cores were collected from this site (Figure 3 in methods). Cores were taken back to the laboratory on two sampling occasions and incubated for 21 days in darkness at *in situ* temperature (11-13°C). At the start of the incubations the sediment cores were added with small amounts of decayed cyanobacterial or diatomic biomass that sank to the sediment surface. The cores were divided into four groups: 1) biomass addition with the water phase overlying the sediment bubbled with air (8-10 mg/L dissolved O_2) denoted as 'Bubbling + Cyano/Diatoms' or 2) gas exchange by diffusion only via the air-water interface 'No bubbling + Cyano/Diatoms' and similar treatments without biomass addition 3) 'Bubbling' and 4) 'No bubbling'. The top 1 cm sediment layer was sliced for pore-water chemistry measurements and DNA extraction

at the start and end of the experiments (as well as the 1-2 cm layer for the diatom experiment). The water phase was sporadically sub-sampled for chemistry measurements and DNA extraction.

Results showed that even though small amounts of cyanobacterial and diatom biomass had been added, no change was observed in regards to oxygen concentrations in the sediment surface. This could be because of the low weight of biomass added (0.2-1.0 dw g C m^{-2}). Interestingly, the 1-2 cm sediment layer was found to be more susceptible to chemistry changes when only aerated via the air-water interface diffusion. This finding highlights the importance of stagnant unaerated bottom water during eutrophication events. Even though no difference was observed on the oxygen depth (~0.5 cm) in the sediment surface changes in chemistry was observed. For example PO_4^{3-} concentrations were higher in the 0-1 sediment layer in the 'Bubbling + Diatoms' treatment compared to the cores with no biomass addition, and NO_2^- $+NO_3^-$ concentrations were lower in the sediment surface in both the diatom and cyanobacteria experiments when biomass had been added (**Paper III - Figure 2**). This could potentially have been due to increased microbial denitrification in the sediment (Conley and Johnstone, 1995). However, contrasting studies have shown that denitrification was suppressed due to phytoplankton biomass (Tuominen et al., 1999) or only temporarily increased before declining after the organic matter had been fully degraded (Hansen and Blackburn, 1992). The total iron concentration in the 1-2 cm sediment layer was found to sharply decrease in the 'No bubbling + diatoms' treatment (**Paper III - Figure 2**). This was likely due to iron reduction and similar findings have been observed after cyanobacterial biomass was added to sediment cores (Chen et al., 2014). As no change was seen in oxygen concentrations in the sediment surface it was suggested that the changes observed in chemistry fluxes were due to the small amounts of cyanobacteria and diatom biomass added. In addition, an increase of NO_2^-+NO_3^- was observed in the water phase throughout the experiments (as well as in **Paper I** oxic controls) with or without phytoplankton biomass addition. Mass balance calculations showed that the available NO_2^-+NO_3^- and NH_4^+ in the initial field sediments were insufficient to account for this increase, either by diffusion or nitrification. It is suggested that microbial nitrogen fixation (Newell et al., 2016a), assimilation, and cycling of nitrogen compounds, e.g. dissimilatory nitrate reduction yielding NH_4^+ in deeper sediment (Kelso et al., 1997; Decleyre et al., 2015) caused this increase.

Differences were observed in the microbial community structure when comparing the cyanobacteria and diatom experiments. It has previously been found that diatom biomass addition induces changes in the sediment microbial diversity (Moraes et al., 2014). However, in the experiment only minor

changes were observed in the microbial community which were mainly related to the class *Betaproteobacteria* and class *Bacteroidetes* species *Burkholderiales* and *Flavobacteriaceae*, respectively (**Paper III - Figure 4**). It was therefore suggested that microbial activity was enhanced rather than causing differences in community structure (as previously observed, e.g. Graue et al., 2012), which explains changes observed in e.g. NO_2^-+NO_3^- concentrations.

In contrast to the diatom experiment, addition of cyanobacterial biomass in the 'No bubbling + Cyano' cores stimulated growth of a single OTU related to the acidophilic archaea family *Ferroplasmaceae* belonging to the class *Thermoplasmatales* (**Paper III - Figure 3 & 4**). This OTU increased in relative abundance from 1.54 ± 2.59% on day 0 to 11.67 ± 15.23% on day 21 (n = 3 and 4, respectively). Interestingly, in almost all other cores in the other treatments the relative abundance of *Ferroplasmaceae* was below 1% (**Paper III - Figure 4**). This family consists of acidophilic species growing in both oxic and anoxic environments, with a metabolic capability to oxidize ferrous iron and organic carbon, and has an optimal pH range of 0.8-1.8 (Dopson et al., 2004). Considering the lifestyle of the *Ferroplasmaceae* it was surprising to find it residing and stimulated in pH neutral Baltic Sea sediments after addition of cyanobacterial biomass. Three possible explanations are 1) cross-contamination in the 16S rRNA gene amplicon library; however, the dual-index approach used is considered to reduce the error rate and risk of cross-contamination (Kircher et al., 2012; Kozich et al., 2013); 2) the *Ferroplasmaceae* cells were residing in acidic microenvironments derived from sulfide oxidation which has previously been observed in biofilms founds on cave walls (Macalady et al., 2007); or 3) novel neutrophilic species most similar to *Ferroplasmaceae* in the database were amplified. Indications of the potential for sulfide oxidation in the sediment included OTUs from the sulfide oxidizing genera *Sulfurovum* spp. and *Sulfurimonas* spp. in the 16S rRNA gene dataset. Development of anoxic microenvironments in sediments has previously been observed after phytoplankton addition (Lehto et al., 2014). Additionally, both acidophilic and halophilic bacteria were some of the most abundant taxa in the sediment (e.g. *Acidithiobacillus* and *Halothiobacillus*; **Paper III - Figure 5**). It is therefore possible that microenvironments existed in the sediment and that the 0-1 cm sediment slices used for DNA extraction contained many different types of microenvironments with complex redox processes.

To conclude, the addition of phytoplankton biomass on the sediment surface induced changes in pore-water chemistry, with and without forced aeration. An increase of NO_2^-+NO_3^- was observed in the water column throughout the experiment which was suggested to originate from microbial assimilation, fixation, and cycling of nitrogen compounds. Microbial community structure

did not change after addition of diatom biomass, while cyanobacterial biomass stimulated growth of acidophilic archaea potentially residing in favorable microenvironments in the sediment surface. The findings from this study suggested that oxygenation of the *in situ* bottom water could potentially keep the microbial community structure in oxic sediments more stable during eutrophication events, while nitrate would be released to the water column which could potentially enhance the growth of phytoplankton in the water.

4.4 Paper IV: Follow up – Oxygen shifts in the sediment surface

A follow up study to **Paper I** was conducted with more frequent sub-sampling and sediment slicing to investigate changes in the sediment surface due to oxygenation. In **Paper IV** only the 'deep site' was sampled, and compared to **Paper I** the bottom water contained ~3 mg/L dissolved O_2. This indicated a more oxygen rich bottom zone compared to previous sampling campaigns (Table 4 in methods). Sediment cores were sampled in the field as described for **Paper I** (as zero time-points); while additional cores were incubated in the laboratory. Of the laboratory incubations, ten cores were continuously aerated by bubbling air (~10 mg/L dissolved O_2; designated as 'turned oxic') and three cores were turned anoxic with the use of N_2-gas (0.00-0.07 mg/L dissolved O_2; designated as 'turned anoxic'). Throughout the incubation period some of the cores were sacrificed, i.e. the water phase was sub-sampled and the oxygen penetration depth determined in the top 1 cm sediment surface before being sliced. After 15 days the remaining cores were sliced including the 'turned anoxic' cores. Chemistry measurements were conducted in the same manner as **Paper I**. In addition, DNA was extracted from the water and sediment to determine both the total and viable 16S rRNA gene OTU communities, as well as RNA from the sediment slices to determine microbial metabolic responses.

Chemistry results showed a sharp decline in redox potential from 109.3 ± 3.7 to -265.6 ± 22.7 mV in the water phase of the 'turned anoxic' cores (n = 3 for both occasions; **Paper IV - Figure 1**), indicating a reduced anoxic environment. This was further confirmed as oxygen was undetectable in the sediment surface of the 'turned anoxic' cores. In the 'turned oxic' sediment the oxygen penetration depth was in the range of 0.88-1.69 mm throughout the experiment. Dissolved PO_4^{3-} decreased in the 'turned oxic' sediment pore-water through the experiment from 23.91 ± 1.72 µM on day 0 to ~3 µM on day 5 and 9, and finally to 9.58 ± 8.06 µM on day 15 (all n = 3 except day 15, n = 4; **Paper IV - Figure 1**). This was likely due to iron oxidation and the formation of Fe-bound PO_4^{3-} preventing it from passing the 0.7 µm filter. In the 'turned anoxic' sediment the PO_4^{3-} remained stable at 22.13 ± 5.55 µM (n = 3). Sulfur cycling in the 'turned oxic' sediment was suggested by pore-water

SO_4^{2-} concentrations of 3.02 ± 0.24 mM on day 0 to 2.48 ± 0.19 mM (day 5), 3.86 ± 0.12 mM (day 9), and 2.98 ± 0.41 mM on day 15. In the sediment pore-water of the 'turned anoxic' cores the SO_4^{2-} decreased to 2.11 ± 0.10 mM at the end of the experiment (**Paper IV - Figure 1**).

The 16S rRNA gene OTUs showed no major differences between total and viable populations (one-way ANOVA tests conducted on phylum, *Proteobacteria* class, and OTU level). The effect of turbidity on PMA treatment has been discussed (Desneux et al., 2015) and RNA-seq has been suggested to better distinguish total DNA and PMA-treated samples (Li et al., 2017). The results indicated that the microbial community was potentially viable and this was further supported by the metatranscriptomic data (**Paper IV - Figure 2**). Therefore, only the viable populations are presented hereafter. Rarefaction curves showed that a large portion of the communities had been sequenced but a portion of undetected OTUs still existed, especially in the sediment. No difference in alpha diversity (Shannon H's index) was observed in the water overlying the sediments. However, in the sediment surface a statistically significant decrease in diversity was observed after nine days of turning the sediment oxic from 7.56 ± 0.08 to 6.42 ± 0.31 ($F = 39.0$, $p < 0.01$), as well as comparing sediment 'turned anoxic' to 'turned oxic' after 15 days of incubation (6.81 ± 0.56 compared to 7.81 ± 0.21, respectively; $F = 8.28$, $p < 0.05$). Bray-Curtis beta diversity also showed that the 'field' and 'turned anoxic' sediment were dissimilar to the 'turned oxic' samples. In the 'turned oxic' water phase *Alpha-*, *Epsilon-* and *Gammaproteobacteria* increased (**Paper IV - Figure 2**), with *Rhodobacteraceae* spp. and *Arcobacter* spp. dominating the *Epsilon-* and *Alphaproteobacteria* classes, respectively. In the 'turned anoxic' water phase *Deltaproteobacteria* increased and this class was dominated by the sulfate reducing genus *Desulfatiglans*.

In the top 1 cm sediment surface of the 'turned oxic' cores the relative abundance of *Epsilonproteobacteria* increased while *Deltaproteobacteria* sharply decreased (from 20.0 ± 9.6% to 4.3 ± 1.1%) along with a decrease in the phylum *Bacteroidetes*. In the 'turned anoxic' sediment both *Deltaproteobacteria* and *Bacteroidetes* remained stable (**Paper IV - Figure 2**). Compared to the partial 16S rRNA gene data, the metatranscriptomes revealed that Archaea (~6%, proportion of FPKM), *Firmicutes* (~18%), and *Gammaproteobacteria* (~23%) had an active role in all sediments throughout the incubation experiment (**Paper IV - Figure 2**). The largest change in the 16S rRNA gene OTUs relative abundance was observed for *Epsilonproteobacteria* in the 'turned oxic' sediment that increased from 20.0 ± 9.6% on day 0 to 45.0 ± 11.1% (day 5), 50.9 ± 3.9% (day 9), and finally 35.4 ± 16.6% at the end of the incubation experiment.

The genus *Arcobacter* was found to dominate the *Epsilonproteobacteria*, as well as the whole microbial community in the sediment (up to 43% of the whole community based on the 16S rRNA gene data; **Paper IV - Figure 5**). The correlation network analysis showed that the presence of *Arcobacter* OTUs positively correlated with *Sulfurovum* and *Sulfurospirillum*, while correlating negatively with *Desulfatiglans*. Different *Arcobacter* species have previously been observed in marine coastal water, including *Arcobacter butzleri* and uncultured species (Fera et al., 2004). Another studied species is *Arcobacter sulfidicus* which can withstand high concentrations of H_2S and is suggested to be highly competitive in an oxic-anoxic interface among sulfide oxidizers (Sievert et al., 2007). In this study, *Arcobacter* spp. in the sediment also correlated positively with *Arcobacter* spp. observed in the water phase ($r > 0.7$, $p < 0.01$), indicating a contemporary increase in the sediment and the overlying water. In more detail, *Arcobacter* spp. in the 'turned oxic' sediment constituted up to 75% of all *Epsilonproteobacteria* and varied in relative abundance throughout the incubation (from 8.2 ± 8.6% on day 0, 42.9 ± 9.1% on day 9, and 29.8 ± 13.1% on day 15; Figure 6). A white film was observed in the oxic-anoxic layer in the sediment at the time of the peak *Arcobacter* spp. abundance (i.e. day 9, Figure 6). Potentially this constituted a film of filamentous sulfur which has previously been observed to be produced by *Arcobacter sulfidicus* (Wirsen et al., 2002), and is the result from incomplete oxidation of sulfide (Sievert et al., 2007). Other sediment *Epsilonproteobacteria* consisted of *Sulfurimonas* spp. and *Sulfurovum* spp. which were less abundant than *Arcobacter* spp. (<13% of the *Epsilonproteobacteria* and <4% of the whole microbial community). OTUs from these genera decreased throughout the experiment from ~7% and ~1% in the field, to ~1% and 0.5% on day 15, for *Sulfurovum* spp. and *Sulfurimonas* spp., respectively (Figure 6). This large increase in *Epsilonproteobacteria* was also observed in the metatranscriptome. *Arcobacter* spp. represented ~0.1% (proportion of FPKM) in the field, and increased to ~7%, ~4%, and ~4% on days 5, 9, and 15 in the 'turned oxic' sediment. In contrast to the 16S rRNA gene data, *Sulfurimonas* spp. represented ~3% in the field and ~11%, ~5%, and 2% on days 5, 9 and 15 in the 'turned oxic' sediment (Figure 6, and **Paper IV - Figure 2**). The relative abundance of *Sulfurimonas* and *Sulfurovum* OTUs from **Paper I** were compared to **Paper IV** and showed less presence in **Paper IV**, instead *Arcobacter* spp. were found to dominate the *Epsilonproteobacteria* (Figure 6).

The *de novo* assembled metatranscriptomic data yielded 431,522 RNA transcripts of which an average of 20 ± 5% could be annotated with a hit in the UniProtKB/Swiss-Prot database. This resulted in an average of 4786 ± 1627 genes per sediment sample ($n = 17$). Genes were functionally categorized into sulfur, nitrogen, and methane metabolism etc. according to the MetaCyc, GO

biology processes, and KEGG databases. In addition, the portion of genes that could directly be linked to the genus *Arcobacter* in the UniProtKB/Swiss-Prot database (closest species *Arcobacter butzleri*) were extracted (**Paper IV - Figure 6**). Differential expression analysis was not reliable due to a high false discovery rate from the high variation among the sediment cores. Therefore the dataset was delimited to genes with >1000 FPKM (in at least one sample) to investigate which genes were maintained in the sediment upon oxygenation. *Arcobacter* activity was observed in the form of genes involved in e.g. energy, respiration and ribosomal activity (i.e. protein synthesis). In more detail, many genes for ribosomal (e.g. *rplD* and *rpsM*), RNA polymerase (e.g. *rpoABC*), and tRNA activity (e.g *argS* and *ileS*) increased from <85 FPKM in the field to >1000 FPKM in the 'turned oxic' sediments (**Paper IV - Figure 6**). Even though the formation of the white film in the sediment was observed, sulfide oxidizing genes could not be detected in the RNA transcript data for the genus *Arcobacter*, which has previously been found in the genome of e.g. *Arcobacter butzleri* (Miller et al., 2007). It was therefore suggested that if *Arcobacter* spp. in this study were active in sulfur cycling it was before the first five days in the experiment.

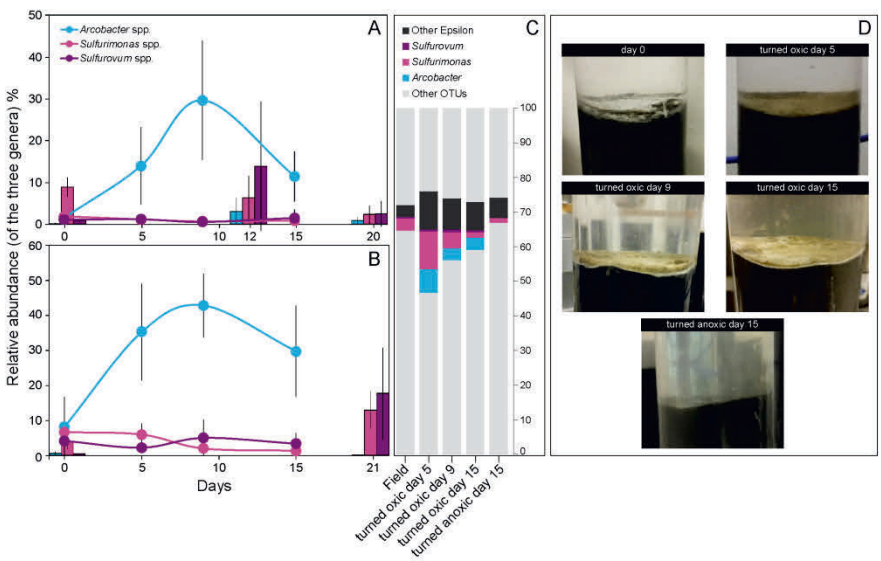

Figure 6 The three most abundant *Epsilonproteobacteria* genera throughout the incubation experiment. A) shows relative abundance in the water phase and B) the top 1 cm sediment surface. Lines are based on data from **Paper IV** (day 0, 9, and 15) while data for the bar graphs (day 0, 12, and 20) are from **Paper I**. All samples consisted of three independent cores (SD = 1) except sediment and water 'turned oxic' day 15 (n = 4) and water 'turned oxic' day 9 (n = 2). C) Shows the dominant *Epsilonproteobacteria* derived from RNA transcript data with a taxonomic affiliation (y-axis shows proportion (%) of FPKM), and D) shows photographs of the sediment surface throughout the incubation experiment.

RNA transcripts involved in sulfur cycling were attributed to genes for e.g. sulfur assimilation (*cysD* and *cysN*; (Fera et al., 2004)), sulfite oxidation (*sat*), sulfide oxidation (*sqr*), and thiosulfate oxidation (*soxA* and *soxB*). RNA transcripts attributed to these genes were maintained in sediment upon oxygenation (**Paper IV - Figure 6**). In addition, the *dsrA* and *dsrB* genes coding for dissimilatory sulfite reductase involved in sulfate reduction or in reverse to oxidize sulfur (Harada et al., 2009) were present in several of the 'turned oxic' sediment cores during all sampled days. Similar to *dsrAB*, the *dsvABC* genes coding for dissimilatory sulfite reductase were maintained in the 'turned oxic' sediment. However, in contrast to *dsrAB*, these genes had a tendency to be higher in the 'turned anoxic' sediment (**Paper IV - Figure 6**). Major taxonomical groups in the UniProtKB/Swiss-Prot database linked to sulfur cycling included archaeal phyla *Crenarchaeota* and *Euryarchaeota* and various bacterial phyla e.g. *Actinobacteria*, *Chlorobi*, *Firmicutes*, and the *Alpha-*, *Beta-*, *Delta-*, *Epsilon-* and *Gammaproteobacteria* classes (**Paper IV - Figure 7**).

A major proportion of the categorized genes were also related to nitrogen fixation. For example, the genes *fdxD*, *fixL*, *frxA*, and *nifDHKP* were maintained in the sediment upon oxygenation (**Paper IV - Figure 6**). Nitrogen fixation in oligotrophic environments rich in oxygen has previously been confirmed (Rahav et al., 2013). Furthermore, recent findings have shown that nitrogen fixation rates in nitrogen rich sediment with active denitrification has been underestimated (Newell et al., 2016b). Other nitrogen cycling genes related to nitrogen regulation (e.g *glnB*, *nrtCX*, and *ptsN*) were also maintained in the sediment upon oxygenation. Major taxonomical groups affiliated with nitrogen cycling in the UniProtKB/Swiss-Prot database included the archaeal phylum *Euryarchaeota* and a large amount of bacterial phyla e.g. *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, and *Proteobacteria* classes *Alpha-*, *Beta-*, *Delta-*, *Epsilon-* and *Gammaproteobacteria* (**Paper IV - Figure 7**).

Species belonging to the genus *Arcobacter* have also been observed to conduct nitrogen fixation, for example *Arcobacter nitrofigilis* (McClung and Patriquin, 1980; Pati et al., 2010) and *Arcobacter sulfidicus* that also produce white mats of filamentous sulfur (Wirsen et al., 2002). Due to the limitation of available isolates in the UniProtKB/Swiss-Prot database it was difficult to conclude which species conducted nitrogen fixation. A likely parameter that contributed to nitrogen fixation was the low amount of nitrate available in the sediment, which has previously been observed in oligotrophic waters (Rahav et al., 2013) and is a characteristic of 'dead zones' (Carstensen et al., 2014b). Considering that maintained nitrogen fixation upon oxygenation was not

observed in **Paper I**, it is possible that *Arcobacter* spp. were either directly or indirectly involved.

Genes involved in methane metabolism were detected in both the 'turned oxic' and 'turned anoxic' sediments. For example, RNA transcripts attributed to the gene *mttC1* coding for trimethylamine-specific corrinoid protein used in e.g. methane production from methylamine was only present in one of the 'turned anoxic' cores. In addition, other genes involved in methanogenesis were maintained in a few of the 'turned oxic' sediment cores, e.g. *mtbA*, *mtrA*, and *mvhA* (**Paper IV - Figure 6**). In the UniProtKB/Swiss-Prot database genes involved in methane metabolism were affiliated with the archaeal phylum *Euryarchaeota* and bacterial phyla *Actinobacteria*, and the *pmoA1A2* and *pmoB1B2* genes for methane oxidation were affiliated to the *Gammaproteobacteria* class. These findings are in accordance to **Paper I** which also showed methanogenesis in both anoxic and oxygenated sediment (likely below the oxic surface layer).

In **Paper IV** the impact of oxygen shifts on the microbial community in oxygen deficient Baltic Sea coastal sediment was studied. By following the 16S rRNA gene and metatranscriptomic data in the sediment surface it was observed that uncultured species belonging to the *Epsilonproteobacteria* genus *Arcobacter* increased in relative abundance and the amount of *Arcobacter* related RNA transcripts increased in just five days (peaking on day 9). In addition, *Sulfurimonas* spp. increased during the first nine days in the metatranscriptome but were not as prevalent in the 16S rRNA gene data. The metatranscriptome data showed that RNA transcripts for sulfur cycling, such as sulfur and sulfide oxidation, were maintained in the sediment upon oxygenation. In addition, RNA transcripts attributed to nitrogen fixation genes were also maintained upon oxygenation.

4.5 Paper V: Diatoms role in hypoxic/anoxic sediment

RNA sequencing data collected from the sampling campaigns during autumn 2013 and spring 2016 of the 'deep site' were used to study the eukaryotic metatranscriptome in the sediment surface both in the field and upon oxygenation during the incubation experiments. The relative proportion of RNA reads in the field and during oxygenation experiments showed that the phylum *Bacillariophyta* (diatoms) dominated the metatranscriptome during the spring sampling, while being abundant during autumn (220,000-800,000 counts per millions (CPM); relative proportion × 1,000,000**; Paper V - Figure 1**). Anoxic incubations after 15 and 21 days compared to the field showed no statistical significance for diatoms (Kruskal Wallis test, $p = 0.044$). Upon a

closer look at the RNA reads linked to diatoms it was revealed that majority were affiliated with the taxonomic order *Thalassiosirales* (790,000-968,000 CPM; representing 95.6 to 98.8 % of the diatom community) with the genera *Thalassiosira* and *Skeletonema* as most common (**Paper V - Figure 2**). Both of these genera are common in the Baltic Sea (Tamelander and Heiskanen, 2004). Diatoms have been found to survive in darkness and anoxia (Lewis et al., 1999; McQuoid et al., 2002; Kamp et al., 2013), including species belonging to the order *Thalassiosirales* such as *Thalassiosira weissflogii* (Kamp et al., 2013).

Further analysis of the RNA data revealed that metabolic processes linked to the diatoms were mainly related to photosynthesis (likely connected to the thylakoid membrane in the chloroplasts) throughout the anoxic and oxic incubations. Previous chloroplast genome sequencing of *Thalassiosira oceanica* (Lommer et al., 2010) indicated that the chloroplast had been sequenced in the 'deep site' sediment. The majority of RNA reads affiliated with diatoms annotated against the InterPro protein database indicated that the sequences were from a chloroplast genome with e.g. ribosome proteins, photosystems I and II, cytochrome b_6/f, and ATP synthase (**Paper V - Figure 4**). Similar results with an active photosystem in darkness and anoxia have been observed in the Landsort deep, the deepest point in the Baltic Sea at 466 m (Thureborn et al., 2016). Furthermore, experiments have shown that diatoms maintain their photosystem during darkness to rapidly generate energy when light becomes available (Nymark et al., 2013; Li et al., 2016). Such studies have been conducted on e.g. *Thalassiosira weissflogii* (Katayama et al., 2015) and *Thalassiosira Pseudonana* (Ashworth et al., 2013) both belonging to the order *Thalassiosirales*. This adaptation to rapid light exposure can potentially be explained by coastal diatoms having a higher concentration of photosystem I and cytochrome b_6/f compared to oceanic diatoms, which are instead adapted to low iron concentrations (Strzepek and Harrison, 2004). This trait would give coastal diatoms an effective means to adapt to changing light conditions which are more common for coastal waters (Strzepek and Harrison, 2004).

A strategy for diatom survival in darkness is linked to nitrate reduction and typically occurs for one day before the organism enters resting stage (Kamp et al., 2011). Considering that no InterPro annotations affiliated with diatoms were related to nitrate reduction it was likely that the diatoms in the hypoxic/anoxic sediment surface were already in resting stage. Oxygenation of the sediment showed no statistical significance in the relative proportion of diatoms (Kruskal Wallis, $p = 0.15$; comparing 'turned oxic' to 'turned anoxic' day 15 sediment, only available for spring 2016 data). Also, the metatranscriptomic data showed no indication that diatom cells in the

sediment germinated upon oxygenation (**Paper V - Figure 4**). To verify the abundance of diatoms in the sediment, an additional sampling campaign was conducted at the 'deep site' during spring 2017. The top 1 cm sediment slice was used for microscope counting and counts confirmed the abundance of diatoms belonging to the order *Thalassiosirales* in the hypoxic/anoxic sediment (Figure 7), with *Skeletonema* being the most common genus (80 ± 54 million cells g^{-1} dw sediment). Furthermore, the viability of the sediment diatoms was confirmed by adding diatoms into f/2 medium kept at 3°C during light exposure (125 µE s^{-1} m^{-2}).

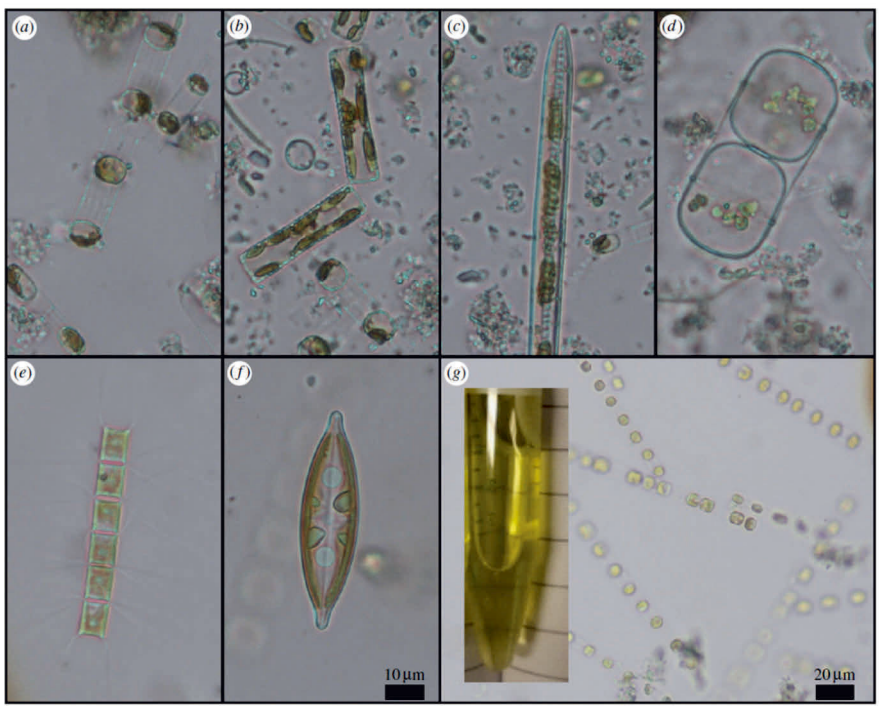

Figure 7 (a) *Thalassiosirales*, (b) *Fragilariales*, (c) *Bacillariales*, and (d) *Melosirales* in the hypoxic/anoxic sediment surface sampled during spring 2017 (in Lugol's solution). After two weeks of growth in medium and exposure to light: (e) *Chaetocerotales* (no preservative) with chlorophyll, (f) *Naviculales*, and (g) the diatom community was dominated by *Thalassiosirales* (*Skeletonema* sp.); inset picture shows extracted chlorophyll. Subfigures (a-f) have a scale of 10 µm and (g) 20 µm. Reprinted with permission from the Royal Society.

A model was constructed to estimate the impact of the diatoms in the sediment upon water column upon mixing (i.e. light exposure). With approximately 80 million *Skeletonema* cells g^{-1} dw sediment and a water content of 94% in the sediment surface (based on autumn 2013 dry and wet weight data, n = 3), the

sediment was considered to have 3,200,000 *Skeletonema* cells per mL sediment. The volume per cell was estimated to be 327 μm^{-3} (Olenina et al., 2006) based on the µm scales from the microscope observation (cell height and dimension: 8 µm and 7.5 µm, respectively; Figure 7). Finally, a 30 pg C per cell carbon factor (López-Sandoval et al., 2014) was used to calculate the *Skeletonema* carbon stock in the sediment, this resulted in 0.96 g C per m^2 sediment (in the top 1 cm layer). Scaling up for the whole water column (200 000 m^2) above the 'dead zone' area of the bay this yielded ~192,000 g of diatom biomass carbon buried in the sediment surface. The euphotic zone in the bay was estimated to reach a 15 m water depth. Therefore, the annual phytoplankton biomass in the water overlying the 'dead zone' would be approximately 57,900,000 g C (based on annual 19.3 g C m^{-3} in coastal Baltic Sea water (Legrand et al., 2015)). The *Skeletonema* cells in the sediment therefore represent 0.3% of the annual phytoplankton carbon biomass in the coastal bay water or 121% if all diatoms would hatch during one day.

In conclusion, diatom cells were found to dominate the eukaryote metatranscriptome during spring while also being abundant in autumn. The most abundant taxonomical order in the metatranscriptomic dataset was *Thalassiosirales* with *Thalassiosira* and *Skeletonema* being the most common genera. Microscope counts confirmed that *Skeletonema* was the most abundant and a modelling approach suggested that the buried cells represent 121% of the daily phytoplankton carbon biomass. This could potentially enhance the spring bloom upon water column mixing (Lashaway and Carrick, 2010). Based on the metatranscriptome data and viability test in medium and light exposure it was found that light seems to be the triggering cue for germination rather than oxygen. The photosystem was maintained in darkness and anoxia likely as an anticipatory circadian mechanism. This is important as the inflow of oxygen rich deep water (Stigebrandt et al., 2015b) or artificial re-oxygenation remediation scenarios (Stigebrandt et al., 2015a) would only trigger germination if the diatoms were exposed to light.

Conclusions and future outlook

The Baltic Sea, including coastal areas, has undergone severe eutrophication during the last 60 years. This has led to an expansion of episodic and permanent hypoxic bottom zones, and because of these drastic environmental changes chemistry fluxes and benthic communities have been altered. The focus of this thesis was to look closer at the early microbial community changes due to degradation of phytoplankton biomass, and the effect of oxygenation on chemistry fluxes, dormant phyto- and zooplankton, the microbial community structure and their metabolic responses. It was found that upon addition of phytoplankton biomass on nutrient rich oxic sediment the changes were partly related to growth of archaea, oxygenation of anoxic sediments decreased stored organic matter, zooplankton eggs hatched potentially increasing the benthic-pelagic coupling, and resting diatoms buried in the sediment continuously expressed the photosystem and were found to be triggered by light rather than oxygen. Additionally, much work went into trying to understand changes in the microbial community structure and their metabolism upon oxygenation of anoxic sediments. It was found that differences in sediment microbial communities to oxygen shifts were dependent on the historical exposure to oxygen and that microbial generalists adapted to episodic oxygenation were favored during oxygen shifts. Facultative anaerobic sulfur/sulfide oxidizing genera were favored upon oxygenation and sulfur cycling and nitrogen fixation genes were abundant in the sediment surface. Finally, it was found that oxygenation regulates the sulfur and methane cycles in the sediment surface. The future outlook of re-oxygenation as a remediation strategy of 'dead zones' looks promising in regards to chemistry studies conducted previously, as well as the zooplankton and microbial studies presented in this thesis. Future re-oxygenation studies could focus on how to efficiently incorporate such artificial or natural remediation strategies in 'dead zones'. It is suggested that such strategies in conjunction with other environmental policies to prevent eutrophication, e.g. regulation of agricultural and wastewater leaching, would help to accelerate the remediation of 'dead zones'.

Acknowledgements

My PhD journey has been quite remarkable because when I came here (Linnaeus University), I had very limited knowledge of microbiology and molecular biology. I had absolutely no skills whatsoever in bioinformatics, and even although I had previously worked with (lake) sediments I had not been exposed to the complexity of sediment genomics, transcriptomics, and the sulfur cycle.

The development of this thesis and the simultaneous increase of my knowledge throughout this journey is much thanks to the possibilities offered by the Linnaeus University Centre for Ecology and Evolution in Microbial model Systems (EEMiS). I wish to express many thanks to my main supervisor Mark Dopson in the EEMiS center who decided to give me this opportunity. He has helped me greatly to develop my scientific comprehension and ability to communicate scientific knowledge either through writing or speaking. In addition to my main supervisor, co-supervisors Jarone Pinhassi and Catherine Legrand have been an undeniable help and an excellent force pushing me through my PhD journey.

Aside from my main supervisor and co-supervisors there have been many helping hands from colleagues here at Linnaeus University. First, I wish to thank Johanna Sjöstedt who taught and instructed me in the lab when I first arrived to Linnaeus University. Second, I wish to acknowledge Samuel Hylander who brought up the possibility of additional studies to investigate zooplankton and diatoms from the sediment experiments. Third, Susanna Fredriksson and Stefan Tobiasson in the Linnaeus University Coastal Group who came out with me to the field and helped with sampling of sediment cores. Their long expertise of field work and kind personalities made field work so much more enjoyable. Fourth, I wish to thank Anders Månsson who struggled with me trying to develop the sediment slicing device. Furthermore, I also wish to thank Henrik Hallberg along with Anders Månsson who helped me set up the incubations for Paper IV. In addition, I wish to thank Sara

Gunnarsson who has helped with many practicalities related to laboratory work. Several students appear as co-authors on the publications attached to this thesis and I wish to thank these students: Lingni Li, Jimmy Fridlund and Varvara Sachpazidou who helped me in the field and laboratory.

Many fellow PhD students and other colleagues have made my time at Linnaeus University so much more enjoyable. First of all the System Biology of Microorganisms research group that I've been part of: Xiaofen Wu, Stephan Christel, Gaofeng Ni, Domenico Simone, Margarita Lopez-Fernandez, Laura Seidel, Abbtesaim Jawad, Magnus Ståhle, Antoine Buetti-Dinh, and Eva Högfors-Rönnholm. Second, all the colleagues and university staff who have joined me on courses, workshops, conferences abroad or during my time spent at LNU. This is a long list and includes e.g. Fredrik Svensson, Oscar Nordahl, Furong Li, Christofer Karlsson, Hanna Berggren, Mattias Fast, Charlotte Marchand, Emil Fridolfsson, Richard Mutafela, Emelie Nilsson, Benjamin Pontiller, Carina Bunse, Anu Helin, Håkan Johansson, Sabina Arnautovic, Joacim Rosenlund, Johanna Sunde, Eva Sörenson, Jon Tinnert, Mireia Bertos-Fortis, Lina Mattsson, Kimberly Berglöf, Ma Shenhong, Mariëlle Van Toor, Josanne Verhagen, Sandra Martinez Garcia, Jonas Waldenström, Terese Uddh Söderberg, Josef Lautin, Karin Holmfeldt, Karen Lebret, Hanna Farnelid, Yahya Jani, Stephanie Turner, Viraja Viru, Daniela Palma, Caroline Littlefield Karlsson, Joanne Chapman, Kristofer Bergström, Stina Israelsson, Conny Tolf, Daniel Lundin, Christina Esplund-Lindquist, Jonas Nilsson, Petter Tibblin, Alexis Avril, Elin Lindehoff, Neus Latorre Margalef, Rahaf Arabi, Sarala Devi Muthusamy, Per-Eric Betzholtz, Maurice Hirwa, Åke Hagström, William Hogland, Markus Lindh, Fabio Kaczala, Martin Olofsson, Lena Wennersten, and Camilla Karlsson. I also want to thank Zhang Lu at Uppsala University who got the information, regarding the available position at LNU from Xiaofen Wu, and shared it further to Jingyang Zhang.

I also want to thank the FUR (Forskarutbildningsrådet; Committee for Doctoral Studies) at the FHL faculty. I was the PhD represent in FUR almost my whole PhD time and learnt a lot regarding the administration organization in the faculty, and especially the organization and administrative work behind the research education. First of all I wish to thank Ymke Mulder who has been a great help during my time in FUR at LNU. In addition I wish to thank the rest of FUR who made it possible for me to be part in the organization of PhD workshops and other tasks: David Brunt, Catarina Gaunitz, Per Larsson, Sven Tågerud, Geoffrey Lemdahl, Anders Forsman, Ian Nicholls, Ulrica Hörberg, Mats Lindahl, Mikael Rennemark, and Kristiina Heikkilä.

I also wish to take this opportunity to thank my supervisor during my master's thesis at Uppsala University, Sebastian Sobek, who was an excellent guide and support. I furthermore wish to thank Lars Tranvik who gave me the opportunity to work in the laboratories of the limnology department at Uppsala University after my master's degree. I also wish to thank my supervisor during my bachelor's thesis at Linköping University, David Bastviken, who made my interest for sediment science and science in general grow. Finally, I wish to thank Bertil Gustafsson while during my studies at Folkuniversitetet in Norrköping, before I started my university studies, made an interest inside me grow for nature science and molecular biology.

To end this journey I wish to thank my family who has always been there for me, min mor Susanne Broman och bonuspappa Roger Nilsson som jag båda älskar. Dom har alltid varit där när jag har behövt hjälp i mitt liv och har alltid stött mina val i livet även om det inte alltid har varit så lätt. Till min mor som tog självständigt hand om mig under en svår tid i livet, vill jag säga: jag älskar dig. Även fast Roger kom in i våra liv när jag var väldigt ung är han i princip min pappa och har alltid tagit hand om mig och min mor under dessa år, och gjort våra liv varmare och mer glädjefyllda. Till dig vill jag säga: jag älskar dig och allt du har gjort för mig och min mor.

我也要感谢张景阳的妈妈孙如平，她的爸爸张维权，和她的弟弟张景利。当我们在中国或瑞典，他们总是支持和帮我们。

Finally I wish to acknowledge one person, Jingyang Zhang. The person I love and have decided to live my life with. She has taught me the importance of appreciating family and friends, and helped me to become the person I am today. She has also convinced me that an adventurous and beautiful life continues after the doctoral journey.

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