From Single Species to the Community Level: Exploring Under-Researched Avenues in Protist Ecology and Protist-Prokaryote Interactions

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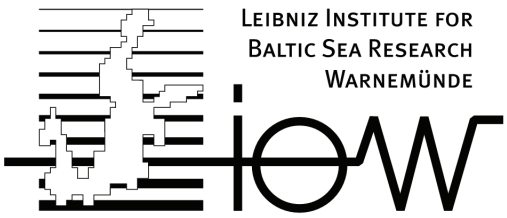

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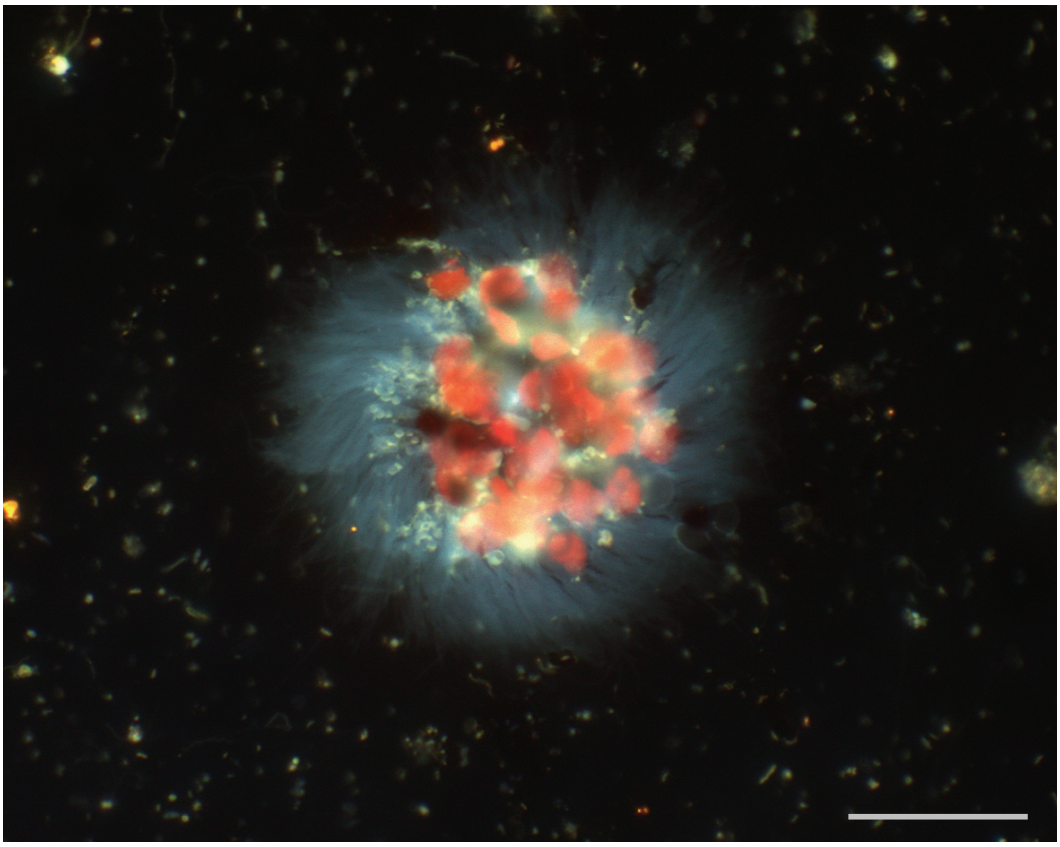
...by the help of Microscopes, there is nothing so small, as to escape our inquiry; hence there is a new visable World discovered to the understanding

Robert Hooke (Micrographia, 1665)



Ciliate from the oxic / anoxic interface of the Baltic Sea. Scale-bar: 20µm

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Abstract

Protists are ubiquitous, extremely diverse eukaryotic organisms with pivotal roles in aquatic microbial food webs and ecosystem functioning. However, research has lagged behind that of prokaryotes, and many open questions remain. Here two case studies of under-researched fields in protist ecology and protist-bacteria interactions are presented and explored:

Case study 1: Vulnerability of carbon starved bacteria to protist grazing. Many heterotrophic bacteria enter the starvation-survival state when subjected to carbon and energy limitation. Through a series of strong metabolic and physiological changes they become very small cells, with little or no metabolic activity, and a high resistance to diverse abiotic stress factors. However, it remains to be seen whether this cross-protection also extends to protist grazing. In the present work, the growth of a model bacterivorous nanoflagellate was compared on different physiological states of three bacterial strains with well studied starvation responses. While all bacterial strains were a good food source when non-starved, marked differences were seen in the growth of the protists on starved cells. This ranged from an equal growth on an equivalent biomass of starved and non-starved prey for one bacterial strain, to unpalatability and adverse effects on protist development for another. The results obtained confirm that carbon-starved bacteria can show a decreased vulnerability to protist grazing, but the response is highly species-specific. Additionally, they demonstrate that feeding on smaller bacterial cell-sizes does not necessarily impose an energetical constraint on a flagellate grazer.

Case study 2: The ecological relevance of bacterivorous protists in pelagic redoxclines. Barrier zones between oxic and anoxic water masses (pelagic redoxclines) host highly active prokaryotic communities with important roles in biogeochemical cycling. However, much less is known on the ecological role of protists in these zones. In the present work, the protist community composition, distribution, and bacterivorous relevance was assessed for two central Baltic Sea redoxclines. A combination of different microscopy techniques revealed strong and consistent shifts in dominating protist groups along the redox gradient. Small ciliates (*Strombidium* c.f.) and dinoflagellates dominated

the protist community at suboxic depths (≤30 µM O_2), while larger ciliates (*Mesodinium* spp., *Metacystis* spp., *Coleps* c.f. and unidentified morphotypes) formed a peak in abundance at the oxic-anoxic interface. In sulphidic waters, protist abundance was very low, represented by only few morphotypes (e.g. *Metacystis* spp.). Grazing rates on prokaryotes were estimated (long-term incubations with fluorescently labelled bacteria (FLB)), and compared to the frequency of virally infected cells (dilution approach). Prokaryotic mortality was dominated by protist grazing in suboxic and oxygen / hydrogen sulphide interface depths (50 – 80 % of prokaryotic standing stock per day). Ciliates and occasionally dinoflagellates, but not heterotrophic nanoflagellates (HNF), were the major bacterivores. In upper sulphidic depths, grazing was below the detection limit and the frequency of virally infected cells was below 5 %, leaving the major prokaryotic mortality factor an open question. Predator exclusion experiments were used to study specific grazing on a chemolithoautotrophic bacterial key-player of Baltic Sea pelagic redoxclines, the *Sulfurimonas* subgroup GD17 (*Epsilonproteobacteria*). Protist grazing on this bacterial group was very high (>100 % of new cell production d^{-1}), thus being able to control its growth and potentially its vertical distribution along the water column. Finally, the principal protist grazers on the *Sulfurimonas* subgroup GD17 were identified by RNA stable isotope probing, employing as prey a ^{13}C-labelled cultured representative ("*Sulfurimonas gotlandica*" strain GD1). This analysis identified 3 flagellate and 2 ciliate taxa as grazers, thus demonstrating that HNF could still play an important role as selective grazers on highly active and biogeochemically significant prokaryotic groups.

Kurzfassung

Protisten sind ubiquitär verbreitete, extrem diverse eukaroytische Organismen, die in aquatischen mikrobiellen Nahrungsnetzen grundlegende Funktionen übernehmen. Im Gegensatz zu den Prokaryoten ist diese Gruppe noch viel weniger erforscht. In dieser Arbeit werden zwei verschiedene Fallstudien im Bereich der Protistenökologie sowie der Protisten-Bakterien-Interaktionen vorgestellt:

Fallstudie 1: Anfälligkeit von hungeradaptierten Bakterien gegenüber Protistenfraß. Viele heterotrophe Bakterien können als Reaktion auf Kohlenstoff- und Energielimitierung in ein Stadium der Dormanz übergehen. Durch Änderungen des Metabolismus und der Physiologie kommt es zu einer starken Größenreduktion der Zellen, sie weisen wenig- bis gar keine Stoffwechselaktivität mehr auf und werden zunehmend resistent gegenüber abiotischen Stressfaktoren. Bisher ist nicht bekannt, ob sich diese Schutzmechanismen auch auf die Anfälligkeit gegenüber Fraß durch Protisten auswirken. In der vorliegenden Arbeit wurden einem bakterivoren Nanoflagellat in Fraßexperimenten drei verschiedene Bakterienstämme, aus wachsenden oder hungeradaptierten Kulturen vorgesetzt. Während alle Bakterienstämme im wachsenden Zustand eine gute Nahrungsquelle darstellten, zeigten sich deutliche Unterschiede im Protistenwachstum auf hungeradaptierten Bakterien. Die Beobachtungen reichten von gleichem Protistenwachstum unabhängig von dem physiologischen Zustand der Bakterien bis hin zu Fraßresistenz hungeradaptierter Bakterien mit negativen Effekten auf die Protistenentwicklung. Diese Ergebnisse zeigen, dass hungeradaptierte Bakterien eine geringere Anfälligkeit gegenüber Protistenfraß aufweisen können, jedoch gibt es deutliche Unterschiede zwischen verschiedenen Arten. Zusätzlich wurde gezeigt, dass der Fraß kleinerer Zellen nicht notwendigerweise eine energetische Einschränkung für bakterivore Flagellaten bedeutet.

Fallstudie 2 Die ökologische Bedeutung bakterivorer Protisten in pelagischen Redoxklinen. Die Übergangszonen zwischen oxischen und anoxischen Wassermassen (pelagische Redoxklinen) beherbergen hochaktive prokaryotische Gemeinschaften, die wichtige Funktionen in biogeochemischen Stoffkreisläufen erfüllen. Über die ökologische

Rolle der Protisten in diesen Zonen ist jedoch bisher viel weniger bekannt. In dieser Arbeit wurde die Zusammensetzung der Protistengemeinschaft, deren Verteilung und die Bedeutung der Bakterivorie in zwei verschiedenen pelagischen Redoxklinen der zentralen Ostsee untersucht. Mittels Mikroskopiertechniken wurde eine deutliche Verschiebung der dominierenden Protistengruppen über den Redoxgradienten nachgewiesen. Kleine Ciliaten (*Strombidium* c.f.) sowie Dinoflagellaten dominierten die Protistengemeinschaften in suboxischen Tiefen (≤30 µM O_2). Große Ciliaten (*Mesodinium* spp., *Metacystis* spp., *Coleps* c.f. sowie unidentifizierte Morphotypen) wiesen ein Abundanzmaximum an der oxisch-anoxischen Grenzfläche auf. In sulfidischen Wassermassen war die Protistenabundanz sehr gering und wurde durch einige wenige Morphotypen (z.B. *Metacystis* spp.) repräsentiert. Frassraten wurden durch Langzeitinkubationen mit fluoreszenzmarkierten Bakterien (FLB) abgeschätzt und mit der Frequenz vireninfizierter Bakterienzellen (Verdünnungsansatz) verglichen. In suboxischen Wassermassen sowie an der oxisch-anoxischen Grenzfläche wurde die Mortalität der Bakterien hauptsächlich durch den Protistenfraß bestimmt (50-80% des Prokaryotenbestandes pro Tag). Die wichtigsten bakterivoren Organismen waren Ciliaten und gelegentlich Dinoflagellaten, nicht jedoch heterotrophe Nanoflagellaten (HNF). In den oberen sulfidischen Tiefen waren die Fraßraten unterhalb des Detektionslimits und die Anzahl vireninfizierter Zellen unterhalb von 5%, sodass der Hauptmortalitätsfaktor weiterhin eine offene Frage darstellt. Der spezifische Fraßdruck auf einen chemolithoautotrophen bakteriellen Schlüsselorganismus, die *Sulfurimonas*-Untergruppe GD17 (*Epsilonproteobacteria*), wurde mittels Räuber-Ausschluss-Experimenten bestimmt. Der Verlust durch Fraß war bei dieser bakteriellen Gruppe sehr hoch (>100% der neu produzierten Zellen d^{-1}), sodass Fraß deren Wachstum und möglicherweise auch deren vertikale Verteilung kontrolliert. Die hauptverantwortlichen Fraßfeinde der *Sulfurimonas*-Gruppe GD17 wurden durch RNA-basierende Stabile-Isotopen-Analyse (RNA-SIP) identifiziert. Dazu wurden ^{13}C-markierte Zellen des isolierten Vertreters der GD17-Gruppe (‚*Sulfurimonas gotlandica*' str. GD1) eingesetzt. Diese Analyse identifizierte drei Flagellatentaxa sowie zwei Ciliatentaxa als Fraßfeinde. Somit zeigt sich dass auch Vertreter der HNF eine wichtige Rolle als Fraßfeinde übernehmen, und zwar mit selektivem Fraßdruck auf einen aktiven und biogeochemisch signifikanten Prokaryoten.

Introduction

Taxonomically, what are protists?

The term protist refers to eukaryotic organisms, defined by the presence of membrane bound nucleus and vesicles, capable of existence as single cells (Caron et al. 2012). This rather simple definition encompasses 1.5 to 2 billion years of evolution (Knoll et al. 2006; Roger & Hug 2006; Javaux 2007) and thousands of different organisms with widely diverging habitats and lifestyles, ranging from 1µm unicellular algae (picoflagellates) in ocean surface waters (Courties et al. 1994) to 40mm testate amoeba living on the abyssal plain (Gooday et al. 2000). This enormous diversity has fascinated scientists since protists were first discovered in the 17th century, but has not been conductive to an easy systematics. Original classification systems separated heterotrophic animal-like protists (protozoa) from plant-like photosynthetic protists (microscopic algae), following the traditional two-kingdom system of Linnaeus 1766. This was maintained until the gradual recognition, starting in the late 19th century (Haeckel 1894), of the basal position held by protists at the origin of eukaryotic multicellularity (reviewed in Taylor 2003). Subsequently, numerous classification systems were developed, based both on morphological, and more recently, small subunit ribosomal DNA (18S rDNA) information (e.g., see reviews by Cavalier-Smith 2002; Corliss 2002), which included several attempts to unify all protists in one kingdom (e.g., kingdom Protista (Whittaker 1969)). However, to date, no absolute consensus has been reached, with even such basic questions as what defines a protist species still under discussion (Caron et al. 2009). At present, protistan taxa are mingled with multicellular forms within 6 supergroups in the domain Eukarya (Adl et al. 2005; Baldauf 2008; Caron et al. 2012). This recognises two fundamental facts: a) that the old kingdoms of animals, plants and fungi (Whittaker 1969) are minor branches among the eukaryotes, with close affiliations to unicellular forms; and b) that protists constitute the bulk of eukaryotic diversity (Caron et al. 2012).

Ecological role of protists

Protists require water for motility, feeding and growth, being therefore strictly aquatic organisms. However, they are not restricted to `traditional´ aquatic systems, such as lakes or oceans, but can also be found at much smaller-scale environments, including the water collected in the pores of soils and certain plant structures (e.g., in tank bromeliads (Foissner & Wolf 2009)), forming thin layers on the surfaces of plants and litter; or in the tissues of `higher´ organisms (Fenchel 1987). These widely differing environments have lead to a variety of different lifestyles and morphological adaptations. Extreme examples are some obligate parasitic protists, like the fungi-related microsporidia, which have strongly reduced their genomes and metabolism as an adaptation to life in their host (Keeling & Fast 2002). In the following text, I will centre on protists inhabiting large-scale water masses (i.e., lakes and oceans), and to a smaller extent, the underlying sediments, due to their larger global relevance in energy and matter cycling.

Protists can be phototrophic or heterotrophic, with both life-forms co-occurring in most major lineages of unicellular eukaryotes (Sherr & Sherr 2002; Jürgens & Massana 2008). The two nutritional modes are additionally not mutually exclusive, with many protists permanently or temporarily employing both in a phenomenon known as mixotrophy (Sanders 1991; Raven 1997; Esteban et al. 2010). Whether phototrophic, heterotrophic or both, protists have been found to be key-components of virtually all aquatic systems studied to date. Photosynthetic protists are major contributors to the standing stock of biomass and primary production in euphotic water masses (Corliss 2002; Sherr et al. 2007; Caron et al. 2009). In marine and oceanic surface waters, where roughly half of global primary production occurs (Field et al. 1998), sporadic blooms of phytoplankton in micro- and nano size-ranges (respectively, 20 to 200 µm and 2 to 20 µm in size), formed i.e. by diatoms, dinoflagellates or coccolithophorids, can dominate primary production and be seen from space (Sarthou et al. 2005; Sherr et al. 2007). Even in non-bloom conditions, picoeukaryotes (<2 µm) can constitute a significant fraction of total oceanic phytoplankton biomass and primary production (Worden & Not 2008). As an example, a study conducted at a coastal Pacific Ocean site showed that an assemblage of picoeukaryotes, including the prasinophyte *Ostreococcus*, was responsible for on average 76 % of picophytoplanktonic primary production, exceeding the contribution of the cyanobacteria *Prochlorococcus* and *Synechococcus* (Worden et al. 2004).

Heterotrophic protists are equally important, acting as consumers of prokaryotic and eukaryotic biomass. The general phenomenon of protist capture and consumption of prey is referred to as protist grazing, and has traditionally been divided into grazing on non-phototrophic bacteria and archaea (bacterivory), on cyanobacteria and `unicellular´ algae (hervibory), on smaller heterotrophic protists (often referred to as predation, though this term is also used for general protist grazing), and on a range of different prey (omnivory). Feeding on colloidal and viral particles has also been observed (González & Suttle 1993; Bettarel et al. 2005), but present knowledge does not indicate that it constitutes a significant carbon source for protists (Miki 2008). The combined effect of bacterivory and hervibory is one of the most important regulating factors for the prokaryotic and phytoplanktonic standing stocks in nearly all aquatic systems studied to date (Sherr & Sherr 2002; Pernthaler 2005; Jürgens & Massana 2008). A 1 : 1 relationship between bacterivory and prokaryotic productivity has been observed for many freshwater and marine systems (Sanders et al. 1992); while hervibory can consume up to 100 % of primary production (reviewed in Sherr & Sherr 2002). However, the importance of heterotrophic protists does not lay solely in their function as a mortality factor, but in their key role as members of complex food webs in aquatic systems.

Significance of protist grazing in aquatic microbial food webs

In 1974 Pomeroy wrote a seminal paper in which he hypothesised that the `classical´ food web (algae – zooplankton – fish) might only account for a small fraction of energy and matter flow in aquatic systems. Instead, heterotrophic bacteria and protists would be the major consumers and account for a large fraction of respiration. This idea was later formalized by Azam et al. 1983 in the `microbial loop´ concept. The premise was that heterotrophic bacteria would primarily be grazed upon by heterotrophic nanoflagellates (HNF), which in turn would be consumed by microzooplankton. As a side effect of protist grazing dissolved organic carbon (DOM) and nutrients would be released, and this would in turn fuel heterotrophic bacterial growth. The result would be a more or less closed system which would be embedded in the `classical´ food web through mesozooplankton grazing upon microzooplankton. The concept was later widened, under the term microbial food web, to include microbial primary producers and herviborous protists (Sherr & Sherr 1994) (Fig. I).

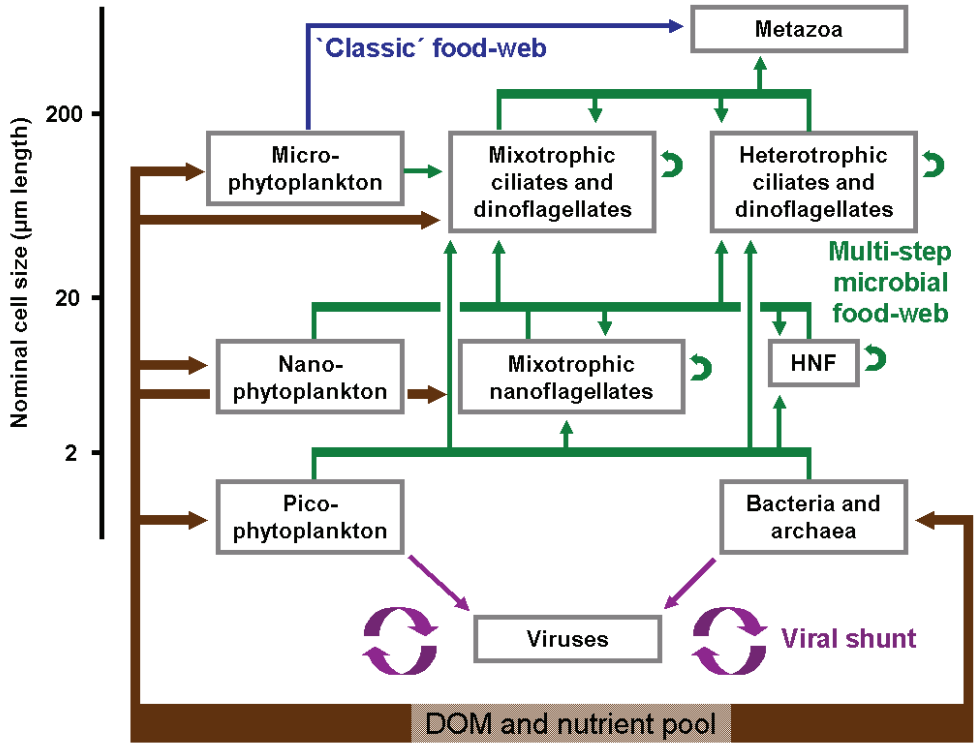

Figure I. *Simplified version of the microbial food web showing the principal flows of carbon and energy: protist mediated multi-step microbial food web, `classical´ algae-metazoa food web, and viral shunt. Processes of uncertain ecological relevance, e.g., protist feeding on viruses, are not included. Only the `bottom-up´ flow of carbon and energy is shown, excluding arrows indicating DOM and nutrient-pool renewal. Phytoplankton take up dissolved inorganic nutrients from the DOM and nutrient pool, while prokaryotes take up both. Nominal cell-size scale bar indicates the typical size-range classification: pico- (<2 µm), nano- (2 - 20 µm) and micro- (20 - 200 µm). HNF: heterotrophic nanoflagellates.*

An experimental body of evidence, collected primarily during the 80s and 90s, confirmed these hypotheses (see reviews in Sherr & Sherr 2002; Pernthaler 2005; Jürgens & Massana 2008). HNF in the 2 – 5 µm size-range were shown to be a primary mortality factor for bacteria in marine and freshwater systems (Fenchel 1982b; Sanders et al. 1992; Vaqué et al. 1994), and serve as food for larger protists (Solic & Krstulovic 1994; Jürgens et al. 1996; Zöllner et al. 2009), which were in turn demonstrably predated on by metazoans (Stoecker & Capuzzo 1990; Jeong 1999; Zöllner et al. 2009). However, numerous shortcuts were also found. HNF were shown to be able to use the DOM pool directly (Sherr 1988; Tranvik et al. 1993), thus by-passing prokaryotes and phototrophic eukaryotes. Ciliates and dinoflagellates were found to also occasionally be significant bacterivores (Sherr et al. 1987;

Šimek et al. 2000; Seong et al. 2006; Karayanni et al. 2008). Finally, the classical `algae – mesozooplankton´ food web still takes place (Carpenter et al. 2001; Stibor et al. 2004); and metazoans were proven to sporadically become important bacterivores, e.g., *Daphnia* spp. in freshwater systems (Jürgens et al. 1997; Callieri et al. 2006).

On the other hand, it was shown that grazers not only affect prokaryotic and phytoplanktonic communities negatively, but can also stimulate their proliferation through the release of nutrients (Goldman et al. 1985; Caron et al. 1990; Eccleston-Parry & Leadbeater 1995; Selph et al. 2003). The very graphically named `sloppy-feeding process´, by which metabolic waste products and undigested prey parts are released to the environment (Lampert 1978), increases the pools of particulate and dissolved organic matter (respectively POM and DOM) (Strom et al. 1997; Ferrier-Pagès et al. 1998; Nagata 2000) and of dissolved inorganic nutrients (Dolan 1997). In the latter case, the production of nitrogen and phosphorous could be particularly important, given that they are limiting elements in many aquatic environments (Sterner 2008; Deutsch & Weber 2012). Prokaryotes have a higher nitrogen and phosphorus concentration per unit of biomass than eukaryotes, owing to the higher ratio of proteins and nucleic acids to total cell-mass (Simon & Azam 1989; Vadstein et al. 1993, 2003; Pernthaler 2005). Protist grazers release these excess nutrients that are not required for growth, thus making them available again for prokaryotic and phytoplanktonic growth.

Overall, protists are integral parts of the microbial community, fostering and consuming prokaryotic and phytoplankton growth. However, they are not the only major prokaryotic regulating factor, viruses can play an equally pivotal role (Wommack & Colwell 2000; Suttle 2007; Breitbart 2012), as will be addressed in the following section

Protists grazing vs. viral lysis

Viruses are the most abundant biological entities in aquatic systems, can equal protists in terms of biomass and are thought to be predominantly composed of bacteriophages (Breitbart 2012). Their contribution to prokaryotic mortality has been studied for a wide range of systems, though unfortunately rarely at the same time as protist grazing, making direct comparisons difficult (Fuhrman & Noble 1995; Miki 2008; Breitbart 2012). It should also be noted that quantifying viral lysis is harder than determining protist grazing rates. However, it is clear that viral lysis can be as, or on occasions more important, than protist grazing in controlling prokaryotic biomass (Fuhrman & Noble 1995; Wommack & Colwell 2000; Suttle 2007; Breitbart 2012), but their effect on the microbial food web is very

different. Virally lysed prokaryotic biomass returns to the POM, DOM and nutrient pools, without transfer of carbon and energy to higher levels in the food web, in the so-called viral-shunt (Fig. I) (Fuhrman 1999; Suttle 2007). Thus, in systems where viral lysis dominates, theoretical models predict that prokaryotic growth and respiration would be enhanced at the expense of biomass at higher trophic levels (Breitbart et al. 2008). The potential for viruses to enhance prokaryotic respiration has been shown experimentally (Middelboe & Lyck 2002), and viral lysates are thought to provide from 4 to 95 % of prokaryotic carbon demand in different aquatic environments (Breitbart et al. 2008). However, the quantitative incorporation of this concept to microbial food web models is difficult, since viral lysates also constitute a nutrient source for phytoplankton production, part of which will be channelled back to higher trophic levels (Breitbart et al. 2008; Breitbart 2012).

A second important aspect of viral lysis is the high host specificity thought to be exhibited by most, though not all, viruses (Breitbart 2012). This lead to the development of the `kill the winner´ theory (Thingstad & Lignell 1997; Thingstad 2000b), which postulated that selective virally induced mortality would suppress numerically dominating prokaryotic groups and allow less abundant bacteria to become dominant. Experimental data supported this theory, albeit with highly variable results between locations and experiments (reviewed in Breitbart et al. 2008), and led to the establishment of a second theory. This proposed that protist grazing would generally control prokaryotic biomass while viral lysis would primarily control prokaryotic diversity (Thingstad & Lignell 1997; Thingstad 2000b). However, this second theory was based on a `black box´ approach to protist grazing. The identity of both the protist grazers and the prokaryotic prey were ignored, on the premise that a given protist grazer would handle all particles in an edible size-range equally. This is generally far from the case, with complex species-specific interactions taking place between bacterivores and prey, which can shape the structure of the prokaryotic community (Hahn & Höfle 2001; Jürgens & Matz 2002; Sherr & Sherr 2002; Pernthaler 2005; Jürgens & Massana 2008).

Interactions between protist grazers and their prokaryotic prey

When considering interactions between bacterivores and their prey, two important facts should be taken into account: (1) It encompasses an enormous diversity of protist grazers and prokaryotes, both of which will be found in different physiological states, from actively growing to dormant. This additionally includes different feeding modes in

protists, ranging from interception-feeders, which handle each prey cell individually, to filter-feeders, which clear water of particles by means of mesh-like structures (reviewed in Boenigk & Arndt 2002; Fenchel 1987). (2) It is an immensely old process, likely constituting one of the first interactions between eukaryotes and prokaryotes (Cavalier-Smith 2002), and therefore has been subject to millions of years of co-evolution. Both facts lead to a very complex array of interactions, which are only just beginning to be understood. A series of studies conducted with laboratory cultures have allowed a first glimpse of this complexity and uncovered some basic principles (Fig. II) (see reviews by Hahn & Höfle 2001; Jürgens & Matz 2002; Sherr & Sherr 2002; Matz & Kjelleberg 2005; Pernthaler 2005). However, the lack of information for certain types of interactions, and the lack of cultures for many relevant groups of bacterivorous protists and prokaryotes in marine and freshwater systems, leaves many open questions in this field.

Interactions between bacterivores and prokaryotes can occur at different stages during the feeding process, and can alter the probabilities of the predator encountering a given prey, and the preys subsequent capture, ingestion and digestion (Jürgens & Matz 2002) (Fig. II). At each stage, different traits of the protist and prokaryote can lead to a `selection´ by the protist. This can be an active selection, with the protist discriminating between different prey (e.g. through the cell-surface characteristics or `taste´ of the prokaryotic prey (Hahn & Höfle 2001; Jürgens & Matz 2002)); or a passive or indirect selection, where traits of a specific prokaryote alter the chances of it being found, captured or consumed without active discrimination by the protists (e.g. resistance to digestion (Boenigk et al. 2001)).

Prokaryotic size and morphology are two factors that have repeatedly and convincingly been linked to changes in the chances of prey encounter, capture and ingestion (Hahn & Höfle 2001; Jürgens & Matz 2002; Sherr & Sherr 2002; Matz & Kjelleberg 2005; Pernthaler 2005). At the encounter phase, simple geometrical models show that the odds of a given protist encountering a prey increase with the size of the prey particle until a certain limit (Fenchel 1982a). Thus, very small prokaryotes are less likely to be `found´ by grazers than larger ones. Upon encounter, small-sized bacteria can avoid capture by filter-feeding protists by being too small to be retained in the filtration structures. Finally, at the ingestion stage, prokaryotes can become too large or morphologically too complicated to be engulfed by the protist food vacuole (e.g., filaments or spiral-shaped cells). Prokaryotic motility, frequently observed in free-living prokaryotes of aquatic systems (Fenchel 2001; Grossart et al. 2001; Herzog & Wirth 2012), is another such factor,

but is considered a `two-sided coin´ (Pernthaler 2005). Higher speeds increase encounter rates with predators (González et al. 1993), but at the same time enable the possibility of escape during capture and ingestion phases (Matz & Jürgens 2005) (Fig. II).

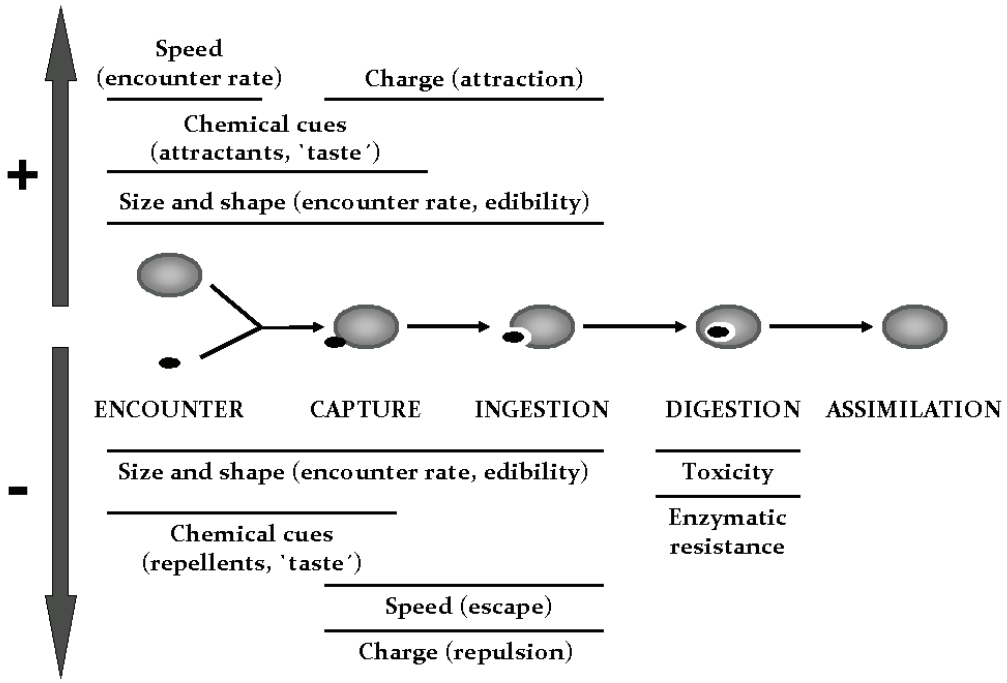

Figure II. *Examples of prokaryotic traits that increase (+) or decrease (-) the probability of a prokaryote being found, captured and consumed by a protist predator. Adapted from Jürgens & Matz 2002.*

Given the large number of stages at which selection can act, it is very likely that all bacterivorous protists will to a certain extent `select´ their prokaryotic prey, either passively or actively. However, for some, especially interception feeding flagellates, it will be a more intensive process than for others. Protist intra-specific variability should also be considered (Weisse 2002). Protists can present complicated life cycles, and will potentially show varying selectivity at different stages. Additionally, experimental data strongly suggests that feeding history can alter the level of selectivity, with protists in prey-limited conditions being considerably less `choosy´ than their well-fed counterparts (Jürgens & DeMott 1995; Boenigk et al. 2002).

In all, these complex patterns in inter-specific and intra-specific traits lead to `prey selectivity´ by protists (here including both active and passive selection). On an individual level, this means that a given protist grazer will not consume all prey equally, and that a given prokaryotic prey will not be consumed equally by all grazers.

Implications of grazer selectivity at a community level

Extrapolated to the community level, prey selectivity implies that protists not only regulate the biomass of prokaryotic communities, but can also affect their phenotypic and phylogenetic composition (Hahn & Höfle 2001; Jürgens & Matz 2002; Sherr & Sherr 2002; Pernthaler 2005; Jürgens & Massana 2008). In freshwater systems, community manipulation experiments convincingly linked high abundances of filamentous bacteria and ultramicrobacteria to high grazing pressure (Šimek et al. 1997; Posch et al. 1999). This development of grazing `resistant´ phylotypes was further proven in chemostat cultures with mixed bacterial communities, were addition of bacterivores lead to a strong bidirectional shift in the prokaryotic size-distribution towards smaller cells and larger filamentous forms or aggregates (Hahn & Höfle 1999; Corno 2008). In marine systems, filamentous bacteria do not tend to occur, but small, often highly motile forms are common (Matz & Jürgens 2003; Pernthaler 2005). Changes in phylogenetic community composition under varying grazing pressure have also been experimentally proven, both through community manipulation techniques (Jürgens et al. 1999; Langenheder & Jürgens 2001; Šimek et al. 2007; Corno 2008) and analysis of food-vacuole content (Jezbera et al. 2005, 2006; Bautista-Reyes & Macek 2012). Moreover, bacterivores leave a direct imprint on the taxonomic composition of prokaryotic communities (Pernthaler 2005), which can be seen e.g., by the numerical importance of putatively grazing resistant Gram-positive *Actinobacteria* in freshwater prokaryotic assemblages (Hahn et al. 2003; Tarao et al. 2009).

Grazing selectivity also has the potential to impact the activity and distribution of given prokaryotic groups (Hahn & Höfle 2001; Jürgens & Matz 2002; Sherr & Sherr 2002; Pernthaler 2005; Jürgens & Massana 2008). Actively growing prokaryotes tend to be larger than non-growing or dormant forms. Thus, in systems where prokaryotes tend towards small cell-sizes, prokaryotic grazing will primarily impact the actively growing portion of the prokaryotic community. As a direct effect, some heavily grazed members of the prokaryotic community are thought to shift away from their zones of growth optimum to areas with reduced grazing pressure (Tittel et al. 2003).

The full *in situ* spectra and implications of selective protist grazing are to a large part still unknown, partly due to the numerous restriction and biases imposed by the existing methodologies to date (McManus & Fuhrman 1988; Vaqué et al. 1994; Dolan & McKeon 2005; Landry & Calbet 2005). However, it's clear that protist grazing is a major regulating factor for prokaryotic and phytoplanktonic communities in aquatic systems, not only controlling biomass production, but also their composition, activity and distribution.

Open questions in protist ecology

As seen above, a considerable body of work has been amassed over the last decades on the ecological role of protists in aquatic environments. These studies have firmly established protists as ubiquitous, extremely diverse eukaryotic organisms with pivotal roles in microbial food webs and ecosystem functioning. However, research has lagged behind that of prokaryotes (Caron et al. 2009), and many open questions remain, ranging from single-cell interactions to the community level (Montagnes et al. 2008). The recent application of 18S rRNA gene based techniques revealed a wealth of novel, environmentally relevant protist groups, only distantly related to cultured representatives (López-Garcia et al. 2001; Moon-van der Staay et al. 2001; Massana et al. 2004; Not et al. 2009). The range and depth of their interactions with in situ prokaryotic communities, and how well the ecological principles obtained through studies with well-characterized laboratory protist strains can be extrapolated to these novel protist groups, are relevant questions that remain to be answered. Also important is to extend our knowledge away from oxygenated surface waters, and include other environments, such as sediments, deep-sea water masses and anaerobic zones. These ecosystems differ strongly in their environmental parameters and constraints, and remain tantalisingly under-explored. It should additionally be noted, as has been mentioned in the preceding text, that even for aerobic conditions and well-studied protist species, many relevant points remain unknown.

For the present thesis, two case studies of under-researched fields in protist ecology and protist-bacteria interactions were selected based on their potential global relevance, and will be expanded on in the following text:

1. A study focusing on interactions between single predator and prey species, analysing the vulnerability of carbon-starved bacteria to bacterivores with regard to its implications for protist grazing in nutrient depleted environments.

2. An environmental study, focusing on grazer-prey interactions at a community level in a hitherto understudied aquatic environment, marine suboxic to anoxic water columns.

The starvation-survival state in bacteria

The fact that all ecosystems are energy driven is a basic ecological principle. The amount of energy entering a system, its quality and bioavailability, and its turnover rate, will determine the biomass and productivity of the system (Kjelleberg 1993; Morita 1997). However, with the recognition in the mid 20th century of the importance of heterotrophic bacteria in aquatic systems (Pomeroy 1974; Azam et al. 1983), came the realization that most of these systems are oligotrophic, and do not receive enough energy to support the average 10^5 to 10^6 prokaryotes per ml^{-1} found there. Thus, if all cells were alive and equally active, they would be starved (reviewed in Kjelleberg 1993; Morita 1997; Giorgio & Gasol 2008). Subsequent research showed that bacterial populations could be divided into actively growing cells, cells that appeared damaged or dead, and a large proportion of apparently intact cells that showed little or no metabolic activity. These latter cells have been referred to as dormant, latent, starved, quiescent or inactive (Giorgio & Gasol 2008).

One of the most obvious causes for latency is the carbon-starvation or starvation-survival state (Giorgio & Gasol 2008). Studies with cultured heterotrophic bacteria have revealed a fairly consistent response to severe carbon and energy limitation (Kjelleberg 1993; Morita 1997; Britos et al. 2011). In a first stage bacterial chemosensory and quorum sensing mechanisms `alert´ the cell that conditions are turning unfavourable and activate a regulatory cascade which starts the starvation-survival response (McDougald et al. 2000, 2003; Brackman et al. 2009). This leads to an initial down-regulation of metabolical activity and an increase in protein degradation. If starvation persists, a series of reorganization events occur, which include changes in membrane composition, reductive division, development of chemotactic responses to diverse solutes, degradation of reserve material, and the onset of development of resistance against a variety of stress factors (Kjelleberg 1993). Finally, a gradual decline in metabolic activity occurs, leaving the cell in a dormant or latent state. The end result is a bacterium with a severely reduced cell-size and metabolic activity, which is strongly resistant to abiotic stress factors and adapted to quickly respond to renewed carbon availability. Oligotrophic ultramicrobacteria can also develop a starvation-survival response, but the changes are less drastic, since they are already small cells (<0.1 μm^3) with a tendency towards high resistance to abiotic stress factors (Cavicchioli et al. 2003; Hahn et al. 2003; Lauro et al. 2009).

The characteristics of the carbon-starvation response lead to it being considered a long-term survival strategy for heterotrophic bacteria (from months to years) (Kjelleberg

1993). A test conducted with several marine isolates showed that cells remained intact and could respond to new nutrient inputs after 8 months of starvation (Amy & Morita 1983). This is partly due to a remarkable resistance to abiotic stress (Kjelleberg 1993). Studies conducted with *Photobacterium angustum* showed that the percentage of surviving cells after heat shock or UV exposure rose from practically 0 when non-starved to almost 100 % when carbon-starved for respectively 10 and 40 h (Nyström et al. 1992). They are additionally thought to be in a sense `immune´ to viral lysis due to the practical absence of metabolic activity (Giorgio & Gasol 2008). However, to date it is not known whether this extensive cross-resistance also encompasses a reduced vulnerability to protist grazing.

The implications of such a resistance could be important for oligotrophic systems. No adequate methodologies exist for quantifying the abundance and heterogeneity of different metabolic and physiological states in situ (Giorgio & Gasol 2008). However, it is thought that two principal strategies exist for surviving in energy limited environments: high affinity continued nutrient uptake with slow growth; or fast growth when nutrients are present followed by entry into the starvation-survival state. Models comparing the two strategies do not always show a benefit for the latter (reviewed in Giorgio & Gasol 2008). Instead, they postulate that only when the periodicity of nutrient input is long compared to the minimum doubling time of a bacterium, does the starvation-survival state present an advantage. However, these models were conducted from a bottom-up nutrient control perspective, and did not take into account potential differences in the magnitude of loss processes for bacteria conducting the two strategies. This latter fact will also affect the standing stocks of available prey for protists. Thus, the resistance or not of carbon-starved bacteria to protist grazing is an important point that should be considered.

Suboxic to anoxic water masses

Suboxic (defined here as <30 µM oxygen (O_2)) and anoxic environments are found in a variety of habitats, from sediments to the intestinal tracts of animals, and are primarily caused by an O_2 demand for organic matter decomposition which exceeds supply. As anoxia develops, anaerobic microbial degradation takes over, and the metabolic end products (e.g., hydrogen sulphide (H_2S) or hydrogen (H_2)) cause chemically reducing conditions (Fenchel & Finlay 1995). An alternative, globally less relevant cause for anaerobiosis is geothermal activity, which releases water rich in reducing solutes such as H_2S (e.g. hot springs and hydrothermal vents) (Fenchel & Finlay 1995). The resulting

transition zones between oxidizing and reducing conditions are termed redoxclines, and in systems where H_2S is produced, the zone of its first appearance is called chemocline. In open water masses, suboxic to anoxic conditions generally occur in highly productive areas where stratification (i.e, due to temperature or salinity) or weak circulation, lead to a limited replenishment of water depleted of O_2 through organic matter decomposition. These zones can be found worldwide in freshwater, brackish and marine systems. Deep, aphotic, pelagic redoxclines are present in e.g., the Black Sea (Yakushev et al. 2008), the Cariaco Basin off the coast of Venezuela (Taylor et al. 2006), and the Baltic Sea (described in detail in later sections). Other systems have shallow euphotic redoxclines, like the Framvaren and Mariager Fjords (Yao & Millero 1995; Zopfi et al. 2001) and certain lakes (e.g. lake Cisó in Spain (Casamayor et al. 2008)). Finally, water masses with permanent or temporary low O_2 concentrations, which can become anoxic and sometimes sulphidic, occur at mid-water depths in coastal oceanic zones located near upwelling areas with high productivity (Oxygen Minimum Zones (OMZ)) (Stramma et al. 2008; Lam & Kuypers 2011) and in lakes (e.g., Mallin et al. 2006; Gobler 2008)).

Life in suboxic to anoxic water masses

Redoxcline prokaryotic communities and biogeochemical cycling

Redoxcline systems are closely interlinked to the highly stratified and active prokaryotic communities they host. Diverse prokaryotic groups require the strong gradients in electron donors and acceptors found there for metabolism and growth. At the same time, by directly or indirectly mediating oxidation-reduction processes, prokaryotes become a major driving force behind element cycling (Pimenov & Neretin 2006), thus shaping the redox gradient in combination with abiotic processes. The role prokaryotic communities play in biogeochemical cycling has long been a focus of intensive studies, especially for nitrogen, sulphur, and carbon cycles (Taylor et al. 2001; Sievert et al. 2007; Jost et al. 2008; Lam & Kuypers 2011; Zehr & Kudela 2011) (Fig. III), revealing important roles in, for example, nitrogen loss processes.

Nitrogen is a critical element for new biomass production in aquatic systems since it is often in short supply relative to other nutrients (Zehr & Kudela 2011). Its availability depends on the balance of input- and loss processes, with major sources being atmospheric deposition, river runoff and nitrogen fixation; and important loss processes occurring in suboxic to anoxic zones of the water column and the underlying sediments

(Lam & Kuypers 2011; Zehr & Kudela 2011). As an example, it has been estimated that 30 – 50 % of the oceans nitrogen losses occur in OMZs (reviewed in (Lam & Kuypers 2011). Studies conducted to date have lead to a relatively good general understanding of nitrogen cycling in redoxcline systems (Fig. III). In suboxic zones, ammonia (NH_4^+) from organic matter remineralization is oxidized via nitrite (NO_2^-) to nitrate (NO_3^-) (nitrification). This processes is conducted by diverse prokaryotic assemblages, but the first step, from NH_4^+ to NO_2^-, is primarily conducted by *Beta-* and *Gammaproteobacteria*, and *Thaumarchaeota* from the marine cluster I (McCaig et al. 1994; Francis et al. 2005; Christman et al. 2011). In suboxic to anoxic zones, the produced NO_3^- is reduced to molecular nitrogen (N_2) by denitrifying bacteria (Zehr & Kudela 2011). Alternatively, in absence of H_2S, N_2 is produced by bacteria belonging to the *Planctomycetes* through the combination NH_4^+ and NO_2^-, in a process called anaerobic ammonium oxidation (anammox) (Kuypers et al. 2003; Lam & Kuypers 2011).

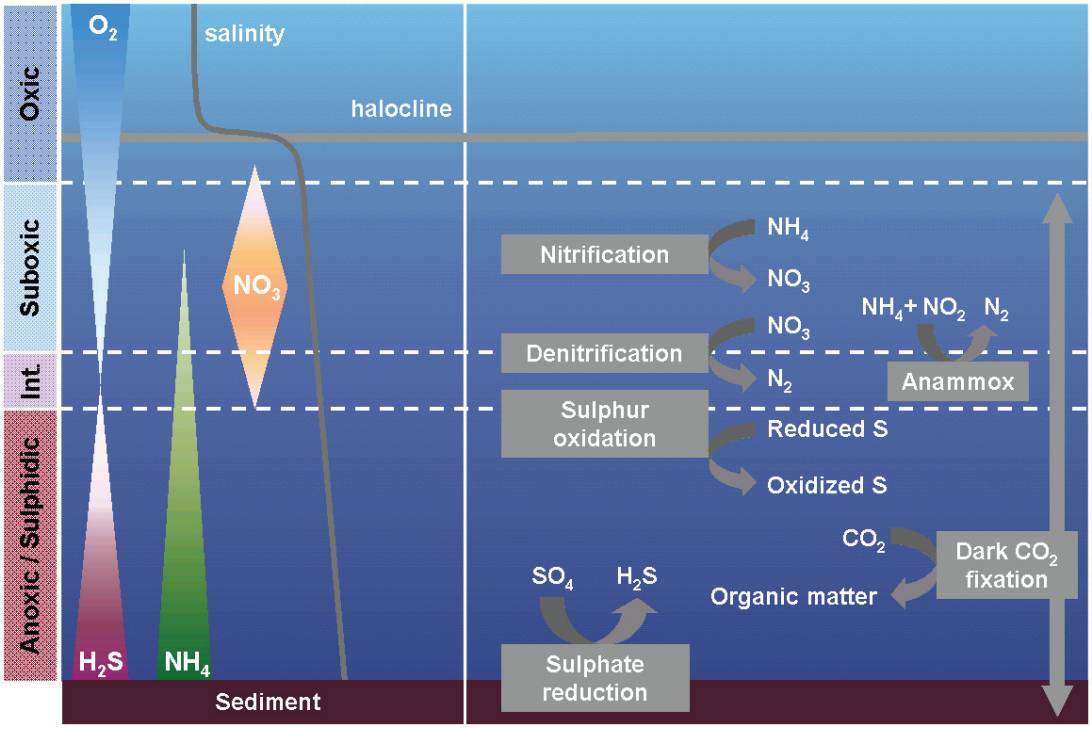

Figure III. *Idealized representation of a marine pelagic redoxcline, showing major biogeochemical processes involved in nitrogen-, carbon- and sulphur cycling.*

Sulphur compounds are the main energy link between aerobic and anaerobic processes (Sievert et al. 2007). In anoxic zones, organic material sinking to the sediment surface is degraded coupled to SO_4 reduction to H_2S (Fig. III). This process is conducted by

an assemblage of prokaryotes, which tends to be dominated by *Deltaproteobacteria* in mesophilic systems (Teske et al. 1996; Nedwell et al. 2004; Ince et al. 2006). H_2S then diffuses upwards and is reoxidized at the oxic / anoxic interface via biological pathways, which can be phototrophic or non-phototrophic, or through inorganic reactions (Sievert et al. 2007). This reoxidation is often coupled to the reduction of other compounds, for example NO_3, linking the sulphur cycle to that of other inorganic elements.

Finally, chemoautotrophy is an important component of the carbon cycle in redoxclines (Fig. III) (e.g., Taylor et al. 2001; Casamayor et al. 2008; Grote et al. 2008). This process, also called `dark CO_2 fixation´, refers to the fixation of CO_2 using the energy from favourable reactions between inorganic electron donors and acceptors instead of from light (photoautotrophy). Chemolithoauthotrophic prokaryotic assemblages tend to be dominated by *Epsilonproteobacteria* and *Gammaproteobacteria* (Glaubitz et al. 2009, 2010; Lavik et al. 2009) though other groups are also involved, such as *Thaumarchaeota* in suboxic zones (Varela et al. 2011; Yakimov et al. 2011), and sulphate reducing prokaryotes in sediments (Neretin et al. 2007).

Overall, successive studies have revealed the importance of redoxcline prokaryotes in biogeochemical cycling across different environments. However, substrate availability and redox optima are not always sufficient to explain the distribution and activity of the specific prokaryotic groups, and to date little attention has been given to other potential regulating processes, such as bacterivorous pressure and viral lysis.

Eukaryotic organisms in redoxcline systems

Suboxic to anoxic conditions are not favourable for `higher´ eukaryotic organisms, which tend to have low tolerance to O_2 deficiency (Marcus 2001; Wu 2002) and an extremely low tolerance to H_2S (Vismann 1991). Thus, higher organisms living permanently in pelagic redoxcline systems appear to be relatively rare, especially for sulphidic zones (Danovaro et al. 2010). Temporary forays to these areas have been seen to occur, for example by the amphipod *Orchomene obtusus* to feed (De Robertis et al. 2001), and could constitute an important link between oxic and anoxic food webs. However, it is clear that protists are the primary, and in some cases only permanent components of eukaryotic communities in suboxic to anoxic aquatic systems.

To date, most studies conducted on protists in pelagic redoxclines have centred on their abundance and diversity, analysed by both classical microscopy (e.g., Bark 1985; Fenchel et al. 1990; Zubkov et al. 1992) and 18S rRNA gene based methods (e.g., Stoeck et

al. 2003; Zuendorf et al. 2006; Orsi et al. 2011, 2012; Wylezich & Jürgens 2011). These studies, carried out for freshwater, brackish and marine environments, demonstrated that redoxcline protist communities can present high cell-abundances at oxic / anoxic interfaces, taxonomically differ from their counterparts in overlaying oxic water masses, and show strong changes in composition along the redox gradient. Additionally, a high proportion of `novel´ protist groups were found, only distantly related to the closest cultured representatives. However, due to the scarcity of cultured free-living redoxcline species, and the methodological constraints of maintaining *in situ* physico-chemical conditions, relatively little is known about the autoecology of redoxcline protist species and their ecological role.

From a physiological point of view, the metabolism of protists which do not use O_2 as a terminal electron acceptor (anaerobes) is practically unknown for pelagic redoxclines (Fenchel & Finlay 1991a, 1995; Ginger et al. 2010). The predominating theory is that free-living anaerobic protists will base their metabolism on fermentation and substrate level phosphorylation (Fenchel & Finlay 1991a). Degenerate mitochondria, called hydrogenosomes, which use the pyruvate:ferredoxin oxidoreductase to oxidize pyruvate to acetate with production of H_2, were discovered in the pathogen *Trichomonas* (Müller 1988; Boxma et al. 2005); and later with a different catabolising enzyme, the pyruvate:formate lyase, in anaerobic chytrids (*Neocallimastix* and *Piromyces*) (Akhmanova et al. 1998; Hackstein et al. 2006). They have been additionally inferred for numerous free-living anaerobic ciliates through ultrastructure studies and / or the presence of methanogenic endo-symbionts, which employ the released H_2 to reduce certain oxidated carbon compounds to methane (CH_4) (Fenchel & Finlay 1995; Hackstein et al. 2006). However, anaerobic respiration is also possible. NO_3 respiration has been found for diatoms, foraminiferans, and the microaerophilic ciliate *Loxodes* (Finlay 1985; Risgaard-Petersen et al. 2006; Pina-Ochoa et al. 2010); while homologues to the prokaryotic nitrite reductase (nirK) have been detected for several groups of protists (reviewed in Ginger et al. 2010). Additionally, SO_4 reduction, though not seen yet in protists, has been found for the fungi *Fusarium oxysporum* (Abe et al. 2007).

Another important factor for protists living in redoxcline systems is how they cope with O_2 and H_2S toxicity. O_2 toxicity arises from the production of O_2 radicals in conjunction with the absence or inadequacy of cellular detoxification mechanisms; and from its ability to destroy or inactivate certain enzymes (Fenchel & Finlay 1991a, 2008). Cultured anaerobic protists have been shown to respire O_2, though the lack of

cytochromes impedes its coupling to energy conservation. Instead, it is thought to act as a detoxification method, maintaining anaerobiosis inside the cells at low O_2 tensions (up to 4 – 5 µmol O_2 l^{-1}) (Fenchel & Finlay 1995). H_2S, on the other hand, acts as a respiratory inhibitor to aerobic organisms, and can also inhibit anaerobic organisms at high concentrations (Fenchel & Finlay 1995). Anaerobic protists studied to date tend to be quite tolerant to H_2S, showing 100 % viability up to 2 - 30 mM, though the underlying mechanisms behind this tolerance are poorly understood (Massana et al. 1994; Fenchel & Finlay 1995; Atkins et al. 2002).

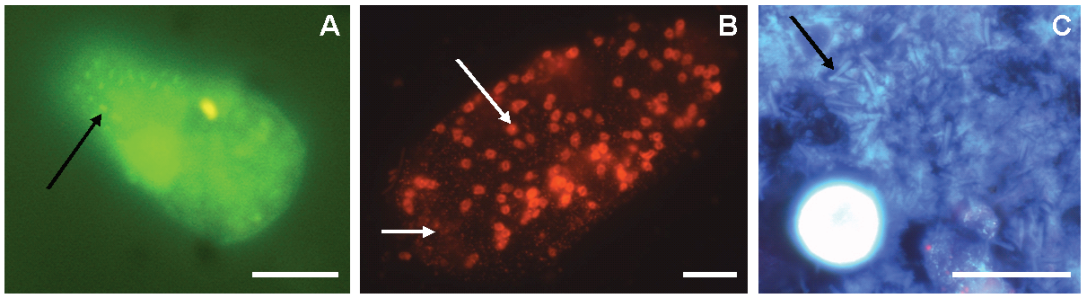

Figure IV. *Epifluorescence microscopy photographs of ciliate cells containing putative symbionts (marked by arrows) from Baltic Sea anaerobic zones. A and B: Fluorescence In Situ Hybridization (FISH) with specific probes for Eubacteria; photograph B courtesy of F. Weber (unpublished data). C: non-specific DAPI staining. Scale-bar represents 20 µm.*

The wide-spread occurrence of prokaryotic endo- and ectosymbionts in anaerobic protists is probably closely linked to both metabolism, and O_2 and H_2S tolerance, and requires further study (Fenchel & Finlay 1991a, 1995; Edgcomb et al. 2011c, 2011d) (Fig. IV). The role of the majority of these symbionts is unknown, but they have been shown to have a profound impact on their hosts. In the case of the methanogenic endosymbionts mentioned above, experimental inactivation or elimination of the symbiont resulted in a strongly reduced growth of anaerobic free-living ciliate hosts (e.g., *Plagiopyla frontata*, *Metopus contortus* and *Trimyema compressum* (Fenchel & Finlay 1991b; Shinzato et al. 2007)). Symbionts have also been shown to modify the behavior of their host, with an intriguing example set by the symbiosis between *Strombidium purpureum* and purple photosynthetic non-sulphur bacteria. The prokaryotic symbiont switches from anoxygenic photosynthesis in the light to oxidative phosphorylation in the dark, thus secondarily transforming *S. purpureum* from an anaerobe in the light to an aerobe in the dark (Fenchel & Bernard 1993). The `other side of the coin´ should be kept in mind too, with protist

hosts constituting favourable microhabitats within the redoxcline, potentially allowing processes to be carried out which would otherwise not be favoured at that depth.

From an environmental point of view, the points discussed above imply that protists could impact biogeochemical cycling though anaerobic respiration and as hosts for symbionts. However, their regulating influence as bacterivores should also not be disregarded, and remains vitally under-explored.

Redoxcline food webs

Redoxcline systems mark the transition from aerobic to anaerobic food webs, and either present chemoautotrophs as sole `primary´ producers (in dark redoxclines), or in combination with phototrophs (in euphotic redoxclines) (here, primary production is shown in brackets since it can also be considered secondary production, using compounds which proceed from organic mater decomposition, such as SO_4). Anaerobic food webs have in principle the same structure as their aerobic counterparts (Fig. I); but are postulated to support at most two levels of consumers, due to lower growth efficiencies (Fenchel & Finlay 1990). However, almost all knowledge of the carbon and energy flows through suboxic to anoxic food webs are based on models using predator and prey biomass and theoretical considerations (Fenchel & Finlay 1995). This is constrained by: (a) the lack of information on predominant metabolic pathways for free-living anaerobic protists, which impacts their efficiency in transforming prey biomass into new protist biomass, and thus the carbon and energy available for the next trophic level; (b) the magnitude of carbon being transported into the protist mediated microbial food web vs. the viral shunt; and (c) the magnitude of import / export processes between oxic, suboxic and anoxic compartments. All of these points remain under-researched to date, largely due to methodological constraints.

Table I summarizes the principal studies conducted on protist grazing and viral lysis for marine, brackish, and freshwater suboxic to anoxic zones. Clearly, no consensus exists on the predominating mortality factor for prokaryotes, even when excluding shallow redoxclines, where a seasonal influence can introduce variability. Weinbauer et al. showed a shift in the predominating prokaryotic mortality factor from protist grazing in oxygenated waters to viral lysis in anoxic / sulphidic zones (Weinbauer & Höfle 1998), but this is not always supported by other studies (Table I). Thus, the relative role played by protist grazers in regulating prokaryotic communities along redox gradients, and their

potential impact on biogeochemical cycling, are important questions that remain to be answered.

Table I. *Review on existing literature on protist grazing impact and viral lysis in suboxic to anoxic water masses; part I: Freshwater systems. HNF: heterotrophic nanoflagellates; PB: prokaryotic biomass;. PM: prokaryotic mortality; PSS: Prokaryotic standing stocks; EM: electron microscopy; PP: prokaryotic production.*

Study site	Target	Method	Zone	Results	Reference
Lake Cisó Spain	*Plagiopyla* sp.	FLB ingestion	Sulphidic	- <0.1 % PB d^{-1}	(Massana & Pedrós-Alió 1994)
Lake Plußsee Germany	HNF and viruses	HNF: literature clearance rates Viral lysis: EM	Suboxic Anoxic (non-sulphidic)	- Summed PM: 22- 57 %; 51- 91 % of PM caused by viruses - Summed PM: 43- 103 %; 88-94 % of PM caused by viruses	(Weinbauer & Höfle 1998)
Lake Eire U.S.A.	Protist community (pre-filtered: 200 µm) and viruses	Grazing : Dilution Viral lysis: EM	Suboxic	- Protists: 71- 78 % PSS; Viruses: 5 – 12 % PSS	(Gobler 2008)
Lake Adyat France (Seasonal study)	Ciliates, HNF and viruses	Grazing: ingestion of fluorescent beads Viral lysis: EM	Suboxic Anoxic (non-sulphidic)	- Summed mortality: 10 - 100 % PP, shared by viruses and HNF - 10 - 50 % PP, dominated by viruses.	(Bettarel et al. 2004)
Lake Alchichica Mexico	Ciliates	Analysis of food vacuole content	Anoxic	- Preference for: *α*-, *γ*- and *δ*-*Proteobacteria* and *Planctomycetes*	(Bautista-Reyes & Macek 2012)
Lake Faro Italy	Whole protist community	FLB ingestion	Sulphidic	- 36 – 72 % picoplankton production d^{-1}.	(Saccà et al. 2009)

Table I (continued). *Review on existing literature on protist grazing impact and viral lysis in suboxic to anoxic water masses; part II: Marine and brackish systems. HNF: heterotrophic nanoflagellates; PP: prokaryotic production; PM: prokaryotic mortality; CC: community composition; interface: zone were both O_2 and H_2S range around the detection limit.*

Site	Target	Method	Zone	Results	Reference
Gotland Deep; Baltic Sea	Protist community (pre-filtered: 100 µm)	Dilution (Note: water for dilution filtered under oxic conditions)	Sulphidic	- 48 % of gross PP cleared d^{-1}	(Detmer et al. 1993)
Gotland Deep; Baltic Sea	Ciliates	Theoretical clearance rates	Suboxic to interface	- 2 - 10 % of water volume cleared d^{-1}	(Setälä & Kivi 2003)
Gotland Deep; Baltic Sea	Viruses	Mytomicin C and electron microscopy	Suboxic Sulphidic	- 11 - 19 % virally mediated PM - 27 - 50 % virally mediated PM	(Weinbauer et al. 2003)
Cariaco Basin	Whole protist community	Predator exclusion (filtration through 1.6 µm)	Interface to sulphidic	- Changes in prokaryotic CC, but generally no strong changes in abundance	(Lin et al. 2007)
Hypoxic Masan Bay	HNF and viruses	Protist grazing: FLB ingestion Viral lysis: Microscopy	Suboxic	- HNF: 0 - 100 % PP; viruses: 2 - 34 % PP	(Park & Cho 2002) (Choi et al. 2003)
OMZ, Chile	HNF	Selective inhibitors ; food vacuole analysis	Suboxic	- HNF: >100 % PP d^{-1}; 0 - 30 % of cyanobacteria production d^{-1}	(Cuevas & Morales 2006)

The Baltic Sea as a model pelagic redoxcline system

The Baltic Sea (Fig. V) is relatively young in geological terms, with its origin dating to about 1500 B.P. (Grasshoff 1975; HELCOM 1981). Today, it extends over 415.266 km^2, and constitutes a land-locked or inland sea. It is not a single basin, but a labyrinth of smaller and larger basins formed by the action of ice movement during and after the last glaciation on different types of rock (Grasshoff 1975). The largest of these basins is Gotland Deep, with 249 m, and the deepest is Landsort Deep, with 459 m. The Baltic Sea is connected to the North Sea through a series of narrow channels called the Danish straits (Øresund,

Great Belt and Little Belt) (Fig. V). Here, water entering or leaving the Baltic must pass through the 7 m deep Drogden Sill or the 18 m Darss Sill, with the consequence that water exchange with the North Sea is generally severely restricted. On the other hand, freshwater input is very strong in the Baltic Sea, both through river run-off and through direct precipitation which is not quite balanced by evaporation, leading to a net outflow of lower salinity water into the North Sea and further restricting the inflow of North Sea water into the Baltic (Reißmann et al. 2009). The result is a horizontal salinity gradient which oscillates between 25 ‰ in the south-western Baltic and 2-3 ‰ in the Gulf of Bothnia, making the Baltic Sea one of the largest brackish water masses in the world. The salinity gradient also extends to the vertical plane, with lower salinity water overlaying denser higher salinity water. This has lead to the formation of a permanent halocline, situated around 60 – 80 m in the central Baltic Sea basins, which restricts vertical mixing between over- and under-laying water masses (Grasshoff 1975).

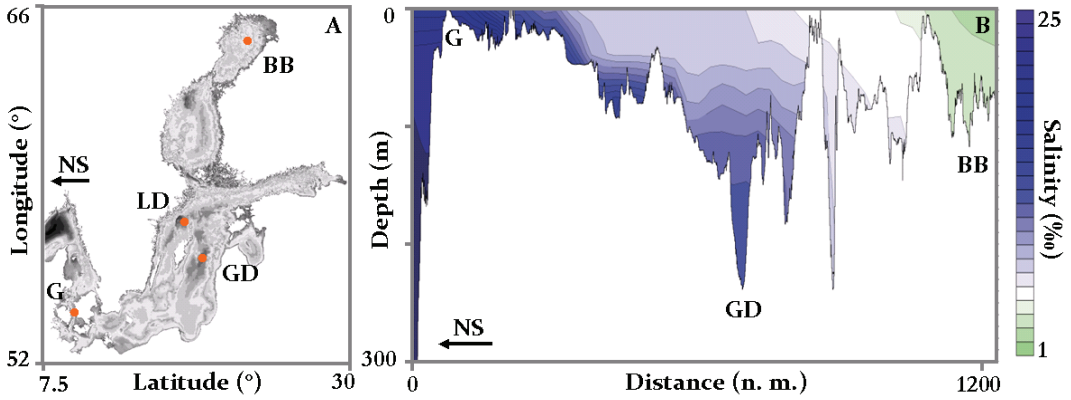

Figure V. *(A) Bathymetric map of the Baltic Sea (maximum depth shown ~600 m, already belonging to the North Sea (NS)). (B) Horizontal profile of the Baltic Sea showing the vertical salinity gradient. The contour plot includes the connection to the NS, the Great Belt from the Danish straits (G), the eastern Baltic Sea proper (including Gotland Deep (GD)) and the Bothnian Bay (BB) (the western Baltic Sea proper, including Landsort Deep (LD), is not shown).*

The restricted vertical and horizontal exchange results in the stagnation of Baltic Sea water masses. However, exceptional meteorological conditions can lead to higher inputs of high salinity oxygenated North Sea water, and consequent renewal of Baltic Sea bottom waters, in processes known as inflow events. These can occur after strong storm events, which cause a sea-level difference between basins located before and after the Danish straits (barotropic inflows (Lass & Matthäus 1996; Reißmann et al. 2009)); or after

prolonged periods of warm calm weather and stratification, caused by density differences across the Great Belt (baroclinic inflows (Feistel et al. 2003; Reißmann et al. 2009)). These sporadic inflows do not occur as single events, but are generally clustered in groups of 4-7, lasting up to 3 years (Matthäus & Franck 1992). Since 1983 there have been only two major Baltic Sea inflow events, in 1993 and 2003, with 10 year stagnation periods in between (Matthäus & Franck 1992). Prior to 1983 inflow events were more regular, but stagnation periods still lasted several years (Matthäus et al. 2008).

The Baltic Sea, through its strong river mediated terrestrial-input is a naturally nutrient rich system. However, since the start of the 20th century anthropogenic impact through industrialization, agriculture and farming have resulted in a strong eutrophication (Zillén et al. 2008). Inputs of phosphorous and nitrogen are especially important since they trigger phytoplankton growth and bloom formation. Photosynthetic primary production is very high in central Baltic Sea surface waters (Schneider & Kuss 2004; Dahlgren et al. 2010), and is characterized by two bloom episodes, one in spring, dominated by diatoms and dinoflagellates, and one in summer, dominated by cyanobacteria (Wasmund et al. 1998; Ploug 2008; Suikkanen et al. 2011). Most of the organic carbon produced is remineralized by heterotrophic bacteria in the oxic zone (Gast & Gocke 1988; Rheinheimer et al. 1989), but a significant fraction still sediments as POM to deeper water layers (Schneider et al. 2002). Decomposition of this organic matter takes place during sinking and at the sediment surface. Since the halocline impedes vertical mixing, O_2 depleted water does not get replaced, with the exception of during inflow events, and deep basins become gradually anoxic. The organic matter degradation process is then taken over by fermenting and SO_4 reducing bacteria, with production of H_2S, leading to sulphidic bottom waters (Piker et al. 1998). The resulting transition zone between oxic and anoxic water masses (i.e. the redoxcline), ranges over several meters, is situated in the aphotic zone and is marked by strong chemical gradients (Fig. VI).

Baltic Sea redoxclines (Fig. VI) host highly active prokaryotic communities that have been well characterized to date (Labrenz et al. 2005, 2007; Brettar et al. 2006; Jost et al. 2008). Successive studies have revealed the presence of key-prokaryotic groups, which can constitute a significant percentage of total cell abundance and are importantly involved in specific biogeochemical processes. These include the nitrifying *Thaumarchaeota* subcluster GD2 (Labrenz et al. 2010); and the *Sulfurimonas* subgroup GD17 (Grote et al. 2008, 2012; Grote 2009), involved in chemolithoautotrophy coupled to denitrification and sulphide reduction. *Gammaproteobacteria* have also been identified as potentially

important chemolithoautotrophs (Glaubitz et al. 2009), but their relevance in Baltic Sea redoxclines is still under investigation. However, despite this wealth of knowledge on the prokaryotes, very little knowledge exists on protist communities of these zones (e.g., Setälä 1991; Detmer et al. 1993; Stock et al. 2009).

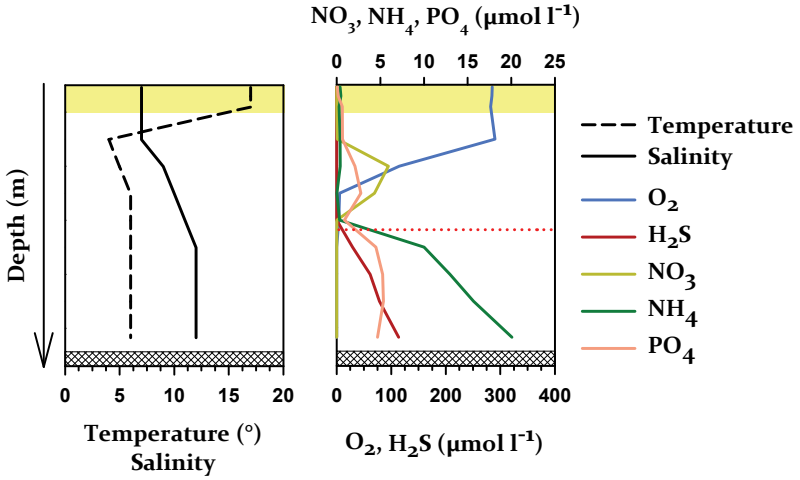

Figure VI. *Typical water column structure and chemical profiles for Baltic Sea redoxclines (measured in September 2009). The dashed bar at the bottom represents the sediments and the light bar at the top the euphotic zone. Dotted red line represents the chemocline, or first zone of detectable H_2S*

Thesis outline

In the present thesis, we aimed to uncover new aspects of protist ecology and protist-bacteria interactions by broaching specific topics of the two cases studies outlined above:

1. The starvation survival state in bacteria and its implications for protist grazers. The principal focus was to establish whether or not an increased resistance of carbon-starved bacteria to protist grazing can occur, therefore forming a base from which further studies on the implications for oligotrophic systems can be built.

In this regard, Chapter 1 of this thesis covers the use of predator / prey model systems to determine the vulnerability of carbon starved-bacteria to protist grazing. The growth of a model flagellate bacterivore is compared when fed starved and non-starved states of different model bacteria. Potential implications and underlying mechanisms are discussed.

2. Ecological role of bacterivorous protists in suboxic to anoxic water masses. The principal aim was to establish the relevance of protist grazers as a mortality factor for prokaryotic communities in pelagic redoxclines, using two deep Baltic Sea basins as model systems. Due to the scarcity of information existing on Baltic Sea redoxcline protist communities a multi-stage approach was developed aimed at uncovering the following points:

1. The diversity, abundance and distribution of the protist community throughout the redoxcline.
2. The impact of bacterivory on the total prokaryotic community, and its relative importance as a prokaryotic mortality factor compared to viral lysis.
3. The role played by bacterivores in regulating the growth and abundance of key-prokaryotic organisms involved in biogeochemical cycling.

In this regard, Chapter 2 of this thesis describes the use of different microscopy techniques to identify and quantify the principal morphotypes found in Baltic Sea redoxclines, and the analysis of the principal protist associations found along the O_2 gradient. The bacterivorous impact of the protist community at depths of differing physico-chemical conditions are assessed by tracer techniques, and compared to the impact of viral lysis, determined through the dilution approach. Finally, the relative role of different protist functional groups is estimated (ciliates, dinoflagellates and HNF), based on data proceeding from food-vacuole analysis and theoretical considerations.

Chapter 3 of this thesis describes the use of predator-exclusion experiments to determine the grazing impact on a prokaryotic key-player of Baltic Sea biogeochemical cycling, the *Sulfurimonas* subgroup GD17 (*Epsilonproteobacteria*). Additionally, the principal grazers on a cultured representative of this strain, "*Sulfurimonas gotlandica*" strain GD1, are identified by means of RNA-stable isotope probing.

Overall, this thesis provides one of the first assessments on the role the carbon-starvation response plays in the bacterial resistance to protist grazers; and it includes by far the most detailed description of the structuring and relevance as bacterivores of Baltic Sea pelagic redoxcline protist communities. It is additionally, the first study which conclusively proves that protists can regulate the growth, and potentially the distribution, of a key-prokaryotic group for redoxcline biogeochemical cycling.

Chapter 1

Species-specific patterns in the vulnerability of carbon-starved bacteria to protist grazing

Abstract

Many heterotrophic bacteria possess adaptations for prolonged survival under carbon and energy limitation, generally involving a reduction in cell size and an increased resistance to environmental stress factors. In order to reveal whether carbon-starved bacteria also become less vulnerable to protist grazing, we compared the growth of a bacterivorous nanoflagellate, *Cafeteria roenbergensis*, on different physiological states of 3 bacterial strains with well-studied starvation responses *(Vibrio vulnificus, Photobacterium angustum* and *Sphingopyxis alaskensis)*. Protists achieved high growth rates on all 3 bacterial strains when they were provided in a non-starved state. However, for carbon-starved bacteria, pronounced differences in the response of the flagellates were observed. *P. angustum* provided similar protist growth for an equal biomass of non-starved and starved cultures, indicating no change in food quality or grazing resistance for carbon-starved cells, despite smaller cell size. In contrast, starved *V. vulnificus* did not support protist growth, even resulting in a strong decrease in flagellate numbers at most concentrations tested; and starved *S. alaskensis* provided only reduced growth rates. The results obtained demonstrate that (1) feeding on bacteria of smaller cell size does not necessarily impose energy constraints on a flagellate grazer, and (2) a pronounced species-specific variability exists in the susceptibility of carbon-starved bacteria to protist grazing.

1.1. Introduction

Suspended bacteria in marine pelagic environments are strongly influenced by both top-down (predation) and bottom-up (resources) controlling forces. Organic substrate and inorganic nutrient concentrations are generally very low, requiring physiological adaptations for efficient substrate uptake and utilization as well as for long-term survival under carbon and energy limitation. In addition, pelagic environments offer little refuge against predation by bacterivorous protists, making necessary the development of strategies to decrease their vulnerability. How bacterial communities cope with these two selection forces has been the focus of numerous studies, revealing a wide range of

resistance mechanisms to protist grazing (Hahn & Höfle 2001; Jürgens & Matz 2002; Pernthaler 2005), and a well characterized carbon-starvation response (Kjelleberg 1993; Morita 1997). However, few studies have analyzed the response to the two forces jointly, centring on the potential ideal for planktonic prokaryotes, an adaptive response able to cope with both substrate limitation and grazing pressure simultaneously.

To date, the physiological and molecular mechanisms of the starvation response have been studied in detail for a number of bacteria, such as *Vibrio* spp (Kjelleberg 1993; McDougald et al. 2001, 2003), Enterobacteria (Jenkins et al. 1988; Galdiereo et al. 1994; Brauer et al. 2006) and others (Wrangstadh et al. 1980; Redon et al. 2005; Johnson et al. 2006). For these model organisms the final outcome is so-called non-growing "ultramicrobacteria" which are highly resistant against diverse stress factors (e.g., UV, chemical oxidants and high temperatures), remain viable for extended periods (from weeks to months or years) and can be recovered to normally growing cells by substrate addition. Interestingly, one of these adaptations, namely miniaturization of the cells, is known to be a major factor leading to a decreased vulnerability towards protist predation, due to a lowered grazer feeding efficiency on smaller bacterial cells (González et al. 1990; Šimek et al. 1994; Posch et al. 1999). This could therefore constitute a mechanism, directly developed or as an indirect benefit of the starvation survival response, for carbon starved bacteria to decrease their vulnerability towards grazers. Additionally, it becomes plausible to consider that the dominance of small or ultramicrobacterial cells among planktonic prokaryotic communities could partly be due to the combination of a high abundance of cells in the starvation-survival state and a preferential elimination of larger cell sizes by predators, particularly bacterivorous protists.

However, despite the potential importance of small cell-size, it alone may not be sufficient for long-term survival of starvation adapted, non-growing cells, in the presence of protist predators. For most interception-feeding flagellates there is no physical limitation for the uptake of small particles, being able to ingest even viruses (González & Suttle 1993) and colloids (Sherr 1988; Tranvik et al. 1993), though not at the same rates as larger particles. Geometric models predict that the clearance rate of interception feeders decreases approximately with the square of the particle radius (Spielman 1977; Fenchel 1982a). This encounter-based size-selectivity has been confirmed in feeding studies with fluorescent beads which revealed that particles of less than 0.5 μm are removed by typical bacterivorous nanoflagellates with a 4-6 times lower efficiency than 1 μm particles (Jürgens & Matz 2002). Extrapolated to the natural environment this would imply that in situations

where protist grazing accounts for the removal of bacterial standing stocks within one or a few days, small cell-size would only prolong survival to 1 - 2 weeks. These first-order estimates show that cell size miniaturization alone would not enable long-term survival in the presence of bacterial grazers.

These considerations, combined with the fact that the starvation-survival programme confers a high, long-term, resistance to abiotic stress factors, raises the question whether carbon-starved cells are also capable of prolonged survival in presence of protist grazers. It has been speculated that the starvation-induced differentiation program which results in cross-protection against different stress factors might also involve an increased protection against predators, for example by resisting the digestive enzymes inside protist food vacuoles (Jürgens & Matz 2002). Similar effects have been observed for bacteria with certain cell wall structures, such as gram-positive bacteria (Iriberri et al. 1994; Tarao et al. 2009); certain pathogens inside protists and macrophages (e.g. *Legionella*) (Barker & Brown 1994); and through the secretion of macromolecules and proteins (Greub & Raoult 2004). Additionally, other resistance mechanisms could act at different stages of the interaction between bacteria and protists (i.e. avoiding capture or ingestion) (Matz et al. 2002; Montagnes et al. 2008).

In the present study we aimed to take the first step of assessing whether the potential exists for carbon starved bacteria to become less vulnerable to protist grazing, thus creating a basis for future studies on long-term survival and potential resistance mechanisms. To this aim we selected three model bacteria with a clearly characterized carbon starvation response, but that differ in their lifestyle strategies. *Vibrio vulnificus*, found mostly in coastal temperate waters, often associated with plankton, shellfish and fish (Oliver 2006) and *Photobacterium angustum*, which was isolated from surface coastal waters, are both copiotrophic organisms with relatively large and fast growing cells, which quickly react to carbon limitation, forming the aforementioned starvation survival ultramicrobacterial cells. Conversely, *Sphingopyxis alaskensis* is a model oligotroph that grows with a constant maximum growth rate on low concentrations of substrates and maintains a relatively small cell volume (Lauro et al. 2009). We used the simple but effective approach of comparing protist growth rates on starved vs. non-starved cells. This provides an integrated measure of the grazers' ability to capture, ingest and digest bacterial prey and the subsequent efficiency in transforming it into protist biomass. Therefore, this methodology, though not revealing specific processes, encompasses both

the possible resistance mechanisms of these bacteria, and the potential differences in nutritional value between starved and non-starved cells.

1.2. Materials and methods

Bacterial strains and pre-cultures

The three bacterial strains used in this study, their specific starvation responses, and their subsequent increase in resistance to abiotic stress factors, have been described previously: *Sphingopyxis alaskensis* RB2256 (Schut et al. 1993; Schut 1994; Cavicchioli et al. 2003); *Photobacterium angustum* S14 (formerly *Vibrio angustum*) (Humphrey et al. 1983; Albertson et al. 1990; Nyström et al. 1992) and *Vibrio vulnificus* M06-24/O (Wright et al. 1990; Morton & Oliver 1994). All strains were obtained from the CMB (Centre for Marine Bio-Innovation, Universitiy of New South Wales, Sydney) and were maintained as glycerol stocks at -80°C.

V. vulnificus and *P. angustum* were routinely grown from glycerol stocks on Luria Broth agar with 2 % NaCl (LB20) and MMM2000 (Marine Minimal Medium (MMM) with 2 g l^{-1} of glucose (Östling et al. 1991); for all solutions 50 ng l^{-1} of Vitamin B_{12} were added after autoclaving (Cavicchioli et al. 1999). *S. alaskensis* did not grow well on LB20 so VNSS agar (Marden et al. 1985) was used instead. In all cases, incubation was at 30 °C, with orbital shaking at 150 rpm for liquid cultures.

Starved bacterial cultures were obtained as previously described (Holmquist & Kjelleberg 1993), with some modification. Bacteria were grown in MMM2000 until an optical density corresponding to mid-exponential phase was reached, namely OD_{610} = 0.2 – 0.3 for *V. vulnificus* and *P. angustum*, and OD_{433} = 0.3 for *S. alaskensis* (D. McDougald &. L. Ting, pers. comm.). Cultures were then washed with MMM, resuspended in the same media, and allowed to starve for 72 hours, to ensure that the cells were fully adapted to long-term starvation conditions (Kjelleberg 1993).

Non-starved bacterial cultures were obtained from overnight cultures grown in MMM2000, corresponding to mid- to late exponential growth phase. Starved and non-starved bacterial cultures to be used in a given experiment were inoculated from the same plate. With *S. alaskensis,* additional cleaning steps with MMM proved to be necessary, both for the non-starved and starved cultures, since strong bacterial growth was observed in the first grazing experiment with *C. roenbergensis*. This is likely due to the fact that this

bacterium has broad-specificity, high-affinity uptake systems, enabling efficient substrate scavenging (Cavicchioli et al. 2003), and can grow on low nutrient levels (Williams et al. 2009) such as those present in the inoculum.

C. roenbergensis pre-cultures

The widespread marine bicosoecid nanoflagellate *C. roenbergensis* (Fenchel & Patterson 1988) was selected as a model predator in tests prior to the experiments, due to its ability to grow well on the selected prey and growth conditions (data not shown). Two different stocks of *C. roenbergensis* were used. For the first experiment with *P. angustum* and the experiment with *V. vulnificus,* an axenic *C. roenbergensis* culture from the CMB was employed (described in (Matz et al. 2005)), while in all other experiments a culture from the Leibniz Institute for Baltic Sea Research was used (reference IOW23). Both were isolated from the Baltic Sea by A. P. Mylnikov. Cultures were routinely grown in MMM, at room temperature (23 - 25 °C) with moderate orbital shaking, using the bacterium to be tested in the experiment as food source.

To eradicate indigenous bacteria in the IOW23 cultures and obtain a protist culture with the desired bacteria as sole prey, serial dilutions in multiwell plates containing MMM as media were performed. Protists were allowed to grow 1-2 days at room temperature, with light orbital shaking and the desired bacteria as added food source. New multiwell plates were then inoculated from the highest dilutions that exhibited protist growth. This process was repeated until only the desired bacterial strain was detected (inspected by fluorescence microscopy and/or colony morphology on plates) and the final cultures were then used to inoculate the protist pre-cultures. The efficiency of the method was tested in the second experiment with *P. angustum* with an immunofluorescence assay using polyclonal antibodies against *P. angustum*, as previously described (Christoffersen et al. 1997).

Growth experiments

For each experiment, batch cultures were inoculated in tissue culture flasks (Sarstedt AG & Co.) containing MMM as medium, using a standard protist concentration of 10^3 flagellates ml^{-1} (obtained from the same protist pre-culture for both treatments), and six different initial bacterial concentrations of starved or non-starved cells, ranging from 10^6 to 10^8 bacteria ml^{-1}. As controls, treatments with an intermediate bacterial concentration of ~ 10^7

cell ml^{-1}, and no protists were prepared. Flasks were incubated at room temperature (23 – 25 °C), with moderate orbital shaking, for 60 – 72 h. Samples were taken every 4 h during the day and immediately fixed with 2 % formaldehyde.

In total, five experiments were carried out, two with *P. angustum* (the first of which tested only growth on starved bacteria), one with *V. vulnificus*, and two with *S. alaskensis*. They shall be referred to respectively as ExpP.a.1 and ExpP.a.2; ExpV.v.; and ExpS.a.1 and ExpS.a.2; throughout this paper. For ExpS.a.1, due to the active bacterial growth during incubation with flagellates, only data for the growth of the flagellate on the non-starved bacterial treatment is shown, and is referred to as flagellate growth on actively growing bacteria. Data from the starved bacterial treatment was excluded from further analysis since the fact that there was bacterial growth was an obvious indication that they were no longer in the starvation-survival state.

Enumeration of organisms and biovolume measurements

For bacterial enumeration, fixed subsamples were filtered onto black polycarbonate filters (0.2 µm pore size; 25 mm diameter; Whatman GmbH) and stained with DAPI (0.01 mg ml^{-1}). For protist enumeration, the same procedure was employed using 0.8 µm pore size filters (Whatman GmbH). Samples were observed under a Zeiss Axioskop 2 mot *plus* microscope (Carl Zeiss MicroImagimg GmbH) and a minimum of 200 bacterial cells (1000 X magnification) and 100 flagellate cells (630 X magnification) were counted per sample using filter set 02 (Carl Zeiss MicroImagimg GmbH).

For bacterial cell size measurements, image acquisition and analysis was performed using the CellP Image analysis software (Soft Imaging System GmbH) and an F-View camera (Soft imaging Systems GmbH). Images were processed as previously described (Posch et al. 2009), with a modification of the morphological filters: Erosion, Morphological Opening and Dilatation. A total of 200 – 250 cells were analyzed (Massana et al. 1997), and cell volumes were calculated using the formula described by Bjørnsen (1986). The accuracy of the method was tested by means of calibration with fluorescent beads of known size.

Size distributions, based on cell volumes, were determined for each bacterial population, and the average length to width ratio was calculated for larger size classes as Ferret max/Ferret min (respectively, the maximum and minimum values between tangents circulating at angle α 0, 10, 20… 180° around the particle). Size distributions were determined for control treatments without flagellates at the start of the experiment and

for treatments with protists, at intermediate to high bacterial concentrations, for later time points during the experiment. This should reveal possible changes in bacterial morphology, e.g., due to nutrient regeneration by the grazers. The time points studied were selected to cover the whole exponential phase of flagellate growth, in some cases extending beyond this time period. Statistical comparison was done by means of parametric tests when possible (t-test or ANOVA, see Table 1.1), but in occasions the distribution of bacterial cell-sizes was not normal, requiring the use of non-parametric tests (U-Mann Whitney).

Data analysis and statistics

For all experiments and treatments, flagellate and bacterial numbers were followed over time at all bacterial concentrations. Analysis was conducted with data obtained from the first 36 – 40 hours of the experiment, and initial bacterial numbers were determined from samples taken immediately after inoculation of the experimental flasks, to avoid errors arising from the carry over of the bacterium from the flagellate inoculum. Total initial biovolumes inoculated in each experimental flask were determined by multiplying the initial concentration by the mean cell-biovolume calculated for the bacterium in the corresponding physiological state.

Net flagellate growth rates were obtained by linear regression of the natural logarithm of *C. roenbergensis* concentration against time for each inoculated concentration of bacterial prey (starved and non-starved) (Fenchel 1982b). Data for flagellate growth rate and initial bacterial concentration or biovolume were fitted by iteration to a hyperbolic function:

$$\mu = x\, \mu_{max} / (K_s + x)$$

where μ: growth rate (d^{-1}); μ_{max}: maximum growth rate (d^{-1}); x: prey concentration or biovolume available; and K_s: half saturation constant for growth. Uncertainties in the estimates of the regression coefficients are expressed by the corresponding 95 % confidence intervals and asymptotic standard errors (see Table 1.2).

As an indication of the minimum number of bacteria needed for flagellate growth, theoretical threshold bacterial concentrations were calculated as previously described (Eccleston-Parry & Leadbeater 1994).

Finally, as a measure of the decrease of bacterial populations in the experimental flasks, bacterial loss rates were determined during the exponential phase of bacterial

decrease (coinciding with the flagellate exponential growth pahse) for each treatment and bacterial concentration as follows:

$$[(\ln N_t - \ln N_o) / (t_t - t_o)] - [(\ln C_t - \ln C_o) / (t_t - t_o)]$$

where N_t and N_o are, respectively, the final and initial bacterial concentration in the test flasks (with protists); C_t and C_o are, respectively, the final and initial bacterial concentration in the control flasks (without protists); and t_t and t_o are, respectively, the final and initial time points.

Statistical analyses were performed with the SPSS 15.0 (SPSS GmbH) and SigmaPlot10.0 (Systat Software GmbH) software packages.

1.3. Results

Bacterial population characteristics

P. angustum in its non-starved state is a rod-shaped bacterium (0.82 x 1.4 µm, median width and length respectively) with a median volume of 0.46 µm³. Starved cells are smaller, with a median volume of 0.18 µm³ and a more cocci-like morphology (0.70 x 0.89 µm). Observing the populations as whole, non-starved bacteria had a more heterogeneous size distribution, covering a wide range of size classes, and possessing higher and more variable length to width ratios (Supplementary Fig. S1.1 Fig. 1.1A, B; only data from ExpS.a.2 is shown for starved bacteria, since cell-size distributions were very similar in both experiments).

V. vulnificus is smaller in terms of volume, median 0.35 µm³, forming longer but thinner rods than *P. angustum* (0.73 x 1.69 µm). As a result of starvation conditions it forms shorter rods (0.66 x 1.19 µm), with a median volume of 0.23 µm³. The cell-size distributions of both populations tended towards a normal distribution with a maximum in the median value (Fig. 1.1C, D; Fig. S1.1). Length to width ratios were higher for the non-starved bacterial population.

S. alaskensis is a rod-shaped bacterium with decreasing sizes in its three physiological states, passing from a median cell-volume of 0.32 µm³ (0.65 x 1.70 µm) when actively growing, to 0.23 µm³ (0.62 x 1.27 µm) when non-starved (but not growing), and, finally to 0.17 µm³ (0.58 x 1.02 µm) when starved (Fig. S1.1). The reduction in length between the three physiological states was more marked than the reduction in width, as

shown by the decreasing length to width ratios (Fig. 1.1.E, F, G). The cell-size distribution of the populations showed a clearer dominance of certain size classes for non-starved (Fig. 1.1.F) and starved bacteria (Fig. 1.1.G), while the actively growing population had a more wide-spread heterogeneous distribution (Fig. 1.1.E).

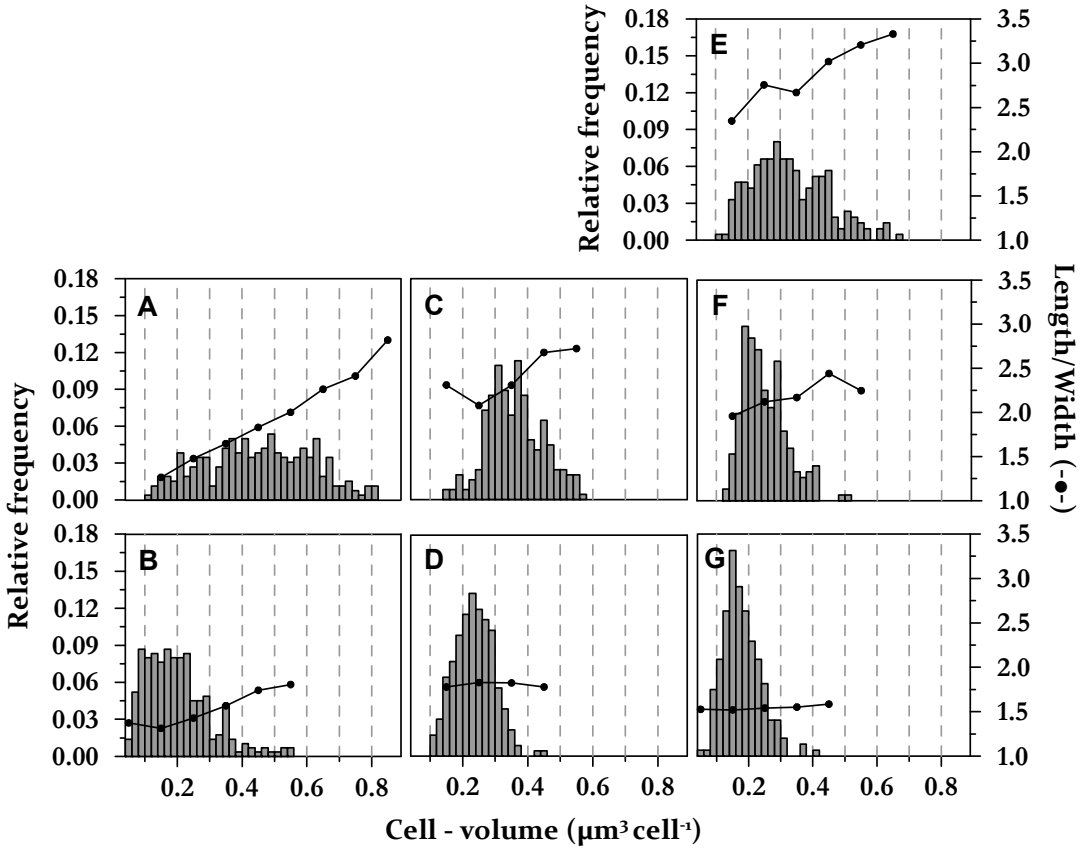

Figure.1.1. *Size distribution of the bacterial prey populations at the start of the experiments. The histograms show the relative frequencies of the different size-classes for P. angustum S14 (ExpP.a.2), A: non-starved (number of cells measured (n): 269) and B: starved (n: 288); V. vulnificus M06-24/O (ExpV.v) C: non-starved (n: 250) and D: starved (n: 234); and S. alaskensis RB2256 (ExpS.a.1 & 2) E: actively growing (n: 235); F: non-starved (n: 211); and G: starved (n: 204). The average length/width ratios for larger size classes (indicated by the dashed lines) are also plotted*

For all three bacterial strains an overlap was observed between the initial cell-size distributions of the different physiological states (Fig. 1.1). However, a significant difference was always maintained between the two populations, starved and non-starved, both at the start of the experiment and at later time points (Table 1.1.; $P < 0.05$). As a

whole, measured median cell-volumes remained constant with time, though some small shifts were observed, i.e. the tendency towards smaller cells in the starved *V. vulnificus* treatment or the appearance of larger cells at the final time-point for non-starved *V. vulnificus* (Table 1.1). Mean and median cell-volumes were normally equal or very similar, though in some cases, such as the final time point for the starved *P. angustum* treatment, divergences between the two occurred due to the appearance of small subpopulations of cells with larger or smaller cell volumes (observed in histograms; data not shown).

Table.1.1. *Mean and median cell-volumes, with the corresponding standard deviation (SD), measured at different time-points for each type of bacterium and physiological state (n: number of measured cells). Data is shown for only one experiment with each type of bacterium, and in each case it proceeds from one experimental flask per physiological state, inoculated with an intermediate to high initial bacterial concentration.*

Non-starved					Starved				
Time		**Biovolume [µm³]**			**Time**		**Biovolume [µm³]**		
[h]	**n**	**Median**	**Mean**	**SD**	**[h]**	**n**	**Median**	**Mean**	**SD**
***P. angustum* ExpP.a.2**									
0 [a+]	269	0.46	0.47[a]	*0.19*	**0** [a+]	288	0.18	0.19[a]	*0.08*
19	185	0.46	0.49[a]	*0.18*	**23**	233	0.17	0.18[a]	*0.07*
36 [a+]	178	0.46	0.48[a]	*0.18*	**36** [a+]	191	0.20	0.25[a]*	*0.13*
***V. vulnificus* ExpV.v.**									
0 [+]	250	0.35	0.36	*0.09*	**0** [+]	234	0.23	0.23	*0.06*
27 [a+]	240	0.36	0.39[a]	*0.15*	**27** [a+]	211	0.21	0.21[a]*	*0.06*
31	199	0.56	0.56*	*0.22*	**56**	196	0.18	0.19[a]*	*0.07*
***S. alaskensis* ExpS.a.2**									
0 [+]	211	0.23	0.25	*0.06*	**0** [+]	204	0.17	0.18	*0.05*
20	185	0.23	0.25[a]	*0.07*	**20**	177	0.16	0.17	*0.04*
24	225	0.20	0.21*	*0.06*	**36** [a+]	169	0.15	0.16*	*0.04*
36 [a+]	212	0.19	0.20[a]*	*0.06*	**44**	147	0.16	0.17	*0.05*

* Significant differences with oh value (ANOVA or U-Mann Whitney ([a]), $p < 0.05$)
[+] time points at which cell-volumes of starved and non-starved bacterial cells were compared. In all cases significant differences were found (t-test or U-Mann Whitney ([a]), $p < 0.05$).

In ExpP.a.2, the percentage of total bacteria corresponding to *P. angustum* was determined by immunofluorescence staining, allowing the quantification of potential contaminant bacteria in the test treatments. These were detected, but accounted for only 2 % or less of the initial total bacterial numbers in most treatments, though slightly higher in those inoculated with the lowest bacterial concentration (data not shown). No contaminant bacteria were detected in control treatments confirming that they came from the protist pre-cultures. The relative proportion of these bacteria did increase as the experiment proceeded and total bacteria numbers were reduced due to grazing, but only constituted a high percentage towards the end of the experiment, when total bacterial numbers were 10 % or less of the original values.

Flagellate growth on starved and non-starved bacteria

C. roenbergensis grew on all offered concentrations of the three bacterial strains in their non-starved or actively growing states, achieving maximal growth rates in the range of 0.2 - 0.3 h^{-1} (Table 1.2), which correspond to doubling times of approximately 2.5 - 3.5 h. In contrast, growth on carbon starved bacterial cultures varied with the different bacterial strains (Fig. 1.2; Table 1.2). The exponential growth phase for the flagellates was observed in most cases between 12 and 24 - 36 h, with the exception of the treatments in ExpS.a.2 supplied with starved bacteria, where it was delayed until 20 h and then continued until 40 h (data not shown).

When fed with *P. angustum*, the increase in flagellate growth with bacterial concentration and biovolume was the same for both experiments and treatments (Fig. 1.2A, B). The hyperbolic fits tended to fall together and the corresponding estimated growth parameters, namely maximum growth rate and Ks values, were similar, presenting in all cases overlapping standard errors and 95 % confidence intervals (Table 1.2). Only theoretical threshold values differed, being higher for flagellates fed on non-starved bacteria (Table 1.2).

In the experiment with *V. vulnificus* as a food source, marked differences were observed between the growth on starved and non-starved bacteria (Fig. 1.2C, D; Table 1.2). An increase in flagellate growth rates with increasing prey concentration and biovolume, equal to that obtained for *P. angustum*, was observed when *C. roenbergensis* was supplied with non-starved cells. However, starved bacteria only supported flagellate growth at the highest concentration, and a marked decrease in flagellate numbers was observed for almost all other concentrations tested. From lowest to highest initial bacterial

concentration inoculated, a respective decrease in flagellate numbers of 60 %, 70 %, 35 %, 84 % and 5 % was observed within the first 27 h of incubation (data not shown).

Table 1.2. *Theoretical threshold values for flagellate growth (Thres.), maximum growth rates (μ_{max}) and half saturation constants (K_s) (in terms of concentration and biovolume) for C. roenbergensis fed on the three bacterial species tested, P. angustum (Experiments P.a.1 & 2), V. vulnificus (Experiment V.v.) and S. alaskensis (Experiment S.a.1 & 2). The last two parameters were calculated by iteration to a hyperbolic function based on the growth rate of the flagellate on the different bacterial concentrations and treatments (n: 6; see Fig. 1.2), and the R^2, 95 % confidence intervals (CI) and computed asymptotic standard errors (SE) for the estimated values are also shown. Treatments: Star.: starved; N-star.: non-starved; A. g.: actively growing.*

	Thres.	**μ_{max}**			**K_s (conc.)**			**K_s (biovolume)**			**R^2**
	(10^5 b ml^{-1})	**(h^{-1})**	**SE**	**CI**	**(10^6 b ml^{-1})**	**SE**	**CI**	**(10^6 µm^3 ml^{-1})**	**SE**	**CI**	
P.a.1											
Star.	4.79	0.19	0.01	(0.17;0.21)	6.45	*1.07*	(4.35;8.55)	1.29	0.21	(0.88;1.7)	0.99
P.a.2											
N-star.	6.77	0.26	0.04	(0.18;0.34)	5.85	*2.92*	(0.13;11.6)	2.93	1.53	(-0.1;5.93)	0.88
Star.	3.57	0.25	0.05	(0.15;0.35)	7.45	*4.34*	(-1;15.9)	1.58	0.89	(-0.2;3.32)	0.88
V.v.											
N-star.	4.26	0.21	0.02	(0.17;0.25)	2.72	*1.29*	(0.19;5.25)	0.99	0.47	(0.07;1.91)	0.86
Star.	-	-	-	-	-	-	-	-	-	-	-
S.a.1											
A. g.	0.05	0.3	0.00	(0.30;0.30)	0.69	*0.08*	(0.53;0.85)	0.23	0.03	(0.17;0.29)	0.98
S.a.2											
N-star.	7.54	0.24	0.02	(0.20;0.27)	7.40	*2.41*	(2.68;12.1)	1.86	0.60	(0.68;3.04)	0.86
Star.	20.7	0.12	0.02	(0.08;0.16)	2.39	*2.07*	(-1.7;6.45)	0.43	0.37	(-0.3;1.15)	0.35

Finally, when fed with *S. alaskensis,* clear differences were observed in the growth of the flagellates on actively-growing, non-starved and starved cells (Fig. 1.2E, F). Calculated maximum growth rates were highest for non-starved bacteria and lowest for starved bacteria (Table 1.2), with standard errors and 95 % confidence intervals in no case overlapping. Theoretical threshold values correspondingly showed the inverse pattern. Ks values however, were conversely higher for the non-starved treatment than for the starved one, but data from the latter bacterial treatment should be treated with caution since the

data points tended to cluster together, gave the only hyperbolic fit with low R^2, and presented estimated Ks values with very high standard errors (Table 1.2).

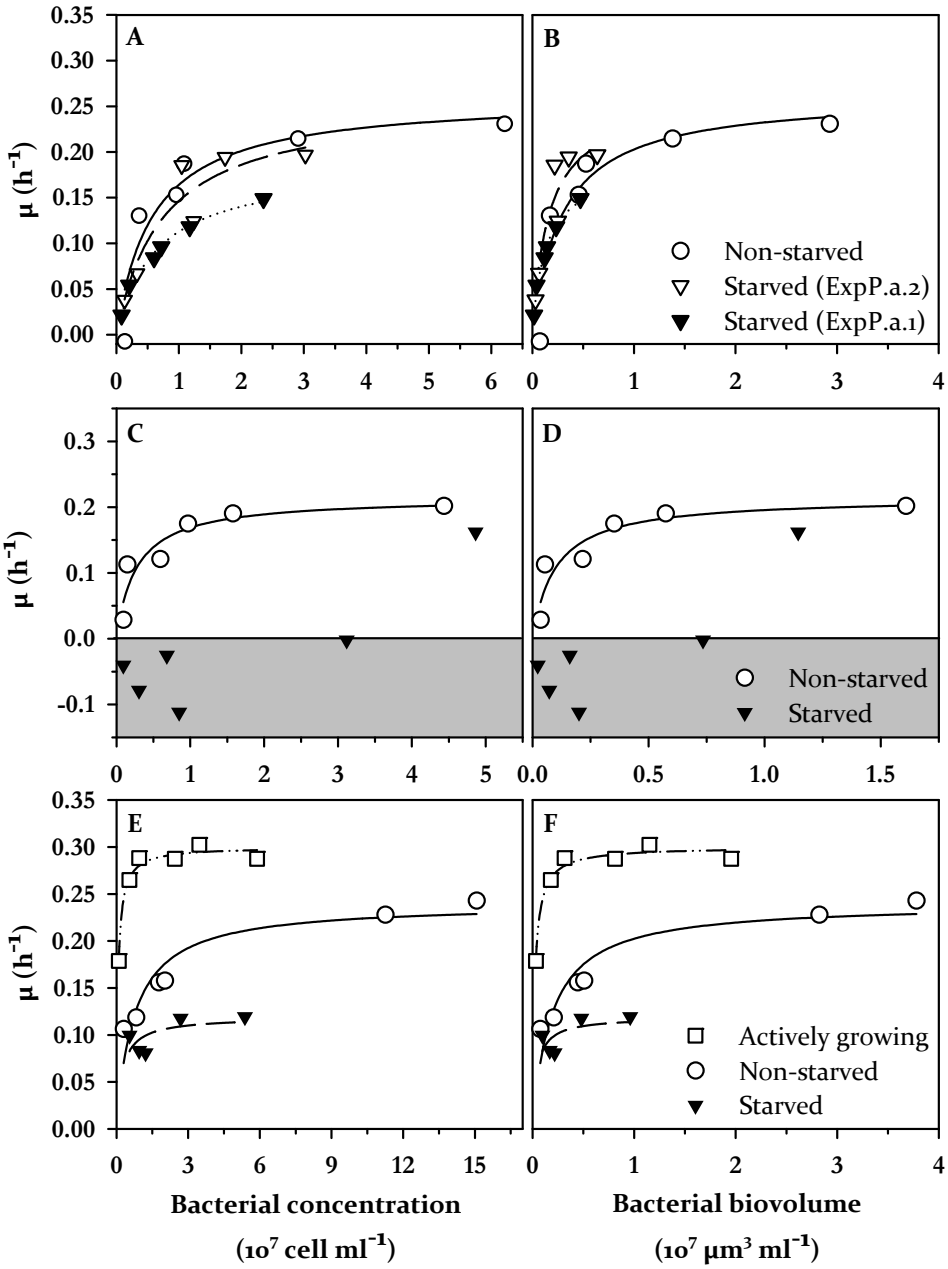

Figure 1.2. *Growth rate of C. roenbergensis fed on different initial bacterial concentrations, and corresponding biovolumes, of the three model bacteria in their different physiological states, P. angustum S14 (A and B; ExpP.a.1 & 2), V. vulnificus M06-24/O (C and D; ExpV.v.) and S. alaskensis RB2256 (E and F; ExpS.a.1 & 2). Shaded zones indicate negative growth rates for the flagellate. Hyperbolic fits are represented by the straight and dashed lines (further information in table 1.2)*

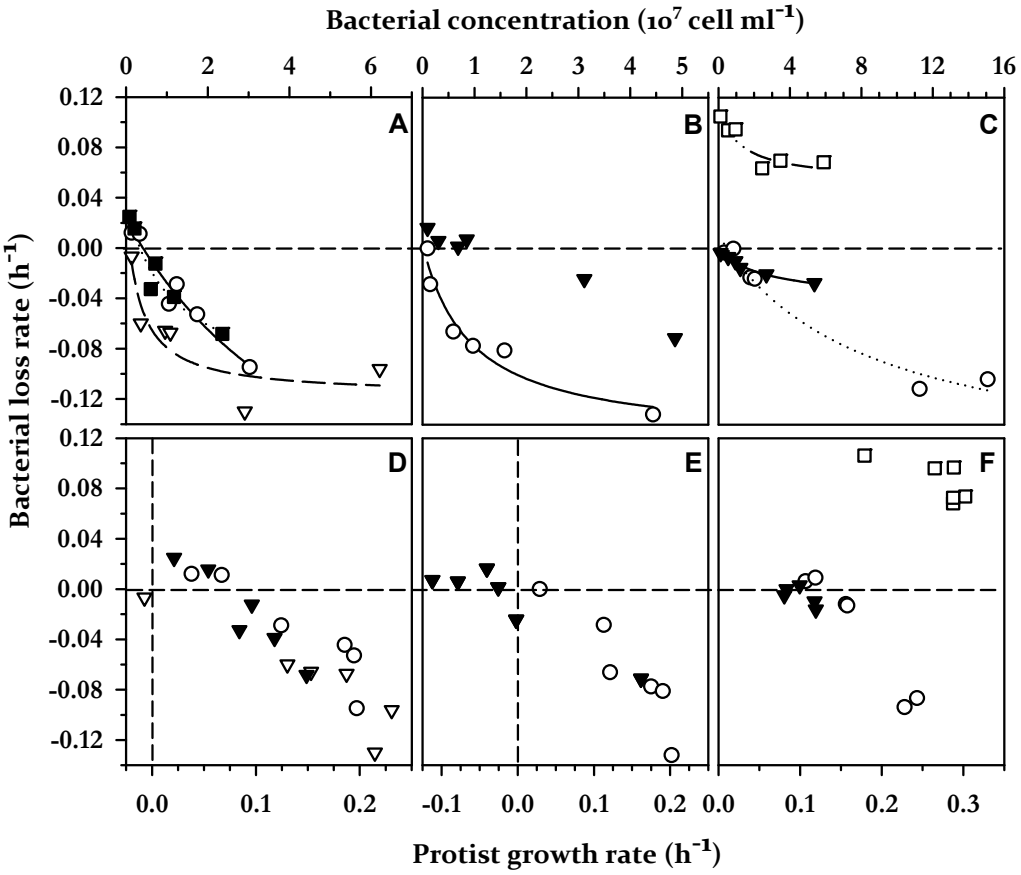

Figure 1.3. *Bacterial loss rates in relation to the initial bacterial concentration inoculated (A, B, C; hyperbolic fits are also plotted (R^2 > 0.81 in all cases)) and the protist growth rate (D, E, F), for the experiments with P. angustum (A and D), V. vulnificus M06-24/O (B and E) and S. alaskensis RB2256 (C and F). Circles represent non-starved bacteria (○), inverted triangles starved bacteria (▼) and squares actively growing bacteria (□). For P. angustum, where two experiments with starved bacteria were conducted, data from ExpP.a.1 are represented by black triangles and data from ExpP.a.2 by white triangles. Dashed vertical and horizontal lines serve as reference to 0 h^{-1} protist growth rates or bacterial loss rates.*

Overall, protist growth resulted in a corresponding decline of bacterial numbers, with bacterial loss rates increasing with protist growth rates (Fig. 1.3). As an exception, in the treatment with actively growing *S. alaskensis* bacterial growth exceeded mortality, though a tendency towards lower bacterial growth with increased protist growth was still observed (Fig. 1.3C, F). In control treatments, with the exception of actively growing bacteria, and test treatments where no protist growth was detected, bacterial loss rates

remained around 0 h^{-1} (the average value for all control treatments was 0.0009 ± 0.005 h^{-1}, and 0.004 ± 0.01 h^{-1} for treatments were no protist growth was detected).

1.4. Discussion

Methodological aspects

In the present study, we compared the growth of a bacterivorous flagellate, *C. roenbergensis*, on three model bacterial strains harvested in different physiological states, carbon-starved, non-growing and exponentially growing. To ensure that bacteria had remained in the desired physiological state throughout the duration of the experiment, analysis was restricted to the first 36 – 40 h sampled and cell-size was employed as an indicator of change in the physiological state during the experiment. We recognize that by employing this measure we potentially ignore certain effects that may occur prior to cell-size increase (i.e. changes in cell-surface characteristics or excretion of certain substances). However, the response of carbon starved bacteria to the appearance of a new carbon source, for example by the regeneration of nutrients through protist feeding, is very fast (Kjelleberg 1993; Morita 1997), and thus would soon be detected as shifts in the cell-size distribution of the population. This measure showed that the bacterial populations in most cases retained a constant cell-volume throughout the experiments and that a significant difference was always maintained between the starved and non-starved treatments (Table 1.1).

At a bacterial strain level, mean cell-volume values from the present experiments were comparable to those reported previously, with the exception of *S. alaskensis*. This strain was shown to have a constant small size of <0.1 μm^3 in prior studies (Schut et al. 1993; Eguchi et al. 1996), whereas we observed a change from 0.16 μm^3 in its carbon-starved state to 0.32 μm^3 when growing. Nevertheless, the identity of the strain used was positively confirmed by the 16S rRNA gene sequences (100% similarity) and similar increases in cell size were also observed in other recent studies with this bacterium (L. Ting, pers. comm.), indicating that there may be certain phenotypic changes in this strain compared to studies performed in earlier years.

In the second experiment with *P. angustum* S14 as food source (ExpP.a.2) small levels of contaminant bacteria were detected at the start of the experiments. However, this contaminant remained after a final dilution of 10^{10} of the initial protist culture (after

successive dilution steps) and the experimental results do not differ from those obtained in ExpP.a.1, which employed an axenic culture. Therefore, we concluded that the contaminant bacteria were likely not preferentially grazed upon by *C. roenbergensis*, and appeared to have no effect on the experimental outcome.

Protist growth on starved vs. non-starved bacteria

The results obtained in this study indicate species-specific differences in the vulnerability of carbon starved bacteria, even among the closely related strains *P. angustum* and *V. vulnificus* (Ruimy et al. 1994). *C. roenbergensis* grew well on all three bacteria in their non-starved state, achieving maximal growth rates which were in the range of those previously reported for this flagellate (González et al. 1993). In contrast, marked differences were observed when comparing protist growth on starved vs. non-starved cells of each bacterial strain.

C. roenbergensis exhibited equal growth when offered a comparable amount of starved and non-starved cells of *P. angustum* (Fig. 1.2A, B), indicating that the bacterium was an equally good food source for the flagellate in both physiological states, and that no apparent resistance mechanisms existed. Higher threshold values for starved bacteria and the tendency towards higher Ks values (Table 1.2) could even point towards a slightly better nutritional quality in this physiological state. A higher growth efficiency of flagellates when feeding on starved bacteria was also demonstrated in a previous study (González et al. 1993). Furthermore, the results obtained with *P. angustum* are also evidence that there is not necessarily an energetic constraint for flagellates feeding on smaller prey particles. This is contrary to observations from an earlier study (Boenigk et al. 2006), which found lower flagellate growth rates on smaller sized bacteria. However, in their study different bacterial strains were compared instead of different sized cells of the same strain, which makes other effects possible, such as inter-specific variations in food quality and grazing resistance. Also, differences in the behaviour of the protist species studied should be considered.

In contrast to *P. angustum*, starved *V. vulnificus* did not support flagellate growth and protist numbers declined strongly in the first 27 h, except at the highest bacterial concentration tested. This drastic negative response at low prey concentrations is too strong to be due to a simple starvation response caused by a decrease in food quality (Fenchel 1987) and suggests the presence of bacterial resistance or repellent mechanisms. As we did not analyze the bacteria-protist interactions in detail, e.g. by live video

microscopy (Boenigk & Arndt 2000), we do not know at which stage of the feeding process *V. vulnificus* resisted grazing. Potentially, ingestion of bacteria could be avoided due to receptor-mediated repellent effects (Matz et al. 2002); there could be postingestional mechanism involved, causing ingested bacteria to be egested after entering the food vacuoles (Boenigk et al. 2001) or allowing them to undergo the vacuole passage without being harmed by digestive enzymes (King & Shotts 1988; Barker & Brown 1994); or antiprotozoal factors could be produced at different stages of the feeding process, as has been seen for *Vibrio cholerae* during biofilm formation (Matz et al. 2005).

The observed exception to the overall pattern with *V. vulnificus* as prey, namely the positive protist growth at the highest bacterial concentration, would need further examination. The drastic negative response of the flagellates at lower bacterial concentrations does not support the explanation of a compensation of low food quality by a much higher food concentration. As a possible alternative, it remains to be examined whether high concentrations of this bacterial strain trigger regulatory shifts, e.g., by components involved in quorum sensing, known to affect both virulence and the starvation survival response in *Vibrio* strains (McDougald et al. 2000, 2001, 2003; Brackman et al. 2009). Nevertheless, this is, to my knowledge, the first documented case of a bacterial strain which can change from a highly edible to an unpalatable food when entering the starvation state.

Finally, *C. roenbergensis* also exhibited differences in growth when fed on different concentrations and biovolumes of the three physiological states of *S. alaskensis*. However, in contrast to the experiment with *V. vulnificus*, no deleterious effect on the protists was observed. The observed reduction in growth likely points to a gradual decrease in food quality from actively growing to carbon starved cells. It has been shown that both ingestion (Shannon et al. 2007) and growth rates (Grover & Chrzanowski 2009) of bacterivorous flagellates are affected by the nutritional quality of the bacterial prey, although, in these studies, mainly a variable C:N:P stoichiometry of the offered bacterial strains was taken into account.

1.5. Conclusions and outlook

In the present study, using predator-prey model systems, we show 1) that miniaturization of cell size alone is not sufficient to lower grazing vulnerability; and 2) some bacterial strains could possess efficient, yet to be examined, defence mechanisms that accompany

the starvation-survival programme and result in drastic changes their edibility. As future work, the following points remain to be elucidated to determine the relevance and universality of the model systems used in this study:

- Further model systems should be tested, including different bacterial phylogenetic groups and grazers, in order to discern general patterns in the effect of bacterial carbon starvation on grazing vulnerability
- Comprehensive studies of the underlying mechanisms should be carried out in cases where decreased vulnerability of carbon-starved bacteria is observed, such as in the present study with *V. vulnificus* as food source.
- Adequate methodologies should be developed to assess the prevalence of the carbon starvation state in natural bacterial communities and its impact on grazer vulnerability. Furthermore, the effect of a varied prey pool (different bacterial strains in different physiological states) on prey vulnerability and prey selectivity should be analyzed in detail to obtain an accurate vision of what occurs in natural environments.

Chapter 2

Protist abundance, diversity and bacterivorous role in suboxic and anoxic waters of the central Baltic Sea

Abstract

Barrier zones between oxic and anoxic water masses (pelagic redoxclines) host highly active prokaryotic communities that mediate important biochemical transformations. However, much less is known on the composition and ecological role of protists in these zones. In the present study we addressed this topic for two central Baltic Sea redoxclines, sampled during cruises in 2005, 2008 and 2009. A combination of microscopy and experimental community manipulation techniques were used to analyse the distribution of different protist groups throughout the redoxcline, and assess the relative importance of protist bacterivory and viral lysis as prokaryotic mortality factors. The results obtained revealed strong and consistent shifts in dominating protist groups along the redox gradient. Small ciliates (*Strombidium* c.f.) and dinoflagellates dominated the protist community at suboxic depths (≤30 µM O_2), while larger ciliates (*Mesodinium* spp., *Metacystis* spp., *Coleps* c.f. and unidentified morphotypes) formed a peak in abundance at the oxic-anoxic interface. In sulphidic waters, protist abundance was very low, represented by only few morphotypes (e.g. *Metacystis* spp.). Prokaryotic mortality was dominated by protist grazing in suboxic and oxygen / hydrogen sulphide interface depths (50 – 80 % of prokaryotic standing stock per day, compared to 2 – 20 % of virally infected cells), with ciliates and, occasionally, dinoflagellates, but not heterotrophic nanoflagellates, acting as the major bacterivores. In upper sulphidic depths, however, grazing was below the detection limit and the frequency of virally infected cells decreased, leaving the major prokaryotic mortality factor an open question.

2.1. Introduction

Protists have long been recognized as fundamental components of microbial food-webs in oxygenated marine and freshwater environments (Sherr & Sherr 2002; Pernthaler 2005). Phototrophic unicellular eukaryotes can contribute a significant fraction of primary production (Worden & Not 2008), whereas phagotrophic protists, especially heterotrophic

nanoflagellates (HNF), regulate the abundance, activity and composition of prokaryotic communities (Jürgens & Matz 2002; Montagnes et al. 2008). Additionally, they act as important agents in nutrient re-mineralization (Sherr & Sherr 2002); and, as prey, serve as a fundamental link to higher trophic levels (Zöllner et al. 2009). However, despite this acknowledged importance, their abundance and ecological role in other environments, such as sediments or suboxic (≤30 µM O_2) and anoxic water masses, remains understudied.

One such environment of special interest is the barrier between oxic and anoxic zones, also termed redoxcline. Here, steep geochemical changes are mirrored by different modes of microbial physiology, with prokaryotes conducting important roles in biogeochemical processes such as chemoautotrophic production (Taylor et al. 2001; Jost et al. 2008), denitrification and ammonia oxidation (Hannig et al. 2007; Labrenz et al. 2010; Lliros et al. 2010), and sulphur cycling (Jannasch et al. 1991; Jørgensen & Bak 1991). Estimated growth rates for chemoautotrophic prokaryotes in these zones are substantial, with doubling times in the range of 1 – 2 days, estimated for both the Cariaco Trench (Taylor et al. 2006) and the Baltic Sea (Jost et al. 2008). However, prokaryotic abundance tends to remain relatively constant, implying loss processes equivalent to new biomass production (Taylor et al. 2006). For oxic water masses it's known that sedimentation is generally negligible (Güde 1986), but biological agents, such as protist grazing or viral lysis, can be major loss factors for prokaryotic production (Suttle 1994; Fuhrman 1999; Sherr & Sherr 2002). However, so far only few studies have been conducted assessing this topic in oxygen-deficient environments.

The existence of protist taxa occurring primarily or exclusively in suboxic or anoxic environments has long been known (Lauterborn 1901; Fenchel et al. 1977). Classical microscopical studies, conducted in marine and freshwater systems, revealed an increased protist abundance in suboxic waters and oxygen / hydrogen sulphide interfaces (Fenchel et al. 1990; Zubkov et al. 1992; Massana & Pedrós-Alió 1994); and a shift in the community towards anoxic / sulphidic waters, with the appearance of different taxonomic groups compared to oxygenated upper layers (Zubkov et al. 1992; Fenchel et al. 1995). Deeper anoxic waters were shown to possess very low protist abundances, with many taxa forming symbiotic relationships with prokaryotes (Fenchel et al. 1977). The subsequent application of 18S rRNA gene-based techniques revealed an unexpected richness of anaerobic protistan communities, and uncovered the existence of novel, uncultivated taxa (Stoeck & Epstein 2003; Behnke et al. 2006; Edgcomb et al. 2011b). However, the functional role and grazing impact of these protists remains largely unknown. The constraints imposed by these

environments on obtaining accurate grazing rates has led to a scarcity of studies, which are additionally hard to compare due to the different methodologies employed and environments analysed. Grazing estimates range from over 100 % of prokaryotic production consumed per day (e.g., Cuevas & Morales 2006)) to below 5 % (e.g., Bettarel et al. 2004)) (see Table I in the introduction) . Thus, no consistent picture appears on the relevance of protist grazing as a loss factor for prokaryotic communities in suboxic and anoxic water masses.

Viral lysis, the other major potential biological loss factor, is even more understudied for oxygen-deficient systems. Existing estimates on mortality due to viruses are scarce and variable, ranging from <5 % to 50 % of prokaryotic production, sometimes even within the same study (Bettarel et al. 2004). However, a tendency has been observed for an increased importance of viral lysis in anoxic waters for certain systems (Weinbauer & Höfle 1998; Weinbauer et al. 2003). This, in combination with studies in other environments (e.g., Breitbart et al. 2004), has led to the general hypothesis that in more extreme environments (with regard to abiotic factors such as oxygen or salinity) there occurs a shift in the major prokaryotic mortality factor from protist grazing to viral lysis (Pedrós-Alió et al. 2000).

In the present study we focused on the redoxclines of the Baltic Sea, one of the worlds´ largest brackish environments. A stable halocline, situated at 60 – 80 m depth, limits vertical mixing and separates the water column into an upper oxygenated layer and a bottom oxygen depleted layer which becomes anoxic and sulphidic in the deepest zones (Reißmann et al. 2009). The euphotic zone of the Baltic Sea proper extends only to 10 – 30 m depth (Sandén & Håkansson 1996; Aarup 2002; Wasmund et al. 2005), with the deeper oxic / anoxic transition zones of the central basins being therefore located at aphotic depths. Occasional inflow events of high salinity water from the North Sea, the last of which occurred in 2002 and 2003 (Feistel et al. 2004), cause oxygenation of the anoxic deep basins. Stagnation periods between inflow events can last more than ten years, and the magnitude varies, generally affecting only some basins (Matthäus 2006). The highly active prokaryotic communities of Baltic Sea pelagic redoxclines have been well characterized (Grote et al. 2007; Labrenz et al. 2007; Glaubitz et al. 2009). However, scarce data exist on the composition of redoxcline protist communities and viral assemblages, with most studies covering only 2 or 3 suboxic or anoxic depths (Mamaeva 1988; Setälä & Kivi 2003; Weinbauer et al. 2003; Stock et al. 2009), and only two studies covering the suboxic and anoxic zone of Gotland Deep in more detail (Setälä 1991; Detmer et al. 1993).

Additionally, to my knowledge, no studies exist for Landsort Deep, the deepest and most stably stratified basin in the Baltic Sea.

The aim of the present study was three-fold. (1) Characterize, quantify and compare the protist communities at the oxic / anoxic transition zone of two deep basins in the central Baltic Sea, Gotland Deep (249 m depth) and Landsort Deep (459 m), during three different sampling campaigns conducted in 2005, 2008 and 2009. (2) Estimate grazing rates and assess the bacterivorous importance of different protist functional groups in three different zones with differing physico-chemical characteristics: suboxic (≤ 30 µM O_2), oxygen / hydrogen sulphide interface (oxygen and hydrogen sulphide coexist or are both below detection), and upper anoxic / sulphidic waters (detectable sulphide and no detectable oxygen). (3) Compare these rates to prokaryotic mortality due to viral lysis.

2.2. Materials and methods

Study sites, sampling and measurement of physico-chemical parameters

Table 2.1. *List of samples and experiments conducted during the three cruises in 2005, 2008 and 2009. W: whole water column, R: redoxcline in higher resolution, C: chemocline, S: sulphidic zone, L: Landsort Deep, G: Gotland Deep (e.g. WRL: whole water column and redoxcline of Landsort Deep)*

Year	2005	2008	2009
Profiling			
Viruses	-	-	WRL, WRG
Prokaryotes	RL, RG	WRL, WRG	WRL, WRG
Protists <10 µm in length	WL	RL, RG	RL, RG
Protists >10 µm in length	RG (x2)	RL, RG	RL, RG
Taxonomic identification			
Flagellates	WL, WG	-	-
Ciliates	CG, SL	-	-
Determination of protist grazing	-	-	RL, RG
Determination of viral impact	-	-	RL, RG

Sampling was conducted in the central Baltic Sea at Landsort Deep (station 284; 58° 35.0′ N; 18° 14.0′ E) and Gotland Deep (station 271; 57° 19.2′ N; 20° 03′ E) on board the R.V. *Alkor* in May 2005, R.V. *Poseidon* in August 2008, and the R.V. *Maria S. Merian* in September 2009. For an overview of the sampling and experiments conducted in each year

and station see Table 2.1. Water was collected in 5 l or 10 l free-flow bottles attached to a conductivity, temperature and depth rosette (CTD) with a coupled oxygen sensor. Inorganic nutrients, oxygen and hydrogen sulphide were measured immediately on board according to standard methods (Grasshoff et al. 1983). Whole water column depth profiling was conducted at 10 – 50 m depth intervals from the surface to just above the sediments, and high resolution redoxcline depth profiles were conducted at 2 – 5 m intervals around the chemocline. In cases were samples were not immediately fixed, only the first 5 l of 10 l free-flow bottles were employed, to avoid oxygen contamination during emptying of the free-flow bottles.

Determination of prokaryotic and virus abundance

For prokaryotic cell counts, 5ml samples were fixed immediately after retrieval with a mixture of paraformaldehyde and glutaraldehyde (1 % and 0.05 % final concentration, respectively), flash-frozen in liquid nitrogen and stored at -80 °C until analysis. For viral counts the same procedure was used, but employing glutaraldehyde (0.5% final concentration; 2-4 ml sample volume) as fixative. Subsequently, thawed samples were stained with SYBR Green I (Invitrogen-Molecular Probes) and abundance was determined by flow-cytometry. For prokaryotes, cell-counts were determined on a FACScalibur (Becton Dickinson) flow cytometer as previously described (Gasol et al. 2004) and data analysis was conducted on Cell Quest Pro software (BD Biosciences). Virus abundance was determined on a FACSAria II (Becton Dickinson) flow-cytometer as previously described (Brussaard 2004) and data analysis was performed using FACSDiva software (version 4.1; Becton Dickinson). Prokaryotic abundance is the result of single measurements while viral abundance is given as the average of duplicate measurements. Prokaryotic biomass was estimated by applying the conversion factor of 20 fg C $cell^{-1}$ (Lee & Fuhrman 1987).

Determination of protist abundance and biomass estimations

For protists <10 µm in length (mainly heterotrophic nanoflagellates (HNF)), 100 ml samples were fixed immediately after retrieval in brown glass bottles with a final concentration of 1 % particle free formaldehyde (in 2008 and 2009) or 1.25 % glutaraldehyde (in 2005) at 4 °C for 2-24 h. Subsamples were then filtered onto black polycarbonate filters (Nuclepore; 0.8 µm pore-size; Whatman) and frozen until analysis. Upon thawing, filters were stained with DAPI (0.01 mg ml^{-1} final concentration) and

observed under a Zeiss Axioskop 2 mot plus epifluorescence microscope (Carl Zeiss MicroImagimg). A minimum of 100 cells per filter were counted at a magnification of 630 X using filter set 02 (Carl Zeiss MicroImagimg). Two major size-classes were distinguished during cell counts, consisting of HNF above and below 3 µm in diameter. Within the first group, choanoflagellates were clearly distinguishable and, thus, were counted as a separate group.

For protists >10 µm in length (mainly ciliates and dinoflagellates), 200 ml samples were fixed immediately after retrieval with acid lugol (Willen 1962) (1 % final concentration) and stored in brown glass bottles at room temperature, in the dark. Protists were later concentrated by sedimentation of the lugol fixed samples in 100 ml sedimentation chambers for a minimum of 24 h (Uttermöhl 1958) and counted at a magnification of 200 X under an Axiovert S100 inverted microscope (Carl Zeiss MicroImagimg). Between ten diagonal stripes and the whole counting chamber were enumerated, depending on protist abundance. Major groups were distinguished during counting, both for dinoflagellates and ciliates, based on morphological characteristics. However, due to the difficulty of proper protist taxonomic identification in lugol-fixed samples, these represent morphotypes resembling known groups or taxa and, in the following, will be abbreviated with c.f..

Protist biomass was estimated for each of the protist groups distinguished in the cruises in 2008 and 2009. For this, size-classes were established, and individual cells volumes were determined through geometric models for representative cells of each morphotype and size-class. Volume to mass conversion factors were then applied of 220 fg C / μm^3 for HNF, 125 fg C / μm^3 for dinoflagellates and 190 fg C μm^3 for ciliates, as previously described for fixed samples (Børsheim & Bratbak 1987; Putt & Stoecker 1989; Pelegri et al. 1999). The total biomass for each group was obtained by multiplying the individual cell mass by the respective cell count.

Protist taxonomic identification

In 2005, samples were taken for the taxonomic identification of flagellates and ciliates. Flagellates were identified through a combination of live observation and electron microscopy, with reference to previous literature (e.g., Bernard et al. 2000). Live samples were collected by concentrating sea water through reverse filtration (0.2 µm membrane filter; Millipore GmbH) in a hermetically-sealed box with a nitrogen atmosphere at 4 °C. Concentrated samples were then placed inside a 1 ml transparent glass chamber,

hermetically sealed with a cover slip, and observed, directly on board, using phase contrast and magnifications of 360X and 630X, under an Axiovert 40 CFL inverted microscope (Carl Zeiss MicroImagimg). Additionally, individual flagellate cells were isolated by means of a specially constructed micropipette (Zhukov & Balonov 1979), and cultured in 96-well plates or petri-dishes, with sterile autoclaved Baltic Sea water as medium and *Pseudomonas putida* MM-1 as food source. Dried whole mount preparations of these flagellates were later examined with a JEM-1011 transmission electron microscope (JEOL Ltd.; Tokyo, Japan) as previously described (Moestrup & Thomsen 1980).

For ciliate identification, 40 – 50 l of sea water were concentrated by filtration through a 10 µm mesh size plankton net, avoiding contact with air, and stored at 4°C. The concentrated material was then transported on ice back to the laboratory, where the taxonomic composition of ciliates was determined. Although it's likely that through this approach only the more abundant and robust taxa remained, sufficient ciliate cells could be retrieved to be studied microscopically by live observations and using silver impregnation methods as previously described (Foissner 1991).

Determination of protistan grazing on prokaryotes

In 2009, protist bacterivory was estimated at different depths throughout the redoxcline at Landsort and Gotland Deep using an approach based on the disappearance of fluorescently labelled bacteria (FLB) during incubation (Marrasé et al. 1992). FLB were prepared before the cruise from *Brevundimonas diminuta* cultures as previously described (Vázquez-Domínguez et al. 1999).

Sampling depths were first selected from oxygen and turbidity profiles recorded by a CTD cast directly prior to that used to obtain water for the experiments. Selected depths corresponded to suboxic, oxygen / hydrogen sulphide interface, and upper sulphidic conditions. Immediately upon arrival of the water on deck, acid cleaned, 500 ml bottles with narrow necks were filled directly from the free-flow bottles, with at least half a minute of overflow. FLB were immediately added to a concentration of 10 - 20 % of *in situ* prokaryotic abundance, and the bottles were quickly closed with glass stoppers, avoiding any head space or air bubbles. Incubations were performed approximating *in situ* conditions (4 °C and in the dark) for 24 hours. To avoid oxygen contamination during sub-sampling, separate sets of triplicate bottles were prepared for the two sampling points (0 h and 24 h). Controls to test for stability of added FLB under the physico-chemical conditions present and in absence of grazers (removed by filtration through a 1 µm pore-

sized filter (Nuclepore, Whatman)), were carried out repeatedly at different depths in both study sites in August 2008.

Fixation for protist and prokaryote samples, and protist quantification were carried out as described above. Prokaryote and FLB enumeration was conducted by epifluorescence microscopy. For this, subsamples were filtered onto black polycarbonate filters (Whatman; pore width, 0.2 μm) and stained with DAPI. Samples were then observed under a Zeiss Axioskop 2 mot plus microscope (Carl Zeiss MicroImagimg), and a minimum of 200 cells per sample or 50 fields were counted at a magnification of 1000 X using filter set 02 for total prokaryotes and 09 for FLB (Carl Zeiss MicroImagimg).

Protist grazing rates on the natural prokaryotic community were derived from the data following the previously described exponential model (Salat & Marrasé 1994), with slight modifications to account for the fact that initial and final replicates proceeded from different incubation bottles. First, an average net prokaryotic growth for the three replicates (*a*) was obtained at each depth as:

$$a = (1 / t) \times \ln (N_t / N_o)$$

where t is the incubation time, and N_o and N_t are the average initial and final prokaryotic abundance, respectively, for the three replicates at each depth.

Next, a specific grazing rate on FLB (*g*) was obtained for each replicate at each depth as:

$$g = - (1 / t) \times \ln (F_t / F_o)$$

where t is the incubation time, F_o is the inoculated FLB abundance and F_t is the final FLB abundance measured for each replicate. The inoculated FLB abundance was obtained as the average FLB abundance for all initial replicates at all depths. This latter measure was possible since all flasks were inoculated with the same volume from the same FLB stock, resulting in the same average initial FLB abundance for all three depths (ANOVA; $P > 0.05$).

Since $a \neq 0$ (see Salat & Marrasé 1994), the grazing rate on the natural prokaryotic community (*G*) was obtained for each replicate at each depth as:

$$G = (1/t) \times (g / a) \times (N_t - N_o)$$

For each replicate, the corresponding *g* value and the *a* value for that depth was used. *G* values were then transformed into prokaryotes consumed ml^{-1} day^{-1}, and the percentage of

the prokaryotic standing stock grazed per day was calculated for each replicate at each depth.

Examination of protist food vacuole contents

The bacterivorous potential of different protist groups was qualitatively assessed through the observation of presence / absence of FLB inside food vacuoles at the end of the grazing experiments, and through Catalyzed Reporter Deposition - Fluorescence In Situ Hybridization (CARD-FISH) targeting prokaryotes inside food vacuoles. FLB detection was carried out using the microscopy samples employed for the quantification of heterotrophic nanoflagellates (0.8 µm filters (described above); filter set 09 for detection). Samples for CARD-FISH (200 ml) were obtained from different depths throughout the redoxcline, parallel to the microbial profile sampling, and fixed immediately after retrieval with the alkaline lugol solution – formalin – thiosulphate method (Sherr & Sherr 1993a; Medina-Sánchez et al. 2005). The complete volume was then filtered onto polycarbonate filters (Nuclepore; pore-size, 2.0 µm; 47 mm diameter; Whatman) which were subsequently frozen. CARD-FISH was later carried out employing a modified version of an established protocol (Medina-Sánchez et al. 2005), which excluded the permeabilization step with achromopeptidase since it severely damaged protist cell integrity. *Eubacteria* were detected using probes EUB 338 I, II and III (1:1:1 mix) (Daims et al. 1999); and *Crenarchaeota* with probe Cren537 (Teira et al. 2004). We targeted *Crenarchaeota* since they are major constituents of the redoxcline prokaryotic communities in the central Baltic Sea, constituting up to 25 % of total cell counts (Labrenz et al. 2010).

Estimation of the frequency of virally infected prokaryotic cells (FIC)

FIC was determined for different depths throughout the redoxcline using a virus-dilution approach (Wilhelm et al. 2002). Specifically, water samples (2 l) were filtered (Isopore; pore-size, 3 µm; diameter, 47 mm; Millipore) to remove larger planktonic organisms. The prokaryotes contained in the filtrate were then concentrated (45 - 60 ml final volume) using a tangential-flow filtration device with a pore-size of 0.22 µm (Vivaflow 200, polyethersulfone, Sartorius Stedim). The viruses contained in this second filtrate were subsequently removed using a tangential-flow filtration device with a molecular weight cut-off of 100 kDa (Vivaflow 200, polyethersulfone, Sartorius Stedim) yielding virus-free water. In order to estimate FIC due to lytic viruses, duplicate aliquots of the prokaryotic

concentrate (10 – 15 ml) were dispensed into tightly sealable polypropylene tubes and filled-up to a total volume of 50 ml with virus-free water from the same station and depth. FIC due to lysogenic viruses was determined by inducing them in a second set of duplicate experiments through the addition of Mitomycin C (1 µg ml-1 final concentration; Sigma Aldrich). Incubations were performed in the dark at 18°C for up to 30 hours and subsamples to determine prokaryotic and viral abundance were taken every 4–5 hours.

Samples for virus abundance were fixed and enumerated as described above. Samples for prokaryote abundance were fixed with glutaraldehyde (0.5 % final concentration) and quantified on a FACSAria II (Becton Dickinson) flow cytometer as previously described (Marie et al. 1999). FIC due to lytic viruses was calculated based on the increase in viral abundance in the unamended incubations, assuming a burst size of 30 viruses per lysed prokaryotic cell for suboxic waters and 90 for oxygen / hydrogen sulphide interface and anoxic waters (Weinbauer et al. 2003; Winter et al. 2004). FIC due to lysogenic viruses was calculated as the difference between the results from Mitomycin C-amended and unamended incubations (Weinbauer et al. 2003). A significant detection of FIC due to lysogeny was only considered when results for Mytomycin C treatments were higher than results from unamended incubations (taking the variation of the duplicate estimates into account).

2.3. Results

Physico-chemical characteristics and prokaryote abundance

During all three cruises both study sites had clearly stratified water columns with anoxic bottom waters (Fig. 2.1). The conditions in both basins in 2008 and 2009, and at Landsort Deep in 2005 (Fig. 2.1; physico-chemical data for Landsort Deep in 2005 are not shown) resembled those generally observed in Baltic Sea redoxclines (e.g. Detmer et al. 1993; Jost et al. 2008). However, Gotland Deep in 2005 was still under the influence of the 2003 inflow event (Feistel et al. 2004), and the chemocline, defined as the zone of first hydrogen sulphide appearance, was located close to the sediment (Fig. 2.1). Conversely, in 2005 the beginning of the suboxic zone (~30 µmol l^{-1} oxygen) was located at similar depths as in the other two years, leading to a much wider redoxcline. Prokaryote abundance, measured in 2008 and 2009 for the whole water column, was highest in surface waters at both study sites, but a second smaller peak could often be observed at or just below the chemocline

(Fig. 2.1, 2.2, 2.3). Typical prokaryotic abundances for the redoxcline ranged between 0.2 – 2 x 10^6 cell ml^{-1}.

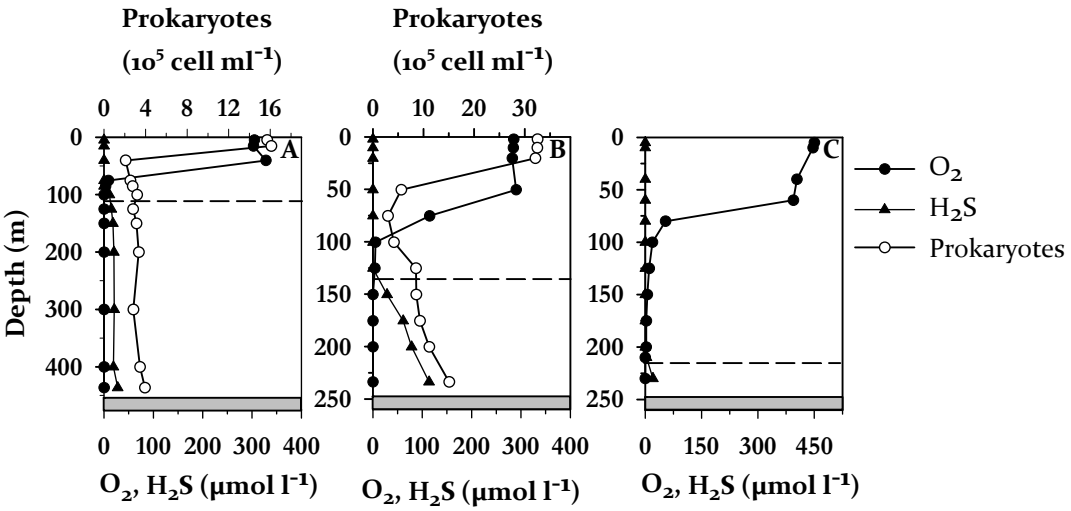

Figure 2.1. *Examples of vertical profiles for oxygen, hydrogen sulphide and prokaryote concentrations from the surface to just above the sediment (indicated by the shaded area), taken at Landsort Deep in 2009 (A) and Gotland Deep in 2009 (B) and 2005 (C). The dashed line represents the chemocline, defined as the zone of first hydrogen sulphide appearance.*

Protist abundance and community composition throughout the redoxcline

In 2008 and 2009, ciliates and/or dinoflagellates dominated protist biomass at both study sites (Fig. 2.2). Ciliates reached their maximum abundance around the chemocline, while dinoflagellates peaked in the suboxic zone, and thereafter practically disappeared. In 2005, detailed redoxcline profiles were conducted only at Gotland Deep, concentrating on depths around the chemocline, thus, missing the upper part of the suboxic zone (Fig. 2.3). Dinoflagellates were not observed and ciliate abundance was lower than in the other two years (maximum abundance 10^3 cells l^{-1}, compared to 4 x 10^3 cells l^{-1} in 2008 and 1.4 x 10^4 cells l^{-1} in 2009).

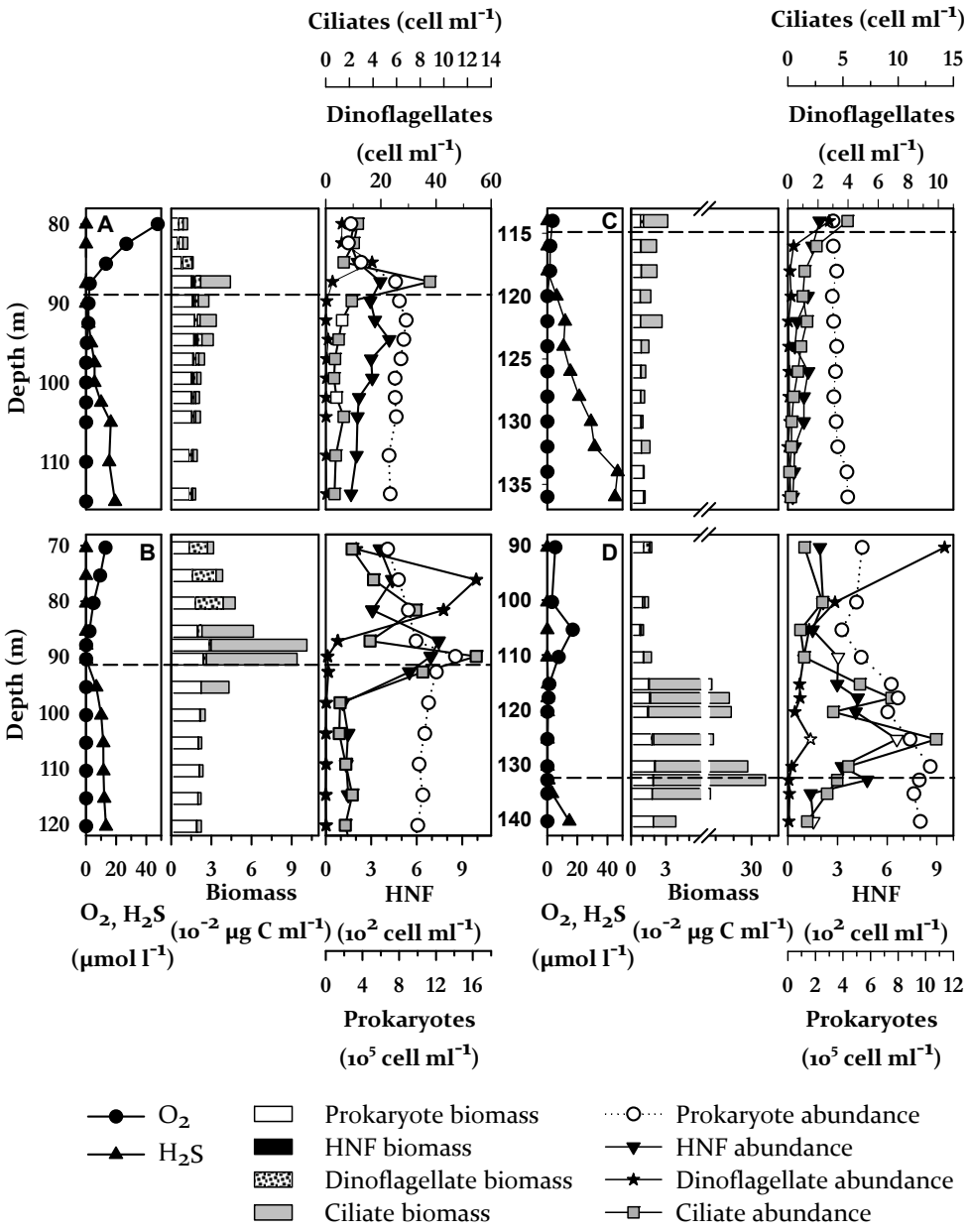

Figure 2.2. *High resolution redoxcline profiles taken at Landsort Deep in 2008 (A) and 2009 (B), and at Gotland Deep in 2008 (C) and 2009 (D), showing oxygen and hydrogen sulphide concentrations, and prokaryote and protist biomass (bars) and cell-counts (line/scatter plots). The horizontal dashed line represents the chemocline. Please note the break in the X axis of C and D middle panels.*

Ciliate and dinoflagellate morphotype composition showed clear changes throughout the oxygen gradient in both study sites (Fig. 2.3 & 2.4; the relative abundance of different morphotypes for 2008 and 2009 were very similar, so only data from 2009 is

shown here). Dinoflagellates were mainly represented in the suboxic zone by rounded morphotypes with a diameter of 10 - 25 µm (major axis) (Fig. 2.4). However, *Dinophysis* spp. (data not shown) and elongated morphotypes (10 - 30 µm in length) could also become important at certain suboxic depths, and larger morphotypes (> 30 µm in length) sometimes appeared at the oxygen / hydrogen sulphide interface and deeper anoxic waters. When observed with epifluorescence microscopy only *Dinophysis* spp. showed autofluorescence, suggesting a heterotrophic lifestyle for the majority of dinoflagellates observed.

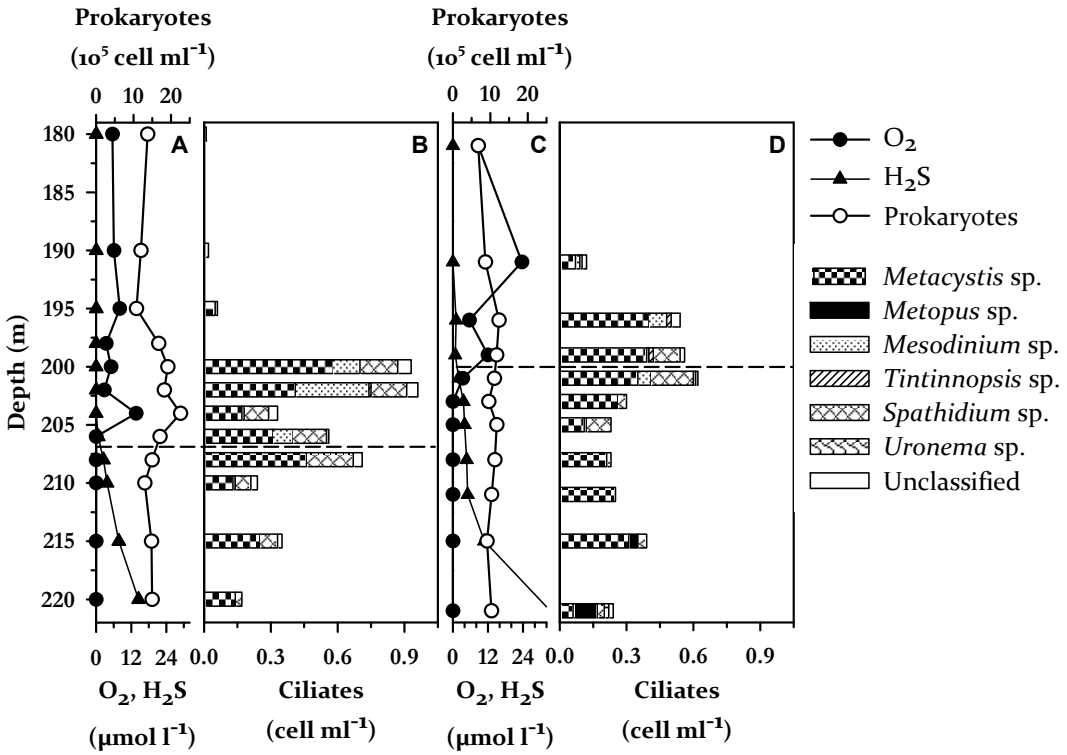

Figure 2.3. *High resolution redoxcline profile of ciliate morphotype composition at Gotland Deep on the 4th (A, B) and the 6th (C, D) of May 2005. Changes with depth in oxygen, hydrogen sulphide and prokaryote concentrations (A, C) and in ciliate cell-counts (B, D) are shown. The horizontal dashed line represents the chemocline.*

Ciliate morphotype composition showed stronger changes within the redox gradient (Fig. 2.3 & 2.4). *Strombidium* c.f. (20 – 30 µm length) dominated the suboxic zone down to oxygen concentrations of 5 – 6 µmol l^{-1} in 2008 and 2009, but was not observed in 2005. At lower oxygen concentrations, and before hydrogen sulphide levels increased, the composition became dominated by *Metacystis* spp. (present as several different morphotypes, ranging from 20 to 150 µm in length), *Mesodinium* c.f., and either *Coleps* c.f.

in 2008 and 2009, or *Spathidium* c.f. in 2005. Once hydrogen sulphide concentrations began to increase, the protist community became numerically dominated by small unidentified ciliates at Landsort Deep and *Metacystis* spp. at Gotland Deep. Additionally, large unidentified ciliates (150 – 350 µm in length) were detected in low numbers (~100 – 400 cells l^{-1}) throughout the oxygen / hydrogen sulphide interface at Landsort Deep and both the interface and upper sulphidic zones at Gotland Deep in 2009, but dominated in terms of biomass at certain depths.

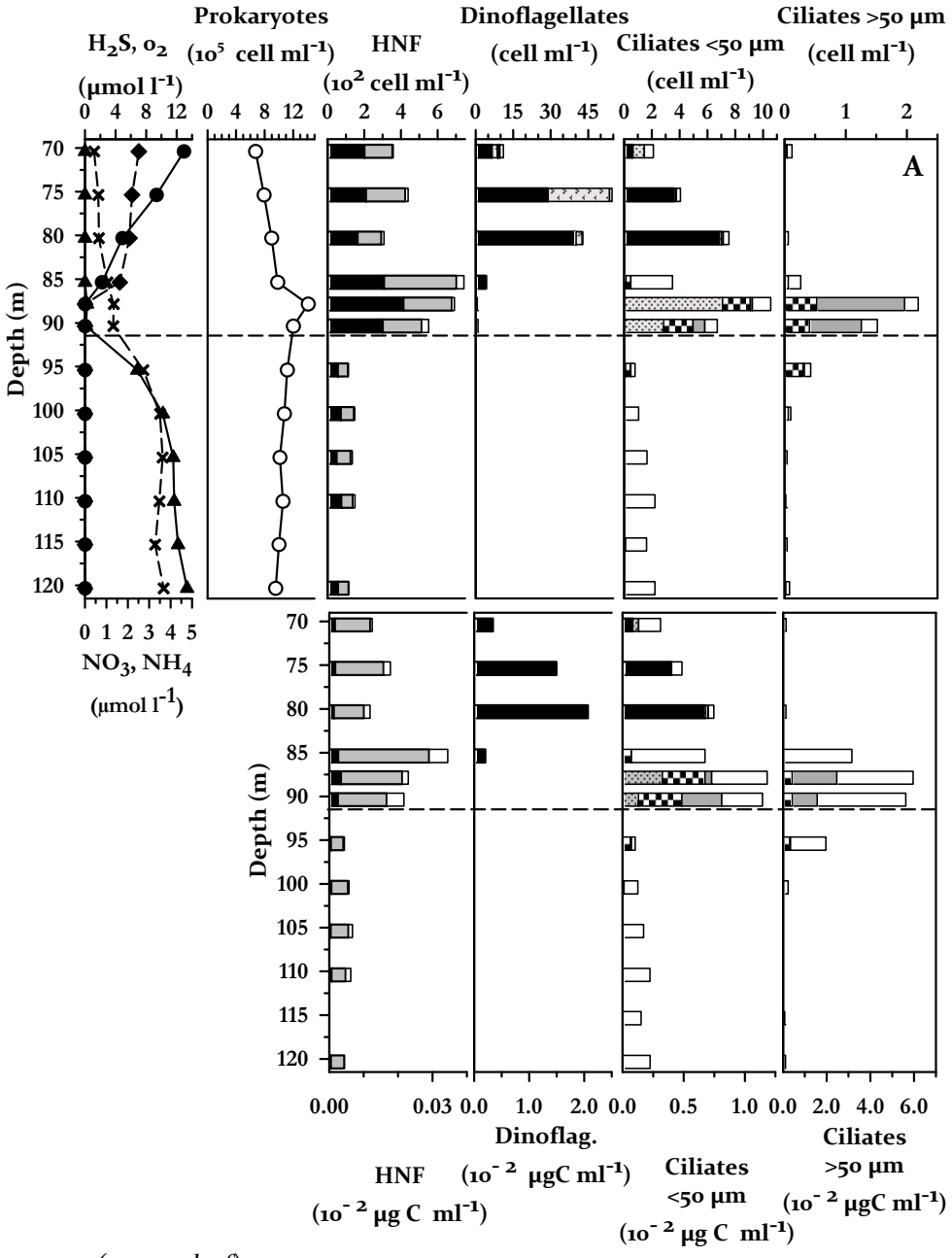

Figure 2.4 *(see overleaf)*

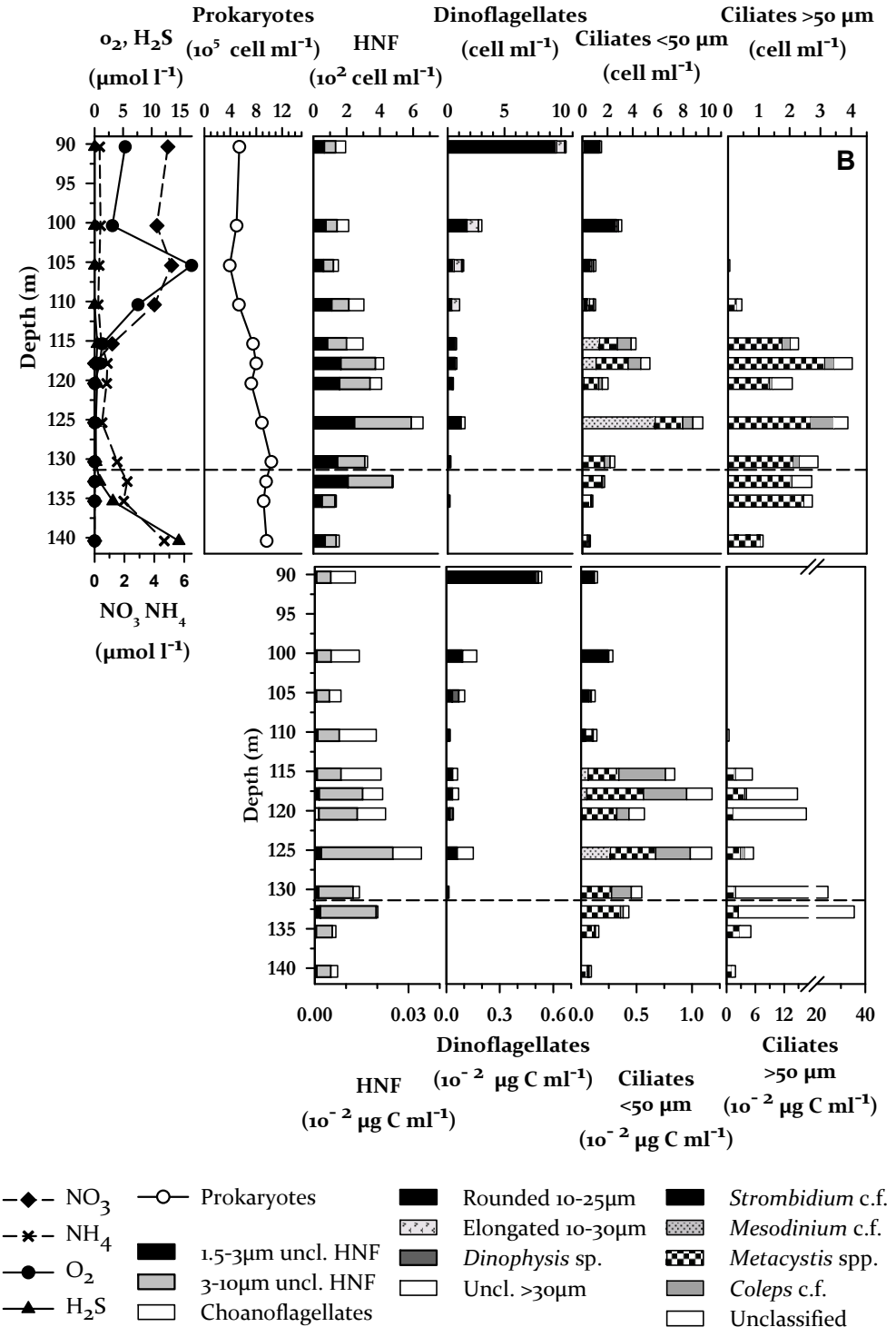

Figure 2.4. *High resolution redoxcline profiles of protist morphotype composition at Landsort (A (above)) and Gotland Deep (B (overleaf)), taken in September 2009. Changes with depth in chemical parameters and in the cell-counts (above) and biomass (below) of the different microbial groups identified are shown. The horizontal dashed line represents the chemocline. Protist sizes denote the cell length.*

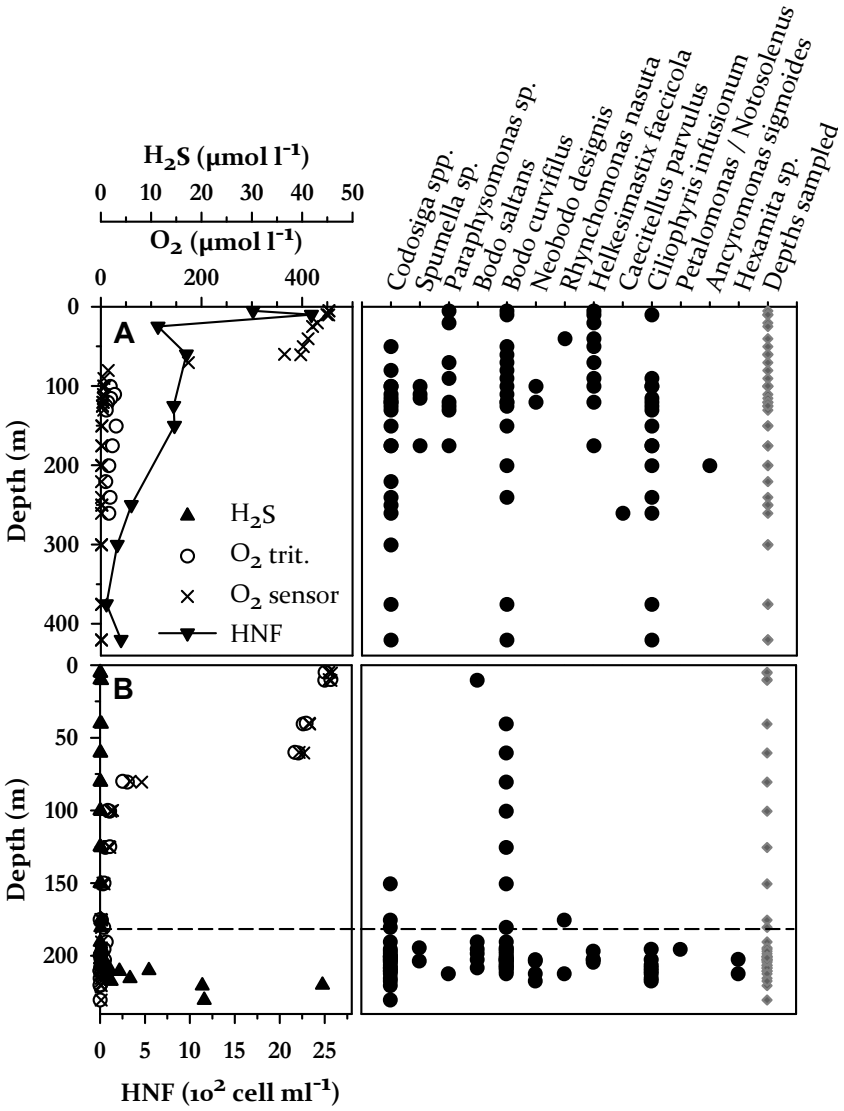

Figure 2.5. *Heterotrophic nanoflagellate abundance and observed taxa in May 2005 throughout the whole water column at Landsort Deep (A) and Gotland Deep (B). The list of flagellate species indentified is shown along with the depths at which they were detected (•), the total sampling depths (gray romboids), and oxygen (measured by tritiation and by the oxygen sensor on the CTD) and hydrogen sulphide concentrations (only available for Gotland Deep). With the exception of HNF abundance, data were pooled for several different CTD casts. The dashed line represents the chemocline.*

HNF at the redoxcline peaked in abundance at the oxygen / hydrogen sulphide interface in all years, but were insignificant in terms of biomass (Fig. 2.2, 2.4 & 2.5). The community was represented equally, in terms of abundance, by larger (generally 3 – 6 µm in diameter (major axis)) and smaller (1.5 – 3 µm) morphotypes, but the former dominated

in terms of biomass (Fig. 2.4). Choanoflagellates were important components both numerically and in terms of biomass at suboxic and oxygen / hydrogen sulphide interface depths at Gotland Deep in 2008 and 2009, but represented only a small fraction of total HNF at Landsort Deep.

Protist taxonomic identification

In 2005, a more precise taxonomic identification of ciliates and flagellates was carried out. 13 flagellate species were identified by means of light and electron microscopy (Fig. 2.5). The examination of their presence / absence throughout different depths of both redoxclines showed no strong differences between the two study sites. Most species had a large vertical distribution, appearing from fully oxygenated waters to bottom anoxic waters (e.g. *Bodo curvifilus*), and only a few appeared to be always restricted to anoxic waters (e.g. *Hexamita* sp.).

Table 2.2. *List of ciliate species identified in May 2005 through silver impregnation techniques, and assessment of their presence (+) at different depths around the chemocline of Gotland Deep and below the chemocline in Landsort Deep. For the genus Metacystis more than one morphotype was identified, shown as Metacystis spp. 1-5*

	Landsort D.		**Gotland D.**					
CTD-cast	**9th May**		**8th May**		**11th May**		**11th May**	
Depth (m)	253	404	200	202	200	203	201	204
***Pleuronema* sp.**	+	+						
***Holophyra* sp.**	+		+		+		+	
***Metacystis* spp.**								
1			+		+	+	+	+
2			+	+	+	+	+	+
3			+	+	+		+	
4			+	+	+		+	+
5	+							
***Cardiostomella* sp.**	+	+	+	+	+		+	
***Mesodinium* sp.**			+		+			
***Euplotes* sp.**	+	+						
***Placus-like* n. gen. n. sp.**	+	+						
***Metopus* sp.**							+	
***Pelagiocama-like* n. gen. n. sp.**					+			

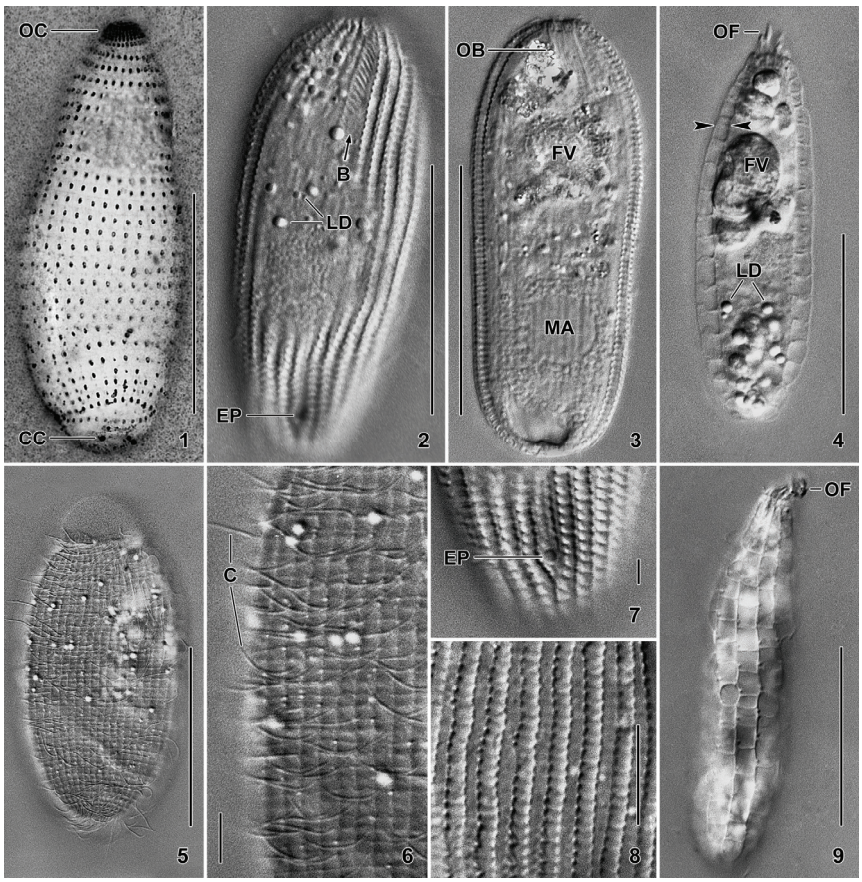

Figure 2.6. *Novel ciliates from the chemocline at Gotland Deep, observed in vivo (2 - 9) and after silver impregnation (1). 1, 5, 6: potential new species belonging to the genus Metacystis. (about 100 µm in length). The genus is easily recognized by the highly ordered arrangement of the cilia/basal bodies, forming quadrangular meshes (5, 6). 2, 3, 7, 8: Surface views (2, 7, 8) and optical section (3) of a new genus related to Placus spp. (about 90 µm in length). The cortex structure of the new genus resembles that of Placus spp. (7, 8), but differs by the structure of the brush (2). 4, 9: Optical section (4) and surface view (9) of a new plagiocampid genus (about 50 µm in length). B – brush, C – cilia, CC – basal body of caudal cilium, EP – excretory pore of contractile vacuole, FV – food vacuoles, LD – lipid droplets, MA – macronucleus, OB – oral basket, OC – oral ciliature, OF – oral flaps.*

For ciliates, silver impregnation techniques allowed the identification of 14 species (Table 2.2). Five belonged to the genus *Metacystis*, with at least one of them representing a new species, similar to *M. elongata* but with a caudal cilium (Fig. 2.6). Two other species represent potentially novel genera. The first appears related to *Placus* spp., with a similar cortex structure but differing in the structure of the brush (Fig. 2.6). The second belongs

to the family Plagiocampidae, as indicated by the characteristic positioning of finger-like oral flaps on the dorsal mouth margin, but differs from known genera of this family by the unusual cortex consisting of large, quadrangular alveoli (Fig. 2.6).

Impact of protist grazing on redoxcline prokaryotic communities

Table 2.3. *Data for the experiments to determine grazing and viral lysis. Prokaryotic and protist abundance correspond to those from the start of the incubations, and are given as average values with the standard deviation in parenthesis. Grazing rates are given as the average of triplicate measurements with the standard deviation in parenthesis, while FIC (frequency of infected cells) is the average of duplicate measurements, with the range in parenthesis. Zones: Sub: Suboxic; Int: Interface; Sul: Sulphidic*

Site	**Landsort Deep**			**Gotland Deep**		
Depth (m)	65	70	80	110	125	130
Zone	Sub.	Int.	Sul.	Sub.	Int.	Sul.
Temperature (°C)	5.2	5.3	5.5	6.2	6.7	6.7
Salinity (‰)	9.6	9.7	10.1	9.3	9.3	9.3
O_2 (µmol l^{-1})	23.67	12.51	-	4.02 [1]	2.68	-
H_2S (µmol l^{-1})	-	0.31	4.71	-	0.16	2.7
NO_3 (µmol l^{-1})	3.26	-	-	1.23	1.51	-
NH_4 (µmol l^{-1})	0.43	0.23	3.31	0.67	0.35	1.58
Prokaryotes (10^5 cell ml^{-1})	6.43 (1.28)	7.35 (0.35)	12.1 (0.47)	7.56 (0.49)	6.77 (0.47)	7.95 (1.29)
HNF (10^2 cell ml^{-1})	3.32 (0.56)	4.56 (0.92)	2.61 (0.26)	5.24 (0.40)	1.51 (0.21)	0.95 (0.25)
Dinoflag. (10^3 cell l^{-1})	3.56 (0.25)	6.05 (1.07)	0.47 (0.16)	0.59 (0.07)	1.89 (0.16)	0.26 (0.08)
Ciliates (10^3 cell l^{-1})	4.03 (0.25)	3.71 (0.32)	2.67 (0.37)	2.17 (0.63)	3.42 (0.32)	3.45 (1.01)*
Net prok. growth (d^{-1})	- 0.23	- 0.18	0.14	0.01	- 0.19	0.31
Grazing rate (10^5 prok. ml^{-1} d^{-1})	5.19 (1.82)*	3.79 (0.80)*	4.58 (3.61)	1.92 (1.66)	3.32 (0.75) *	1.15 (1.45)
% prok. standing stocks d^{-1}	80.8 (28.3)	51.5 (10.9)	38.0 (22.0)	25.4 (22.0)	49.0 (11.1)	14.0 (18.2)
FIC (%)	19 (13–25)	3 (1.5–4)	2 (1.5–2.5)	6 (5–7)	2 (1.5-2.5)	3 (2.5-3.5)

[1] Due to errors in the titration measurements, O_2 values from the CTD sensor are shown instead; *Significant differences between initial and final cell-counts (t-test, $P<0.05$). In the case of the grazing rate, this refers to the initial and final abundance of FLB; (-) Parameter was expected to be below detection limit and was not measured at this depth.

FLB experiments were carried out at Landsort and Gotland Deep in 2009 in suboxic, oxygen / hydrogen sulphide interface, and sulphidic waters (Table 2.3). The protist communities observed resembled those seen in the profiles for zones of similar physico-chemical characteristics, with the exception of suboxic waters at Gotland Deep, where *Metacystis* spp. was also detected (data not shown). No significant changes in protist abundance occurred during the incubation, excepting at the sulphidic depth at Gotland Deep (Table 2.3; t-student, $P<0.05$), where a 50% reduction in protist numbers was observed, mainly due to a decrease in *Metacystis* spp. abundance.

For FLB abundance, significant differences were detected between the initial and final cell-counts at the oxygen / hydrogen sulphide interface of both study sites and the suboxic depth of Landsort Deep (Table 2.3; ANOVA, $P<0.05$). In all three cases calculated grazing rates were high, resulting in the removal of 50 – 80% of the prokaryotic standing stock per day. For the other three depths, the decrease in FLB abundance was not significant due to small changes in cell-counts (suboxic and sulphidic waters at Gotland Deep) or a high variability between replicates at the final time point (sulphidic waters at Landsort Deep). Control treatments showed no significant differences between initial and final FLB abundance. Net growth of the natural prokaryotic community in presence of grazers was only observed for sulphidic depths (Table 2.3). In suboxic and oxygen / hydrogen sulphide interface waters rates were negative, indicating a decrease in prokaryotic abundance over time, or were close to zero.

To assess potential bacterivory of the different protist functional groups, a qualitative assessment of the presence / absence of FLB, *Eubacteria*, and *Crenarchaeota* (the latter two detected by means of CARD-FISH) was carried out in 2009 at both stations. High percentages of HNF (22 – 63 %) contained FLB or *Eubacteria* and *Crenarchaeota* throughout all depths of the redoxcline tested (Table 2.4). For ciliates and dinoflagellates, not enough cells could be detected to obtain accurate percentages of cells containing FLB or cells labelled through CARD-FISH, but a qualitative assessment could be made. In this sense, *Strombidium* c.f and large unidentified ciliates (150 – 350 µm long) detected at the oxygen / hydrogen sulphide interface and sulphidic waters always contained large numbers of FLB or *Eubacteria* and *Crenarchaeota* in their food-vacuoles, dinoflagellates generally contained *Eubacteria* and *Crenarchaeota* (FLB detection was not possible due to fast fading of the fluorochrome signal and strong DAPI signal from the cells), *Coleps* c.f. and *Metacystis* spp. contained less often FLB or *Eubacteria* and *Crenarchaeota* in their food-vacuoles, and none were ever detected inside *Mesodinium* sp.. However in this last

case, cells always contained large quantities of autofluorescent pigments, which could have masked the fluorescence of ingested, labelled cells. Additionally, large numbers of potentially symbiotic *Eubacteria* and *Crenarchaeota* were detected in most ciliates on the surface and / or in the cytoplasm at interface and sulphidic depths (data not shown).

Table 2.4. *Percentage of HNF containing FLB or prokaryotes hybridized with Eubacteria- (EUB) or Crenarcheota- (Cren) specific CARD-FISH probes. The total number of HNF cells analyzed is shown in parenthesis.*

	Landsort Deep					Gotland Deep			
	Depth (m)	**FLB**	**EUB**	**Cren**		**Depth (m)**	**FLB**	**EUB**	**Cren**
Profile	**75**	-	63.7 (80)	57.5 (80)	**Profile**	**100**	-	39.2 (79)	38.8 (80)
	80	-	58.7 (80)	41.2 (80)		**117.5**	-	37.3 (75)	30.3 (67)
	87.5	-	56.2 (80)	38.7 (80)		**125**	-	52.5 (80)	45.0 (80)
	120	-	57.5 (40)	21.9 (41)		**132.5**	-	45.0 (60)	32.2 (62)
FLB Exp.	**65**	48.2 (83)	-	-	**FLB Exp.**	**70**	53.0 (83)	-	-
	70	46.2 (80)	-	-		**80**	55.0 (80)	-	-
	80	22.9 (61)	-	-		**110**	35.5 (76)	-	-

Viral abundance and impact on the prokaryotic communities

Viral counts were highest in surface waters (Fig. 2.7), coinciding with the maxima in prokaryotic abundance (Fig. 2.1) and then decreased and remained relatively constant throughout the water column. However, no significant correlations were observed at Gotland Deep between prokaryotic and viral abundance, and only a weak positive correlation was observed at Landsort Deep (r: 0.410; Kendal-Tau P<0.01). High-resolution profiles of the redoxcline showed no marked changes in total viral abundance (Fig. 2.7) and again no significant correlations were observed with prokaryotic abundance. Virus to prokaryote ratios ranged from 10 – 60 throughout the whole water column, with highest values above the redoxcline. At the redoxcline itself, values remained relatively constant (ratios 15 – 30).

The dilution experiments conducted in parallel to the grazing studies revealed prokaryotic cells to be exclusively infected by lytic viruses. FIC was highest at suboxic

depths (average of 19 % in Landsort and 6 % in Gotland) and then decreased to 2 – 3 % in oxygen / hydrogen sulphide interface and anoxic depths (Table 2.3). Additional dilution experiments to determine FIC at other depths throughout the redoxcline showed similar results to those conducted in parallel to the grazing experiments (Fig. 2.7). Lysogenic viral infection was constantly below the detection limit, and the frequency of virally infected cells was highest in suboxic zones (10 – 20 %), and then decreased to <5 % in interface and anoxic depths.

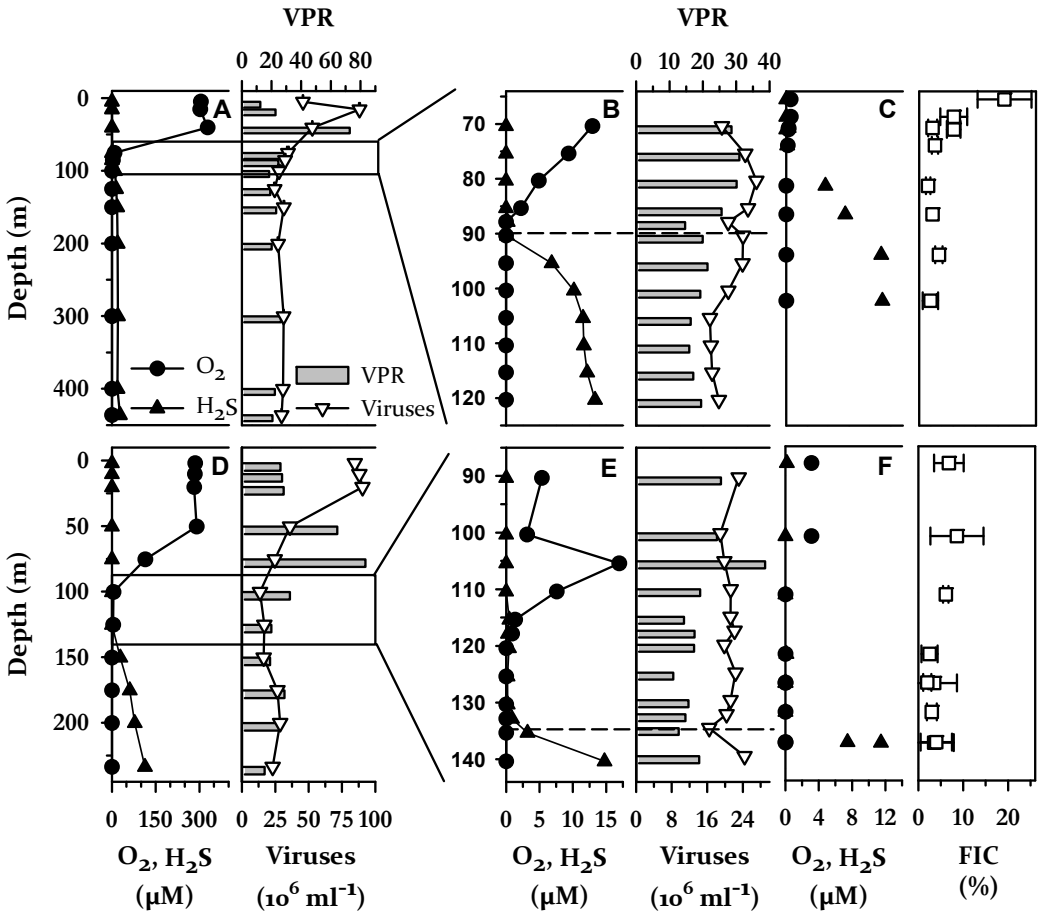

Figure 2.7. *Whole water column (A, D) and redoxcline high resolution profiles (B, E) for viral abundance and virus to prokaryote ratio (VPR), with the accompanying oxygen and hydrogen sulphide values for each depth, taken at Landsort Deep (A, B, C) and Gotland Deep (D, E, F) in 2009. For the redoxcline, average frequencies of infected cells (FIC) were determined on different casts (error bars represent the range), and are shown with the corresponding oxygen and hydrogen sulphide values for that depth (C, F).*

2.4. Discussion

Protist community composition analysis: methodological considerations

In the present study, we pooled results from three different sampling campaigns, conducted in 2005, 2008 and 2009, to obtain a first view of the structuring and function of Baltic Sea redoxcline protist communities. This was possible due to the overall stable nature of deep pelagic redoxclines, with low influence of seasonality, allowing us to compare results proceeding from different years and seasons. However, it should be taken into account that in Baltic Sea redoxclines lateral intrusion of different water masses and small-scale mixing events occur (Lass et al. 2003), which can produce conditions not normally found at the sampling depth tested (e.g. peak in oxygen at 105 m at Gotland Deep in 2009 (Fig. 2.4)). Additionally, inflow events of North Sea water occur periodically, oxygenating the anoxic basins and causing the redoxcline to retreat into the sediment (Feistel et al. 2004). Both physical processes should be considered when comparing data proceeding from different years, but, at least in the case of inflow events, constitute the exception rather than the norm in these systems.

The analysis of the protist community composition was carried out using a combination of microscopy techniques. Fixative solutions and quantification methods were selected with regard to previous literature (reviewed in Sherr & Sherr 1993b), though an optimal balance between protist preservation and quantification could not always be maintained. In this sense, the lugol concentration employed in this study could have lead to an underestimation of the abundance of certain ciliates (Stoecker et al. 1994). The effectiveness of the selected methodologies in capturing protist diversity could be compared in 2005 to the commonly used 18S rRNA gene techniques (e.g. Behnke et al. 2006; Stoeck et al. 2009; Edgcomb et al. 2011a; Orsi et al. 2012), since 18S rRNA gene clone library results from the same sampling campaign have already been reported (Stock et al. 2009). Both methodological approaches showed clear changes in the protist community throughout the physico-chemical gradient and detected similar protist groups, but the clone libraries captured, as expected, a larger fraction of the diversity. However, *Metacystis* spp. and *Mesodinium* spp., dominant species detected via microscopy, were absent from the clone libraries. These results reflect two of the major problems of applying 18S rRNA gene based approaches to suboxic and anoxic environments: the need for universal primers that recover the complete diversity (Wintzingerode et al. 1997; Stoeck et al. 2006; Edgcomb et al. 2011b) and the lack of sequenced representatives for many

microaerophilic and anaerobic ciliates (i.e. *Metacystis* spp.). Thus, for the present study, aimed at observing the major morphotypes and assessing their abundance and distribution, the selected microscopy techniques proved more adequate, despite capturing a lower fraction of the diversity.

Protist community composition: diversity and distribution

In the present study, we observed a clear dominance in terms of biomass of ciliates, and, at certain depths, dinoflagellates, with a peak in total protist abundance at the oxygen / hydrogen sulphide interface, and strong shifts in major morphotypes throughout the redoxcline. This allowed the distinction of three clear zones: 1) suboxic: dominated by *Strombidium* c.f. and dinoflagellates; 2) oxygen / hydrogen sulphide interface: dominated by *Metacystis* spp., *Mesodinium* c.f. and either *Coleps* c.f. (2008 and 2009) or *Spathidium* sp. (2005); and 3) upper anoxic / sulphidic, dominated by *Metacystis* spp. and / or unidentified ciliates. HNF were present in abundances an order of magnitude lower than in Baltic Sea surface waters (Kuuppo-Leinikki 1990; Weber 2008) and were always negligible in terms of biomass (0.05 – 3 % of total protist biomass). These results were similar for both study sites and the two sampling campaigns conducted under normal, non-inflow, conditions (2008 and 2009) and are consistent with previous observations from Gotland Deep suboxic and anoxic waters (Mamaeva 1988; Setälä 1991; Setälä & Kivi 2003), thus potentially representing the characteristic protist community of Baltic Sea redoxclines.

The shifts observed in the protist community composition along the redox gradient are in accordance with observations from other suboxic and anoxic systems (e.g. Fenchel et al. 1990; Zubkov et al. 1992; Orsi et al. 2011), and will depend on the autecology of the individual species involved (e.g, hydrogen sulphide tolerance (Vismann 1991; Fenchel & Finlay 1995) and prey availability. The strongest change in morphotype composition was detected in the present study at ~ 4 – 5 µM oxygen, likely marking the shift from a mainly aerobic to a mainly anaerobic metabolism. Physiological studies have demonstrate that oxygen optimums for microaerophilic ciliates are generally higher than this range (Finlay et al. 1986; Fenchel & Finlay 1989), while ~ 4 - 5 µmol oxygen l^{-1} has been shown to be the upper limit where oxygen can be beneficially used by anaerobic ciliates as a sink for reducing power (Fenchel & Finlay 1995).

The protist morphotypes found in the present study are also consistent with those often detected in suboxic to anoxic systems (e.g. Fenchel et al. 1995). However, some

differences in community composition do exist when comparing to similar redoxcline systems, such as those from the Black Sea, likely reflecting differences in salinity and hydrological regimes, among other factors. Notably, *Metacystis* spp., though found previously in diverse suboxic and anoxic environments (e.g. Foissner 1984; Fenchel et al. 1995), is recorded here for the first time as a major constituent of deep pelagic redoxclines. Additionally, the HNF community composition, as revealed by live microscopy in 2005, showed no obvious shifts throughout the oxygen gradient, differing from results obtained at Danish fjords (Fenchel et al. 1995). Flagellates with a wide distribution throughout different oxygen conditions have also been detected in other suboxic and anoxic systems, such as certain uncultured marine stramenopiles (MAST taxa) at the Cariaco Trench (Orsi et al. 2011). However, it remains unclear whether the flagellates found in the present study indeed constitute species with a wide physico-chemical tolerance or encompass genetically different strains with a highly similar morphology (Scheckenbach et al. 2005).

A broad comparison of the protist community during all three years studied showed no strong influence either of seasonality, or of the 2003 North Sea water inflow event, which affected Gotland Deep in 2005. However, in this latter year, as we did not analyse the full suboxic water column, we cannot properly assess the effect of the inflow event on the protist community in this zone. Some differences in community composition could be observed at oxygen / hydrogen sulphide interface and sulphidic depths in 2005, but these mainly related to ciliate morphotypes present in low numbers (< 200 cell / l) and the absence of *Coleps* c.f. It remains to be investigated whether Baltic Sea pelagic anaerobic ciliates are capable of living in the sediments during periods of water column oxygenation, similar to those inhabiting lakes (Finlay 1981), or have developed resistance forms to withstand it.

Role of protists and viruses in Baltic Sea redoxcline food webs

The FLB experiments conducted revealed significant and high grazing at the suboxic depth of Landsort Deep and at the oxygen / hydrogen sulphide interface depths of both study sites, accounting for 50 – 80 % of the prokaryotic standing stock per day. These bacterivory rates are among the highest measured for suboxic and oxygen / hydrogen sulphide interface systems (Park & Cho 2002; Bettarel et al. 2004; Saccà et al. 2009) and are considerably higher than those estimated in a previous study for suboxic and anoxic waters at Gotland Deep, through biovolume-dependent theoretical clearance rates for ciliates (Setälä & Kivi 2003). However it should be noted that in this last study, ciliate

abundance was lower than in the present study (< 10^3 cell l^{-1}). The rough application of the same theoretical clearance rates to the estimated ciliate biovolumes and abundances found in the present study, combined with the use of literature clearance rates for dinoflagellates and flagellates (Kuuppo-Leinikki 1990; Neuer & Cowles 1995), resulted in grazing estimates that fell within the range of experimentally measured rates (Supplementary Table S2.1). Studies in oxygenated water masses demonstrate that protist selection for or against FLB can lead to over- or underestimations of grazing rates (Landry et al. 1991; Mischke 1994). This potential bias will in all likelihood also apply to suboxic and anoxic water masses. However, the comparison of measured and estimated grazing rates allows us to conclude that despite this potential selectivity, the use of FLB did not shift the experimentally obtained grazing rates away from reasonable ranges for the protist community present.

At the other 3 depths tested (suboxic zone of Gotland Deep and the two sulphidic depths), no significant grazing was detected. The first of these depths, however, showed anomalous water chemistry (nitrate values were lower than expected, and ammonia was higher) and a mix of typically suboxic (*Strombidium* c.f. and dinoflagellates) and oxygen / hydrogen sulphide interface / anoxic protists (*Metacystis* spp.), likely indicating the effect of a small-scale mixing event (e.g. due to lateral intrusions). Thus, the estimated grazing rate would belong to a perturbed system, and not be a representative rate for this zone. For the other two depths, the low protist abundance and the tendency of one of the dominating protists, *Metacystis* spp., to decrease during the incubations, suggest that we were at the detection limit of the methodology, leading to low and variable rates, with no statistical significance. Overall, the very low protist abundance at sulphidic depths in this and previous studies (Mamaeva 1988; Setälä 1991; Detmer et al. 1993) supports the view that the bacterivorous impact on the prokaryotic community is low in this zone.

The analysis of food-vacuole content revealed that nearly all protists observed had the potential for bacterivory, though some of the larger ciliates (e.g. *Coleps* c.f.) contained prokaryotes less often, potentially indicating a more predatorial role on smaller protists (i.e., observed FLB could belong to the food vacuoles of protist prey). For HNF, the consistency of the percentage of flagellates containing FLB or bacteria detected by EUB probes, indicates that around 50 % of HNF were active grazers at most depths. However, their low abundance excludes them from being major predators. Rough calculations, using HNF abundances determined throughout the redoxclines of both study sites in all 3 years, a clearance rate of 5 nl flagellate^{-1} h^{-1}, determined for HNF in surface waters of the Baltic

(Kuuppo-Leinikki 1990), and assuming that 50 % of the HNF community is active, showed that HNF grazing accounted in all cases for less than 8 % of the prokaryotic standing stock day^{-1}, generally being below 5 %. Thus, as opposed to surface waters where grazing is dominated by small bacterivorous flagellates (Sherr & Sherr 2002; Jürgens & Massana 2008), ciliates and, under suboxic conditions, dinoflagellates, would constitute the major grazers of Baltic Sea oxic / anoxic transition zones.

The analysis of the frequency of virally infected prokaryotic cells showed that lysogeny remained below detection throughout the redoxcline, while lytic infection was highest in the suboxic zone (reaching occasionally up to 25 %), and decreased to <5 % at the oxygen / hydrogen sulphide interface and in sulphidic zones. The rationale behind the virus-dilution approach (Wilhelm et al. 2002) is to dramatically reduce viruses and therefore prevent new viral infections during the incubation. Thus, an increase in viruses is only due to viral infections which had already occurred in the source water, prior to the start of the incubation, and is not an artefact of the incubation itself. However, this methodology requires the water to be filtered, and, for redoxcline samples, every filtration alters the *in situ* physico-chemical conditions (e.g., oxygen or sulphide concentrations). Thus, FIC estimates may have suffered from this disturbance. However, prokaryotic abundance in the incubations was stable over time, exhibiting no drastic decreases in abundance such as would occur through a strong toxic effect of oxygen. Additionally, FIC from suboxic and oxygen / hydrogen sulphide interfaces should be less impacted than for sulphidic depths. A previous study conducted at Gotland Deep (Weinbauer et al. 2003) detected similar or slightly higher FIC for suboxic zones, but also found increased FIC (up to 25 %) in the anoxic zone. Weinbauer et al. (2003) did not reduce viral abundances through dilution, which excludes the problem of oxygen contamination. However, new viral infection during their particularly long incubation times of up to 3 days may have increased their estimates in all zones. This is especially important in the Mitomycin C-induced treatments used to determine the impact of lysogenic viruses. Mitomycin C causes DNA damage affecting DNA replication and cell division, resulting in greatly elongated cells. Because viral contact rates increase with the size of host cells (Murray & Jackson 1992), estimates of FIC due to lysogenic viruses by Weinbauer et al. (2003) may be higher than *in situ*.

Overall, prokaryotic mortality decreased from suboxic to upper anoxic / sulphidic depths, with the reduction in bacterivorous pressure not being accompanied in the depths analysed by a corresponding increase in viral to prokaryote ratios or FIC. This could also

be observed in the net prokaryotic growth rates, which were negative or close to zero in suboxic and oxygen / hydrogen sulphide interface waters, but positive for upper sulphidic zones (Table 2.3). Thus, for upper sulphidic layers, where a high chemoautotrophic prokaryotic productivity is still maintained (Jost et al. 2010), neither protist grazing nor viral lysis appear to be major mortality factors. A similar phenomenon has been observed for some lakes (Bettarel et al. 2004), where summed mortality in the metalimnion and seasonally in the hypolimnion, remained below 50 % of prokaryotic production. This discrepancy between prokaryotic productivity and loss is a major open question requiring further study. Potential biases in the measurement of productivity and mortality should be determined, along with the assessment of other potential mortality factors (i.e, physical (e.g., sedimentation) or biological (e.g., apoptosis) processes). A significantly higher grazing impact than measured here is unlikely at these depths, due to the very low protist abundance present, but viral lysis could have been underestimated (as discussed above). It additionally should be considered that for some prokaryotic groups specific mortality by selective protist grazing or viral infection (Jürgens & Matz 2002; Yoon et al. 2011) might result in significantly higher losses than averaged for the total prokaryotic community.

2.5. Conclusions

In the present study, using a combination of microscopy techniques and incubation experiments, we demonstrate the existence of a characteristic protist community structure for pelagic redoxclines of the central Baltic Sea, which is remarkably stable over different years, different study sites, and different water-column conditions. Furthermore, we showed the potential of said community to exert a strong grazing control on prokaryotic communities at suboxic and oxygen / hydrogen sulphide interface depths, but not in sulphidic zones. Viruses, on the other hand, did not appear to constitute a strong loss factor for prokaryotic communities at the depths tested. Thus, there is a discrepancy between the high prokaryotic productivity previously measured at upper sulphidic depths and low mortality. Finally, we uncovered differences to surface water food-web structuring, with HNF playing only a marginal role, and ciliates and, at some depths, dinoflagellates acting as the major bacterivores.

Chapter 3

Protist grazing impact on a chemoautotrophic bacterial key-player of Baltic Sea redoxclines

Abstract

Barrier zones between oxic and anoxic water masses (pelagic redoxclines) host highly active prokaryotic communities that mediate important biogeochemical transformations. In central Baltic Sea pelagic redoxclines, *Epsilonproteobacteria* of the genus *Sulfurimonas* (subgroup GD17) have been shown to dominate dark CO_2 fixation and be responsible for denitrification via sulphide oxidation. However, the loss processes balancing this chemoautotrophic production are unknown. In the present study, the protist grazing impact on the *Sulfurimonas* subgroup GD17 was determined using predator exclusion assays and bacterial amendment with a cultured *Epsilonproteobacteria* from Baltic Sea redoxclines ("*Sulfurimonas gotlandica*" strain GD1). Experiments were conducted for two physico-chemically differing depths of Baltic Sea pelagic redoxclines (suboxic and oxygen / hydrogen sulphide interface). Additionally, the principal bacterivores involved were identified by RNA-Stable Isotope Probing (RNA-SIP), employing labelled "*S. gotlandica*" strain GD1 as substrate. The results obtained demonstrate a strong growth of the *Sulfurimonas* subgroup GD17 at the oxygen / hydrogen sulphide interface (doubling time of 1 – 3 days), which could be completely accounted for by protist grazing (>100 % of new cell production d^{-1}). At suboxic depths, little or no growth of *Sulfurimonas* subgroup GD17 was observed, but grazing was still high, indicating that this was a suboptimal habitat for this bacterial group. RNA-SIP identified 5 active grazers on "*S. gotlandica*" strain GD1, belonging to typical redoxcline ciliate groups (Prostomatea and Oligohymenophorea) and wide-spread marine flagellates (Chrysophyta, MAST-4 and Cercozoa (novel clade VI)). Overall we demonstrate, for the first time, that protist grazing can control the growth of a chemolithoautotrophic key-player of oxic / anoxic interfaces.

3.1. Introduction

Prokaryotic communities inhabiting barrier zones between oxic / anoxic water masses, also termed redoxclines, play vital roles in biogeochemical cycling. Successive studies have demonstrated their importance for i.e. chemoautotrophic production (Taylor et al. 2001;

Jost et al. 2008), denitrification and ammonia oxidation (Hannig et al. 2007; Lam et al. 2007; Labrenz et al. 2010), and sulphur cycling (Jannasch et al. 1991; Jørgensen & Bak 1991). Based on dark CO_2 fixation measurements, it has been postulated that these prokaryotic communities will be highly active and achieve relatively high growth rates, with doubling times in the range of 1 – 2 days estimated for both the Cariaco Basin (Taylor et al. 2006) and the Baltic Sea (Jost et al. 2008). However, little is known on the loss factors that balance this productivity.

The potential for protist grazing to regulate the biomass, activity and composition of prokaryotic communities is well known for oxygenated marine and freshwater systems (Jürgens & Matz 2002; Sherr & Sherr 2002; Pernthaler 2005). The recent *in situ* application of techniques that take into account grazer identity, such as RNA-Stable Isotope Probing (RNA-SIP) (Lueders et al. 2006; Frias-Lopez et al. 2009; Moreno et al. 2010) or Fluorescent In Situ Hybridization (FISH) (Massana et al. 2009; Piwosz & Pernthaler 2009), has additionally lead to an increased understanding of how preferential grazing by specific protist groups can affect the distribution of their prokaryotic prey. As an example, *in vitro* experiments (Christaki et al. 1999; Guillou et al. 2001) and *in situ* studies employing RNA-SIP (Frias-Lopez et al. 2009) revealed differences in the protist assemblages that graze on the highly abundant marine cyanobacteria *Prochlorococcus* and *Synechococcus*, which could partly explain their different distribution throughout the water column. Thus, protist bacterivory, and the abundance of specific protist grazers, can be as important as abiotic environmental factors (i.e., nutrients or temperature) in explaining the distribution and activity of a given prokaryotic group in the water column (Thingstad 2000a; Gasol et al. 2002).

However, very few studies have addressed this topic for suboxic to anoxic water masses, despite the fact that protists can be abundant and active bacterivores in these zones (Chapter 2; Fenchel et al. 1995; Bettarel et al. 2004; Gobler 2008). Lin et al. (2007), and Bautista-Reyes & Macek (2012), both demonstrated preferential protist grazing on certain broad phylogenetic prokaryotic groups in redoxcline systems, respectively *Gamma-* and *Epsilonproteobacteria* in the marine Cariaco Basin, and *Alpha-*, *Delta-* and *Gammaproteobacteria* in the hypoxic hypolimnion of a stratified lake. Glaubitz et al. (2009) additionally demonstrated by means of RNA-Stable Isotope Probing (RNA-SIP), that chemolithoautotrophically produced biomass could be assimilated by bacterivorous ciliate of Baltic Sea anoxic water masses. However, studies quantifying the magnitude of

protist grazing on specific key-prokaryotic groups of suboxic to anoxic water masses are lacking.

In the present study we focused on the pelagic redoxclines of the Baltic Sea, one of the worlds´ largest brackish environments. A stable halocline, situated at 60 – 80 m depth, limits vertical mixing and separates the water column into an upper oxygenated layer and a bottom oxygen depleted layer which becomes anoxic and sulphidic in the deepest zones (Grasshoff 1975). The prokaryotic communities inhabiting this pelagic redoxcline have been studied in depth, uncovering several groups of special importance, among them the *Sulfurimonas* subgroup GD17 (Grote et al. 2007, 2008, 2012). This phylogenetic cluster represents almost the totality of *Epsilonproteobacteria* in the oxic / anoxic transition zone of the Baltic Sea, and can constitute up to 25 % of total cell counts. It has been shown to dominate chemoautotrophic production and is actively involved in hydrogen sulphide oxidation coupled to denitrification, thus potentially constituting an important barrier for the spreading of the sulphidic zone in Baltic Sea redoxclines (Grote et al. 2012). However, information on regulating processes for the *Sulfurimonas* subgroup GD17 other than substrate availability is lacking to date, and could be vital in understanding the *in situ* activity, abundance and distribution of this prokaryotic group. Meanwhile, protist grazing has been shown to be very high at the oxic / anoxic interface of Baltic Sea redoxclines, with up to 100 % of bacterial standing stocks grazed per day (Chapter 2; Table 2.3). Thus, a strong bacterivorous pressure could act as an important regulating factor for the growth of the *Sulfurimonas* subgroup GD17 at the oxic / anoxic interface of Baltic Sea pelagic redoxclines.

To determine the potential grazing impact on the *Sulfurimonas* subgroup GD17, we applied a combination of predator exclusion and bacterial addition assays (employing a cultured representative of this phylogenetic cluster, "*Sulfurimonas gotlandica*" strain GD1 (Grote et al. 2012)). Experiments were conducted at two physico-chemically differing depths: lower suboxic zone (low oxygen concentrations (3 - 5 µM O_2 in the present study) and presence of nitrate); and oxygen / hydrogen sulphide interface (oxygen and hydrogen sulphide around the detection limit, and trace levels of nitrate). Additionally, RNA-SIP was applied at the oxygen / hydrogen interface to identify grazers on this bacterial group, employing [^{13}C]-labelled "*S. gotlandica*" strain GD1 as substrate.

3.2. Materials and methods

Study sites, sampling and measurement of physico-chemical parameters

Sampling for predator-exclusion and bacterial addition assays were conducted in the central Baltic Sea at Gotland Deep (station 271; 57° 19.2′ N; 20° 03′ E) on board the R.V. *Maria S. Merian* in September 2009. Sampling for the identification of grazers was carried out under similar physico-chemical conditions (Table 3.1) in Landsort Deep (station 284; 58° 35.0′ N; 18° 14.0′ E) on board the R.V. *Alkor* in March 2011. Water was collected in free-flow bottles attached to a conductivity, temperature and depth rosette (CTD) with a coupled oxygen sensor. Inorganic nutrients, oxygen and hydrogen sulphide were measured immediately on board according to standard methods (Grasshoff et al. 1983).

Table 3.1. *Physico-chemical parameters measured for each of the depths analyzed in the different experiments, and for the three depths used for comparison to the RNA-SIP experiment (Depth 1, 2 and 3) (Pred. Exc.: predator exclusion without addition; Pred. Exc. Add.: predator exclusion with addition of "Sulfurimonas gotlandica" strain GD1; RNA-SIP: RNA Stable Isotope Probing; Study sites, G: Gotland Deep, L: Landsort Deep; Zones, S: suboxic; I: oxygen / hydrogen sulphide interface.*

Experiment	Study Site	Zone	Depth (m)	O_2 (µM)	H_2S (µM)	NO_3 (µM)	NH_4 (µM)
Pred. Exc.	G	S	105	4.91	-	2.71	0.55
	G	I	135	0.44*	0.2*	-	2.88
Pred. Exc. Add.	G	S	120	3.13	0	-	0.06
	G	I	135	-	0.25*	-	2.64
RNA-SIP	L	I	90	2.23*	-	1.47	2.55
Depth 1	L	I	85	3.71	-	3.38	1.09
Depth 2	L	I	87	1.83*	-	2.02	2.02
Depth 3	L	I	89	1.12*	-	2.76	1.59

- Parameter not measured at this depth.

* Value around the detection limit of the methodology (2 µM for O_2 and 0.2 µM for H_2S).

Predator-exclusion assays

Experiments were carried out with water from two depths selected to correspond to suboxic and oxygen/hydrogen sulphide interface zones. For the preparation of treatments with no predators, water from the free-flow bottles was collected, avoiding air contact, in 4 l bags pre-gassed with nitrogen (UD 3 TUR-BAG, Sarstedt; modified to seal any non required openings). Only the bottom 5 l of 10 l free-flow bottles was used to avoid oxygen contamination during emptying of the free-flow bottles. Filled bags were taken to a 4 °C climate chamber and placed inside a glove bag with a nitrogen atmosphere (AtmosBag™; Sigma-Aldrich). Predator exclusion was achieved as shown in Fig. 3.1, by filtration through 1 µm pore-size polycarbonate filters (Isopore; diameter, 47 mm; placed inside a Swin-Lock™ filter holder; all Whatman), with the help of a peristaltic pump (Sci-Q-323; Watson Marlow; 100 rpm). Gas tight tubing was employed to avoid oxygen contamination (Iso-Versinic, Saint-Gobain Performance Plastics). Filtered water was transferred via needles (Sterican; 0.8 mm x 120 mm; B. Braun) into acid-cleaned glass bottles with septum lids, which had been pre-gassed with argon gas. Non-fractioned control treatments (with predators) were prepared by filling 1 l acid cleaned glass bottles directly from the free-flow bottles, with at least half a minute of overflow, and immediately closing them avoiding air bubbles. Incubation was conducted approximating *in situ* conditions (4°C, in the dark). Samples were collected at 0, 27 and 50 h, with separate sets of triplicate incubation bottles prepared for each time point to avoid oxygen contamination during sampling.

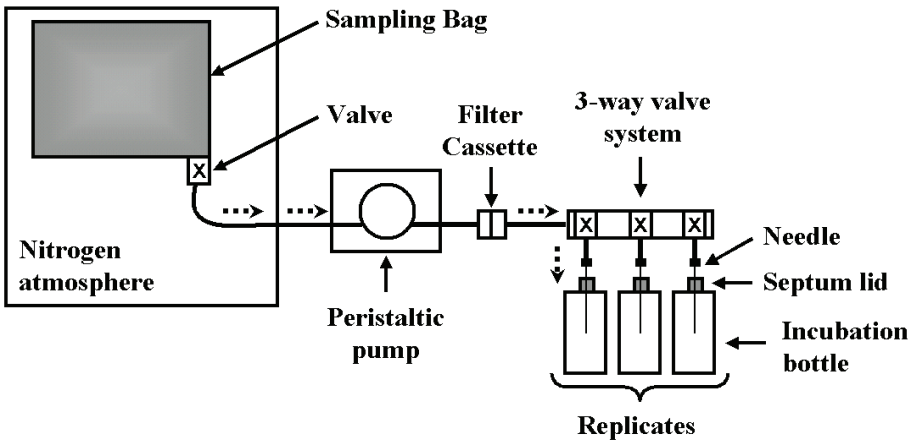

Figure 3.1. *Set-up used to obtain filtered treatments in predator exclusion experiments. Dashed arrow indicates direction of water-flow.*

Predator exclusion with addition of "*Sulfurimonas gotlandica*" strain GD1

In a second set of experiments, the relative proportion of the *Sulfurimonas* subgroup GD17 population was increased by addition of "*S. gotlandica*" strain GD1, so that it constituted the principal available prey (~ 50 % of total prokaryotic cell-counts). To obtain cultures for the amendment, "*S. gotlandica*" strain GD1 (Grote et al. 2012) was grown anaerobically for around 15 days, at 15 °C and in the dark, using as media artificial brackish water medium (ABW) (Bruns et al. 2002) modified as previously described (Grote 2009). Experiments were carried out with water from two depths selected to correspond to suboxic and oxygen / hydrogen sulphide interface zones. Predator exclusion and control treatments were prepared as described above (Fig. 3.1) with the incorporation of a bacterial addition step. Immediately after filling, bottles were placed in an anaerobic chamber (Coy Laboratories) with a forming gas atmosphere, and "*S. gotlandica*" strain GD1 was added to a final concentration of ~ 5 x 10^5 bacteria ml^{-1}. Incubation was conducted approximating *in situ* conditions (4°C, in the dark). Samples were collected at 0, 27 and 50 h, with separate sets of triplicate incubation bottles prepared for each time point to avoid oxygen contamination during sampling.

Prokaryote and protist quantification

Total prokaryotic cell-counts were obtained by flow-cytometry as previously described (Labrenz et al. 2007). Heterotrophic nanoflagellate (HNF), dinoflagellate and ciliate abundance and morphotype composition were obtained by epifluoresence and light microscopy following the protocols described in detail in Chapter 2.

Sulfurimonas subgroup GD17 abundance was obtained by Catalyzed Reporter Deposition-Fluorescence In Situ Hybridization (CARD-FISH) employing probe SUL90 for detection as previously described (Grote et al. 2007). A minimum of 200 hybridized cells were counted at 1000 X using filter set 09 for detection under an Axioskop 2 mot plus epifluorescence microscope (Carl Zeiss MicroImaging). In cases of uneven filter surface distribution two or more filter sections from opposing sides of the filter were counted and averaged, or, in severe cases, filters were discarded. The SUL90 probe can not distinguish between the *Sulfurimonas* subgroup GD17 and its cultured representative, "*S. gotlandica*" strain GD1 (Grote et al. 2007). Thus, all counts are referred to as *Sulfurimonas* subgroup GD17 abundance.

Estimation of *Sulfurimonas* subgroup GD17 growth and grazing losses

Growth and grazing rates were determined from the predator exclusion experiments as follows. Exponential growth was assumed, and net growth rates of *Sulfurimonas* subgroup GD17 were determined for each treatment as:

$$\mu = (\ln N_t - \ln N_o) / (t_t - t_o)$$

where N_o and N_t are respectively the cell abundance at the start of the experiment (t_o) and at the end of the time interval measured (t_t). At t_o, all cell-counts for the same depth were averaged, giving a pooled initial abundance for *Sulfurimonas* subgroup GD17 (N_o). Net growth rates were subsequently used to calculate doubling times for a given time interval as:

$$\ln (2) / \mu$$

Finally, grazing rates were calculated for each time interval and depth as:

$$G = \mu_{\text{filtered treatment}} - \mu_{\text{unfiltered treatment}}$$

Incubations for RNA-stable isotope probing (RNA-SIP)

"*S. gotlandica*" strain GD1 was grown prior to the cruise as described above, using [^{12}C]-$NaHCO_3$ (Merck) for `normal´ culture conditions (unlabelled, [^{12}C]-"*S. gotlandica*" strain GD1) or as [^{13}C]-$NaHCO_3$ (Cambridge Isotope Laboratories) to obtain [^{13}C]-labelled "*S. gotlandica*" strain GD1.

Experiments were carried out with water from a depth selected to correspond to the oxygen / hydrogen sulphide interface. 2 l acid-cleaned narrow-neck glass bottles were filled from the free-flow bottles, with at least half a minute of overflow, and immediately closed with tightly fitting glass stoppers. The bottles were incubated without further amendment in the dark at 4 °C for 5 days to achieve an enrichment in protists naturally abundant *in situ*, following the principle of unamended sea-water incubations (Massana et al. 2006). Once in the laboratory, part of the bottles were left unamended (referred to as N-treatment), and part were inoculated in an anaerobic chamber (Coy Laboratories; forming gas atmosphere) with either [^{13}C]-labelled "*S. gotlandica*" strain GD1 (referred to as [^{13}C]-treatment) or unlabeled "*S. gotlandica*" strain GD1 (referred to as [^{12}C]-treatment). Incubation was conducted at 10 °C in the dark. Samples were collected at 0 and 50 h, with separate sets of triplicate incubation bottles prepared for each time point to avoid oxygen

contamination during sampling. Samples were obtained for determining protist, total prokaryote and *Sulfurimonas* subgroup GD17 abundance as described above. The remaining volume (~ 1.4 l) was filtered onto membrane filters (Isopore; pore size, 0.2 µm; diameter, 47 mm; Millipore), which were then shock-frozen and stored at -80°C for the subsequent analysis of 16S and 18S rRNA.

Nucleic acid extraction, isopycnic centrifugation, and 18S rRNA gene quantification

DNA-free total RNA was obtained following the protocol in Weinbauer et al. (2002), modified according to Glaubitz et al. (2010). Gradient preparation, isopycnic centrifugation, and gradient fractionation were performed as previously described (Lueders et al. 2004b), with minor modifications as described in Glaubitz et al. (2009). Each gradient consisted of 5.1 ml of CsTFA (~ 2 g ml^{-1}, GE Healthcare), 185 µl of formamide and 1 ml of gradient buffer (100 mmol Tris-HCl l^{-1} (pH 8.0), 100 mmol KCl l^{-1}, 1 mmol EDTA l^{-1}) including 500 ng of DNA-free RNA. Prior to centrifugation, the average density of the centrifugation medium was controlled refractometrically and adjusted to an average density of 1.80 g cm^{-3} if necessary. The samples were centrifuged in 5.1 ml Quickseal polyallomer tubes in a VTi 65.2 vertical rotor using an Ultima L-100 XP centrifuge (all Beckman Coulter). Centrifugation was carried out at 20 °C for > 65 h at 35 000 rpm (105 000 g). Gradients were fractionated as previously described (Neufeld et al. 2007) and the density of each collected fraction was measured by determining the refractory index. Subsequently, the RNA contained in each fraction was precipitated using isopropanol; the pellet was washed once with 70% ethanol, and was then dissolved in 25 µl of diethylpyrocarbonate (DEPC) treated water (Calbiochem).

Quantitative reverse-transcription-PCR (RT-qPCR) of density-resolved RNA was carried out following the protocol described in Glaubitz et al. (2009) employing a one-step RT-PCR kit (Access Kit, Promega) and a quantitative PCR cycler (iCycler; Bio-Rad). Domain-specific primer sets for eukaryotes Euk 350f (5′- TCC GGA GAG GGW GCC TGA G – 3′) and E561-R (5′ - TTA CCG CGG STG CTG GCA CC – 3′) were used, obtained by modifying the primers in Zhu et al. (2005) to afford a better coverage of cercozoa and some ciliate groups (e.g., *Mesodinium* spp. or *Cyclidium* spp.). For absolute quantification of rRNA molecules, *in vitro* RNA transcripts of cloned *Tetrahymena pyriformis* were used in defined dilution steps from 10^8 to 10^2 copies per qPCR reaction. PCR was started with reverse transcription (45 min at 48°C), followed by an initial denaturation step for 5 min.

Amplification was performed with a 40-cycle PCR (30 s 95 °C, 30 s 56 °C, 30 s 68 °C, 10 s 80 °C) followed by a terminal elongation step (5 min). After each run, a melting curve between 50 °C and 89 °C was collected to differentiate between specific amplicons and unspecific signals.

16S and 18S rRNA fingerprinting analysis

The microeukaryote community present in each of the density resolved fractions was analyzed by denaturing gradient gel electrophoresis (DGGE). cDNA was synthesised using the iScript Select Kit (Bio-Rad) according to manufacturer's instructions. 1µl of the cDNA obtained served as template for a PCR using the eukaryotic primers EukA and Euk516r-GC (Díez et al. 2001) and 2U Taq DNA polymerase (5 PRIME). The PCR started with an initial denaturation step at 94°C for 2 min followed by 30 cycles of denaturation at 94 °C for 30 s, annealing at 56 °C for 45 s and elongation at 72 °C for 2 min. The terminal elongation step was performed at 72 °C for 6 min. Samples were electrophoresed on 6% polyacrylamide gel as described in Díez et al. (2001), with a linear denaturating gradient of 25 – 55 %, and using the Ingeny PhorU system. Gels were run for 16 h at 100 V and 60 °C, with 0.5 X TAE as electrophoresis buffer, and then stained for 60 min with 1X TAE buffer containing SybrGold. The bands were visualized by UV radiation and excised under illumination with blue light.

As a control for transfer of the [^{13}C]-signal within the prokaryotic community, the 16S rRNA contained in the density resolved fractions was analysed by single stranded conformation polymorphism (SSCP) as previously described (Glaubitz et al. 2009).

The DGGE and SSCP fingerprinting gels were digitalized and the resulting images processed using GelCompar II (Applied Math). The relative contribution of each band to the total band intensities of each lane was calculated based on densiometric curves after normalization and background subtraction.

Sequence analysis

Sequencing efforts were centred on the gels proceeding from the [^{13}C]-treatment, though representative bands were also analysed for control gels. Bands from SSCP gels were excised and cleaned as previously described (Glaubitz et al. 2009). Bands from DGGE gels were excised, eluted in nuclease-free water, and reamplified, using the same primer set and PCR conditions as described above. The purity and correct mobility of the reamplified

product was tested in a second DGGE (denaturing gradient; 25 – 45 %). Bands excised from the second DGGE were re-amplified in a semi-nested PCR with the primer sets 18SForN2 (5′ - GAT CCT GCC AGT AGT CAT AYG C – 3′) (Wylezich et al., submitted) and Euk516r (with no GC clamp; see Díez et al. 2001), and purified with the Nucleospin II kit (Macherey and Nagel). Sequencing was performed by Qiagen (Hilden, Germany). All sequences were quality checked using the programme BioEdit (Hall 1999) and preliminary estimates of phylogenetic affiliations of the 16S and 18S rRNA sequences were obtained by BLAST (Altschul et al. 1997).

Phylogenetic analysis

Sequences obtained were aligned with those retrieved from GenBank using the CLUSTAL_X program (Thompson et al. 1997) and then edited manually. MrBayes (Huelsenbeck et al. 2001) and PhyML 3.0 (Guindon & Gascuel 2003); http://www.atgc-montpellier.fr/ phyml/) were used for the phylogenetic analyses. This was carried out with the GTR model of substitution (Lanave et al. 1984) and a gamma-shaped distribution of substitution rates among sites, with eight rate categories and a proportion of the invariable sites. Bayesian analysis was performed for 1 000 000 generations and sampled every 100 generations for four simultaneous chains. For the likelihood analysis, all model parameters were estimated from the dataset. To estimate branch support, 100 bootstrap replicates were performed in maximum-likelihood analyses.

Statistical analysis

Significant differences in protist and prokaryote abundances at different time-points during incubations were detected by means of ANOVA or t-test, using the programme SPSS 15.0 (SPSS).

3.3. Results

Grazing impact on *Sulfurimonas* subgroup GD17

Two experiments were conducted comparing the growth of *Sulfurimonas* subgroup GD17 in presence and absence of grazers, and under similar suboxic and oxygen / hydrogen sulphide interface conditions (Fig. 3.2; Table 3.1). In the first, no further amendment was

conducted (henceforth called non-addition experiment) and, in the second, "*S. gotlandica*" strain GD1 was added (henceforth addition experiment). In both experiments, only trace abundances of the three functional protist groups could be found at the end of the incubation in filtered treatments (<10 %, and generally <5 %; Fig. 3.3B, D; Fig. 3.4B, D), indicating that predator exclusion was successful.

The initial abundance of the natural *Sulfurimonas* subgroup GD17 population was similar for depths of comparable physico-chemical conditions in the two experiments (respectively for non-addition and addition experiments, 2.54 ± 0.34 x 10^4 cell ml^{-1} and 2.24 x 10^4 cell ml^{-1} at the suboxic zone, and 6.52 ± 0.86 x 10^4 cell ml^{-1} and 8.06 x 10^4 at the oxygen / hydrogen sulphide interface). In the addition experiment, the inoculation of "*S. gotlandica*" strain GD1 raised the proportion of the *Sulfurimonas* subgroup GD17 from 2 to ~ 65 % of the total prokaryotic community at the suboxic depth, and from 6 to ~ 45 % at the interface.

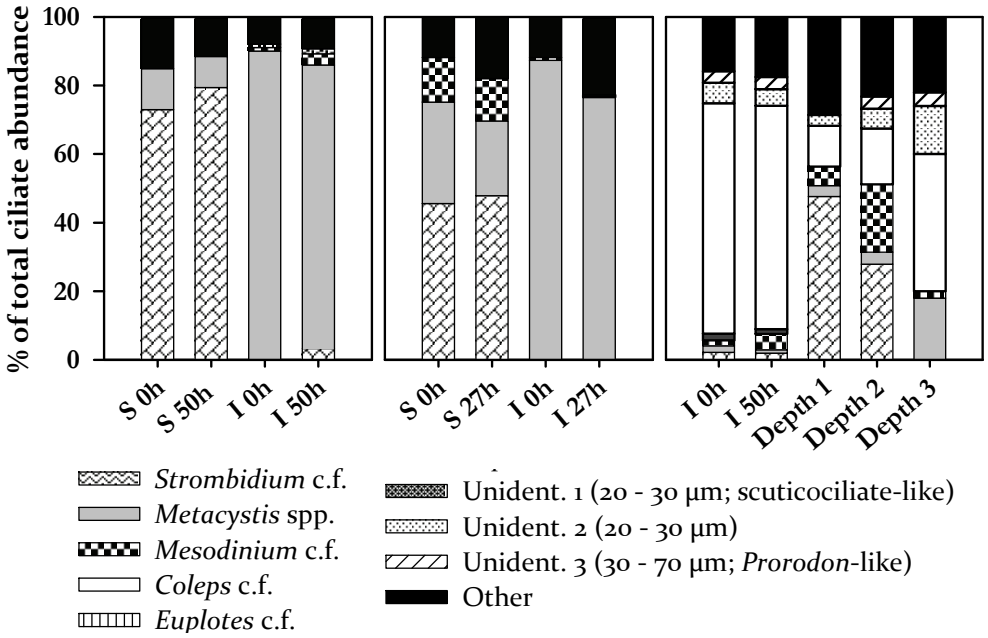

Figure 3.2. *Relative abundance of the different ciliate morphotypes identified at the beginning and end of the predator exclusion experiments, (A) without and (B) with addition of "S. gotlandica" strain GD1; and (C) at the beginning and end of the ^{13}C enrichment experiment compared to in situ depths of similar physico-chemical characteristics sampled during the same cruise (Depth 1, 2 and 3). S: suboxic depth; I: oxygen / hydrogen sulphide interface depth. For the RNA-SIP experiments, three abundant morphotypes were distinguished through morphological characteristics, but could not be classified (Unident. 1, 2 and 3).*

In both sets of experiments, *Sulfurimonas* subgroup GD17 showed different dynamics over the incubation time at the two depths analysed (Fig. 3.3 and 3.4, Table 3.2). At suboxic depths, the *Sulfurimonas* subgroup GD17 generally showed no growth in treatments with no predators (Fig. 3.3 and 3.4, Table 3.2). The exception was the first 27 h of the non-addition experiments, where significant growth was observed (ANOVA $P<0.05$), followed by a strong decrease to an abundance equal to that at the start of the experiment (ANOVA $_{27h\ vs\ 50h}$ $P<0.05$, $_{0h\ vs.\ 50h}$ $P>0.05$). In treatments with predators at the suboxic zone, *Sulfurimonas* subgroup GD17 abundance did not vary over time in the non-addition experiment (t-test; $P>0.05$), and decreased in the addition experiments (t-test; $P>0.05$). This resulted in grazing rates of 0.21 d^{-1} for non-addition experiments, and 0.38 d^{-1} for addition experiments, being in both cases higher than the growth rates (Table 3.2).

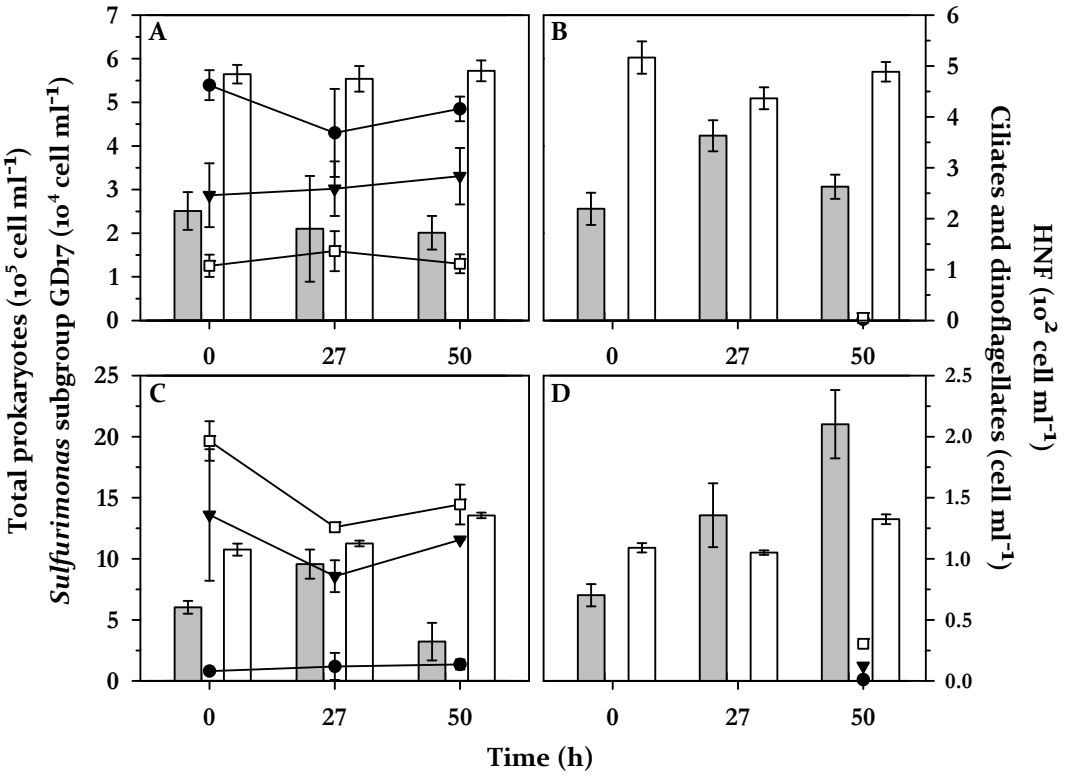

Figure 3.3. *Changes during the predator exclusion experiments (with no bacterial addition) in total prokaryote (white bars), Sulfurimonas subgroup GD17 (grey bars) and protist abundance (line plots; ciliates (□), HNF (▼), and dinoflagellates (●)), under suboxic conditions in presence (A) and absence (B) of grazers; and under oxygen / hydrogen sulphide interface conditions in presence (C) and absence (D) of grazers. The corresponding standard deviations are shown.*

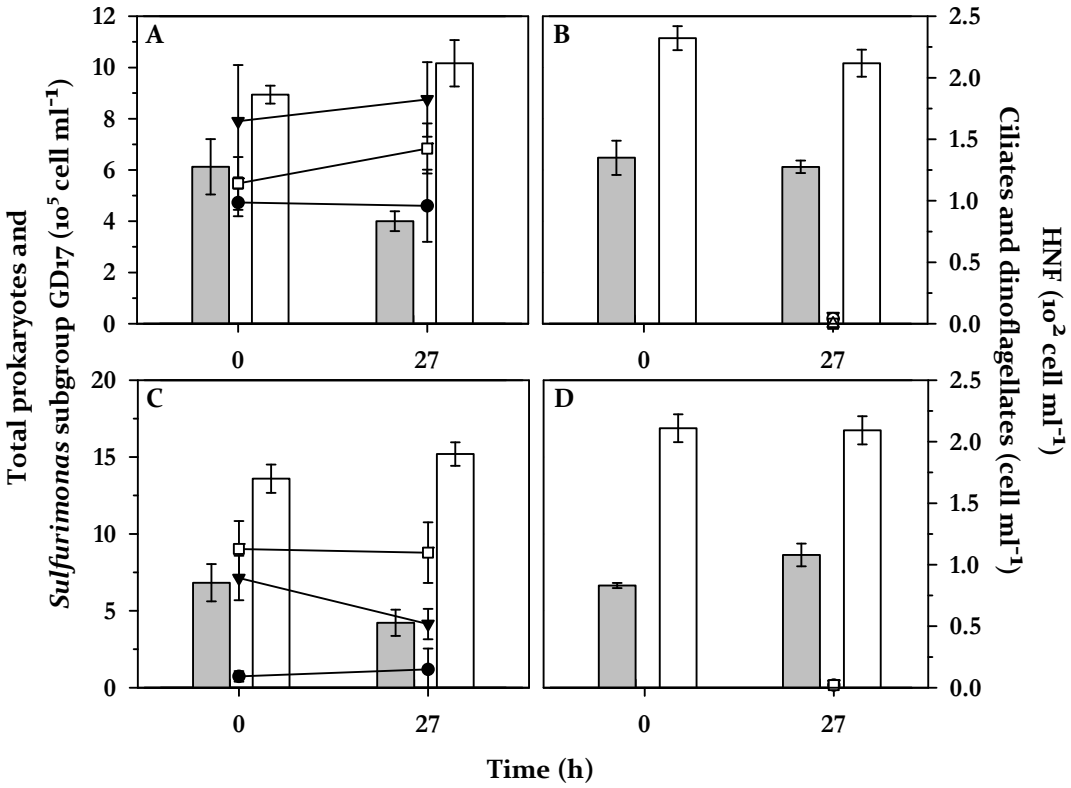

Figure 3.4. *Changes during the predator exclusion experiments with addition of "Sulfurimonas gotlandica" strain GD1, in total prokaryote (white bars), Sulfurimonas subgroup GD17 (grey bars) and protist abundance (line plots; ciliates (□), HNF (▼), and dinoflagellates (●)), under suboxic conditions in presence (A) and absence (B) of grazers; and under oxygen / hydrogen sulphide interface conditions in presence (C) and absence (D) of grazers. The corresponding standard deviations are shown.*

At oxygen / hydrogen sulphide interface depths, *Sulfurimonas* subgroup GD17 increased in abundance in treatments without predators (Fig. 3.3 and 3.4; t-test; $P<0.05$), resulting in growth rates of 0.56 d^{-1} and 0.22 d^{-1} for non-addition and addition experiments respectively (Table 3.2). For treatments with predators, a significant decrease in abundance was observed for the addition experiment (t-test; $P<0.05$). In non-addition experiments a initial increase was observed between 0 and 27 h (t-test; $P<0.05$), coinciding with the significant decrease in ciliate abundance (ANOVA; $P<0.05$), followed by a strong decrease in *Sulfurimonas* subgroup GD17 abundance between 27 and 50 h, once protist abundance stabilized (ANOVA; $P>0.05$). This resulted in grazing rates of 0.65 d^{-1} for the addition experiment, and of 0.94 d^{-1} for the full 50 h incubation time of the non-addition experiment (Table 3.2), being again higher than the growth rates. Total prokaryotic abundance did not significantly differ between treatments (presence or absence of grazers)

for either depth or experiment for all time points analysed (Fig. 3.3 and 3.4; ANOVA; P>0.05).

Table 3.2. *Growth and grazing rates (standard deviation in parenthesis) on Sulfurimonas subgroup GD17 measured for the full incubation time of predator-exclusion experiments with and without addition of "Sulfurimonas gotlandica" strain GD1.*

	No predators		Grazing rate (d-1)	% cell production grazed d^{-1}
	Growth rate (d-1)	doubling time (d)		
Suboxic				
no addition	0.09 (0.04)	9.40 (5.42)	0.21	231.75
addition	a	a	0.38	b
Interface				
no addition	0.56 (0.06)	1.25 (0.20)	0.94	166.03
addition	0.22 (0.07)	3.42 (1.07)	0.65	296.02

[a] no growth detected
[b] could not be determined since no cell-production was measured

Identification of "*Sulfurimonas gotlandica*" strain GD1 grazers

For the [^{13}C]-incorporation assays water was collected at an oxygen / hydrogen sulphide interface depth (Table 3.1), and incubated without further amendment for 5 days prior to the start of the experiment. At the end of this incubation time, an approximately 10-fold increase in ciliate abundance was observed compared to *in situ* depths with similar physico-chemical conditions), while dinoflagellate abundance remained constant (HNF were not quantified for the *in situ* depths). All ciliate morphotypes observed at the end of the enrichment were also found in the *in situ* samples, though the proportions changed, with small ciliates (25 – 40 µm in length) becoming more abundant than other larger morphotypes (Fig. 3.2). At the start of the [^{13}C]-enrichment incubations, protist abundances were: 621 ± 54 HNF ml^{-1}, 1.42 ± 0. 36 x 10^3 dinoflagellates l^{-1} and 9.24 ± 0.75 x 10^3 ciliates l^{-1}. These abundances did not vary significantly over the 50 h experimental incubation (ANOVA P>0.05).

The natural *Sulfurimonas* subgroup GD17 population had an initial abundance of 7.17 x 10^4 cell ml^{-1}, constituting about 10 % of the total prokaryotic community. Through addition of "*S. gotlandica*" strain GD1, this proportion was raised to 26 ± 3% of total prokaryotes in both the [^{12}C]- and [^{13}C]-treatments (data not shown). After 50 h incubation, *Sulfurimonas* subgroup GD17 average cell-counts had decreased by 25 – 30 %

in [^{12}C]- and [^{13}C]-treatments, and by 14 % in the N- control treatment without "*S. gotlandica*" strain GD1 addition (data not shown). As a control for the transfer of the [^{13}C]-signal to other members of the prokaryotic community, 16S rRNA SSCP fingerprinting was conducted for the three density gradients. All bands with enhanced relative intensities in heavier fractions of the [^{13}C]-gradient were identified as "*Sulfurimonas gotlandica*" strain GD1 with 100% sequence identity (data not shown).

RT-qPCR analysis of the density resolved RNA originating from the 50h samples, with 18S rRNA specific primers, showed a slight but clear density shift towards heavier rRNA in the [^{13}C]-treatment when compared to the two control treatments ([^{12}C] and N) (Fig. 3.5). The copy number maximum was found at a buoyant density of 1.787 g ml^{-1} for all three treatments. However, 46 % of total 18S rRNA copies were distributed at higher densities in the [^{13}C]-treatment, compared to 33 and 28 % respectively for the [^{12}C]- and N-control treatments, indicating an enrichment in the total rRNA pool. This was clearest at densities between 1.798 g ml^{-1} and 1.805 g ml^{-1} which contained 30 % of 18S rRNA copies in the [^{13}C]-treatment, but only 4 and 7 % in the [^{12}C]- and N treatments controls, respectively.

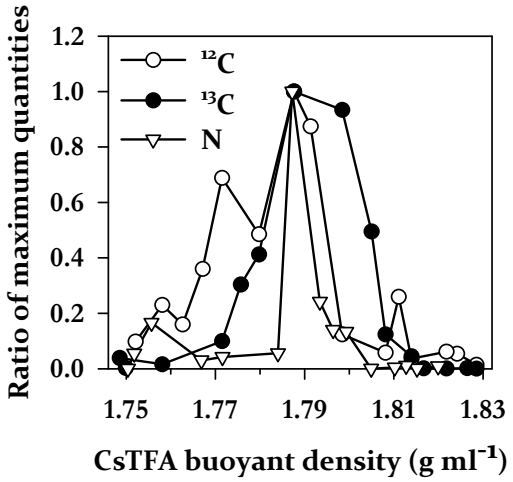

Figure 3.5. *Quantitative distribution of 18S rRNA in the CsTFA density gradients for the treatments with addition of ^{13}C-labelled (^{13}C) and non-labelled (^{12}C) "S. gotlandica" strain GD1, and the treatment with no addition (N).*

The 18S rRNA DGGE fingerprinting of the three density gradients and subsequent comparison of relative band intensity, showed 5 clearly enriched bands (C, E, G, I and J), and three potentially enriched bands (Q, L, and R) (Fig. 3.6; Supplementary Fig. S3.1). These increased in band intensity towards heavier buoyant densities in the [^{13}C]-treatment,

but not in either control (Fig. S3.1). Sequenced bands belonged mainly to the alveolates (12 OTUs (operational taxonomic unit; here defined as DGGE band position)), though stramenopiles (3 OTUs), choanoflagellates (1 OTU) cercozoa (1 OTU) and euglenozoa (1 OTU) were also present (Fig. 3.7; the euglenozoa OTU (band R) is not included since it was difficult to assign in BLAST databases). All groups, with the exception of the choanoflagellates, contained enriched or potentially enriched OTUs. Clearly enriched bands were identified as two ciliate OTUs, the first belonging to the Oligohymenophorea, and closely related to *Cardiostomatella vermiformis* (98 % sequence identity) (Band I), and the second belonging to the Prostomatea (Band C); two stramenopile OTUs, belonging to the MAST-4 group (Band E) and the chrysophytes (Band J); and the cercozoan OTU, which belonged to the novel clade VI (Bass & Cavalier-Smith 2004). Potentially enriched bands included one additional ciliate OTU, belonging to the Armophorea and closely related to *Metopus contortus* (98 % sequence identity) (Band L), a dinoflagellate OTU (Band Q) and the euglenozoan OTU (Band R).

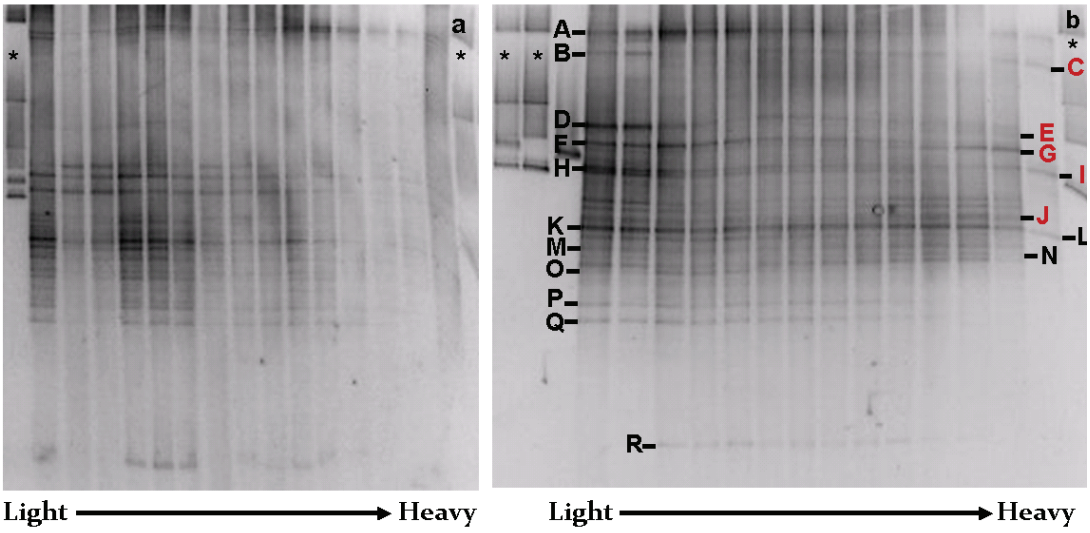

Figure 3.6. *Fingerprints of density-resolved eukaryotic SSU rRNA templates from the CsTFA density gradient fractions obtained for the treatments with (a) no addition (N-treatment control) and (b) addition of [^{13}C]-labelled "S. gotlandica" strain GD1 ([^{13}C]-treatment). The lanes of each gel represent, from left to right, fractions of increasing buoyant density. Relative band intensities were evaluated for all fractions besides the lightest and the heaviest, which usually contain erratic community patterns. Bands identified by sequencing are specified (A - R), and bands marked in red are those clearly enriched (based on relative band intensities). Reference lanes are indicated with an asterisk.*

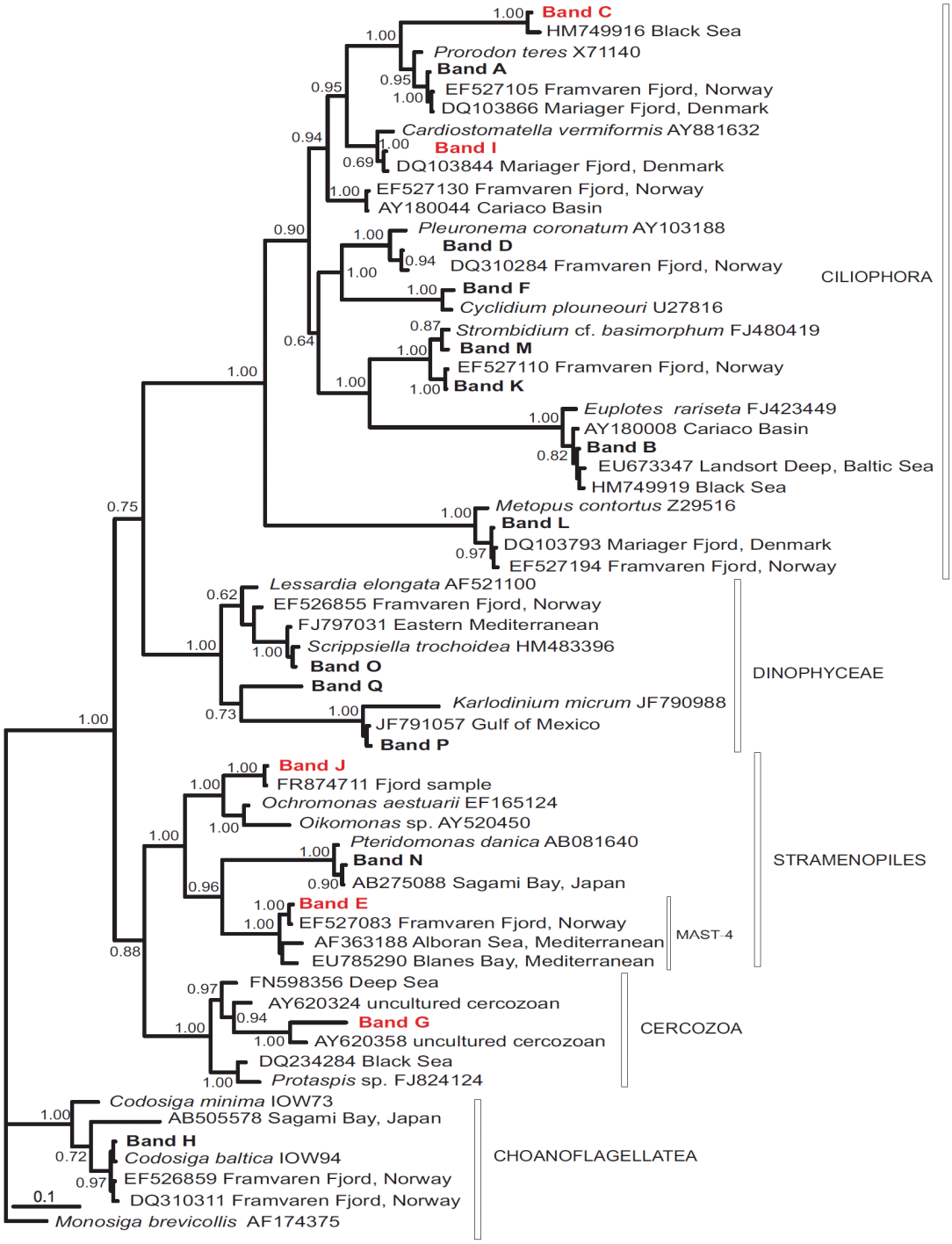

Figure 3.7. *Phylogenetic tree showing the relationship of eukaryote 18S rRNA sequences retrieved from DGGE bands as shown in Fig. 3.6. Clearly enriched bands are marked in red. Bar indicates 0.1 fixed point mutations per nucleotide. Numbers at nodes represent the posterior probabilities of the Bayesian analysis.*

3.4. Discussion

Protist grazing impact on the *Sulfurimonas* subgroup GD17

In the present study we observed a high grazing impact on the *Sulfurimonas* subgroup GD17 at suboxic and hydrogen sulphide interface depths of a Baltic Sea pelagic redoxcline (Gotland Deep), capable of exceeding the growth of this prokaryotic group. The *Sulfurimonas* subgroup GD17 grew strongly in absence of grazers at the oxygen / hydrogen sulphide interface (Fig. 3.3 & 3.4; Table 3.2). Calculated doubling times (1 – 1.5 d) were comparable to the maximal rates estimated for chemolithoautotrophic production in the Baltic Sea (Jost et al. 2008) and the Cariaco Basin (Taylor et al. 2006)) based on ^{14}C-bicarbonate incorporation. In suboxic zones however, *Sulfurimonas* subgroup GD17 growth was only detected in the first 27 h of the non-addition experiment, indicating that this zone was likely sub-optimal for the growth of this bacterial group. This in accordance with previous studies on the *Sulfurimonas* subgroup GD17 and its cultured representative "*S. gotlandica*" strain GD1, which show that this group will primarily grow by coupling denitrification to the oxidation of reduced sulphur compounds and dark CO_2 fixation (Labrenz et al. 2005; Grote et al. 2008, 2012). Thus, the oxygen / hydrogen sulphide interface, with overlapping nitrate and hydrogen sulphide concentrations, constitutes the optimal habitat for this bacterial group, while the suboxic zone, where nitrate is present but hydrogen sulphide is absent, would not support significant growth.

The high grazing rates obtained, however, raise the question of how the relatively high natural abundance of the *Sulfurimonas* subgroup GD17 population is maintained. Potentially the experimental set-up could lead to over- or underestimations of one or both parameters, through, e.g., substrate exhaustion or confinement. However, both rates fit well with previous estimates for high grazing (Chapter 2; Table 2.3) and chemoautotrophic production (Jost et al. 2008; Grote et al. 2012) in this zone. It is additionally interesting to note that maxima in *Sulfurimonas* subgroup GD17 abundances and dark CO_2 fixation rates are often found once hydrogen sulphide levels begin to increase (Grote et al. 2008, 2012; Jost et al. 2010), below the theoretical `optimum growth zone´ for this bacterial group (Bruckner et al. submitted). While several possible physiological explanations to this phenomenon have been postulated (e.g., use of alternate electron donors (Jost et al. 2010)), the ecological background remains unknown. It has been recently shown that protist abundance and grazing rates on the total prokaryotic community strongly decrease once hydrogen sulphide levels rise (Chapter 2; Fig. 2.2 & Table 2.3). Thus, protist grazing could

potentially constitute a strong factor influencing the shift in the *Sulfurimonas* subgroup GD17 population distribution away from its depths of growth optimum and towards a zone which constitutes a refuge from protist predation.

As an additional point of interest, specific grazing on the *Sulfurimonas* subgroup GD17 was always higher at the oxygen / hydrogen sulphide interface than at the suboxic zone (Fig. 3.3 & 3.4; Table 3.2). This was especially evident in the addition experiment were a comparable initial abundance of protists and prey were present (Fig. 3.4; Table 3.2). A parallel study conducted on the same sampling cruise revealed that grazing on the total prokaryotic community was conversely higher for the suboxic zone, and that the protist communities in the two zones differed in composition (Chapter 2; Fig. 2.4 & Table 2.3). Thus, it appears that at the oxygen / hydrogen sulphide interface zone, where the *Sulfurimonas* subgroup GD17 is actively growing, the protist community present is better adapted to consuming this bacterial group, than the protist association found in suboxic zones

Finally, total prokaryotic abundance was not significantly influenced by presence or absence of grazers (Fig. 3.3 & 3.4), contrary to observations for the *Sulfurimonas* subgroup GD17. However, as we did not conduct any further analysis on the dynamics of other prokaryotic groups, we cannot properly asses the changes induced by grazing on the remaining prokaryotic community. A similar phenomenon was observed at the oxic / anoxic interface of the Cariaco Basin, with changes in the relative abundance of different prokaryotic groups in absence / presence of grazers not necessarily being accompanied by changes in total prokaryotic abundance (Lin et al. 2007). Potentially, the interplay between vulnerability to grazers and effectiveness in competing for substrates leads to a fast turnover of different prokaryotic groups under changing grazing pressure (i.e., a high grazing pressure on the *Sulfurimonas* subgroup GD17 could indirectly `free´ nutrient for other less vulnerable prokaryotes that would otherwise have been outcompeted).

Overall, I demonstrate, to my knowledge for the first time, that protist grazing can balance cell production of a chemolithoautotrophic bacterial key player of pelagic redoxclines. An increased *Epsilonproteobacteria* growth in absence of grazers has also been seen at the oxic / anoxic interface of the Cariaco Basin (Lin et al. 2007), indicating that bacterivory could constitute an important regulating factor for this key prokaryotic group of suboxic to anoxic systems (Taylor et al. 2001; Campbell et al. 2006; Lavik et al. 2009; Glaubitz et al. 2010).

RNA-Stable Isotope Probing (RNA-SIP): methodological considerations

In the present study we successfully applied a combination of RNA-SIP (Manefield et al. 2002; Lueders et al. 2006) and unamended sea water incubations (Massana et al. 2006) to enrich protists naturally present *in situ* in a Baltic Sea redoxcline (Landsort Deep) and identify grazers on `*S. gotlandica*´ strain GD1, a cultured representative of the *Sulfurimonas* subgroup GD17 (Grote et al. 2012). Ciliate morphotypes present at the end of the enrichment period were consistent with those found at the *in situ* depths used for comparison (Fig. 3.2), and existing data for Baltic Sea redoxclines (Chapter 2; Fig. 2.3 & 2.4). This, combined with the fact that the closest relative to most sequences recovered proceeded from suboxic to anoxic water masses, indicates that the incubation conditions favoured naturally abundant protists and did not result in a severe change in the community composition.

The [^{13}C]-enrichment obtained in the present study was clear, but not strong (Fig. 3.5). Maximum copy numbers were found at the a buoyant density of 1.787 g ml^{-1} for all treatments, falling within normal ranges for [^{12}C]-rRNA (Lueders et al. 2004a). However, a considerably higher proportion of rRNA copies was found at densities above the copy maximum in the [^{13}C]-treatment than in the two controls (Fig. 3.5). One problem arising from this `weaker´ enrichment, is that in cases where the DGGE bands already naturally extended across the full gradient when unlabelled (e.g. Band A; Fig. 3.6), any enrichment in the [^{13}C]-treatment is likely masked and hard to detect. Thus, under the current protocol, it is not possible to determine for all protists whether they incorporated the labelled substrate or not. The distribution of unlabeled RNA along the gradient to the extent that it contaminates denser fractions has been observed and discussed previously, and is the reason why careful comparison with control gradients is recommended (Manefield et al. 2002; Lueders et al. 2004a; Singleton et al. 2005; Neufeld et al. 2007). An increase in the amount of labelled prey or incubation time could potentially resolve this problem, but this could lead to other experimental artefacts, i.e., an excess in the labelled bacteria could alter the natural prey selection process of a given grazer. Thus, the application of RNA-SIP alone to the environment analysed in the present study may not be able to fully resolve all specific grazers on a particular prokaryotic group.

16S rRNA analysis indicated that no significant transfer of the [^{13}C]-signal was occurring to other members of the prokaryotic community, for example through use of labelled by-products of protist grazing. Additionally, alternative sources of enrichment for the protist community other than bacterivory can be disregarded. The incubation times

used should be too short for predators on smaller protists to assimilate enough labelled prey biomass (considering that the protist prey itself needs enough time to become significantly labelled through bacterivory), and an enrichment through osmotrophy with [^{13}C]-bicarbonate proceeding from the *"S. gotlandica"* strain GD1 culture media would also be observed in the prokaryotic community. Thus, we can conclude that all enrichment in the protists community occurred exclusively through the ingestion and assimilation of "*S. gotlandica*" strain GD1 biomass.

Identification of active grazers on "*Sulfurimonas gotlandica*" strain GD1

In the present study we identified 5 clearly and 3 potentially enriched protist taxa, indicating that they were active grazers on "*S. gotlandica*" strain GD1. Clearly enriched taxa belonged to typical ciliate groups for hypoxic water masses: Oligohymenophorea and Prostomatea (Fenchel et al. 1990; Guhl et al. 1996; Stock et al. 2009; Stoeck et al. 2009), and flagellate groups widely spread in marine systems, including hypoxic water masses (e.g., Stoeck & Epstein 2003; Behnke et al. 2006; Orsi et al. 2011), which are increasingly recognized as major bacterivores for surface waters: MAST-4 (Massana et al. 2006, 2009), Chrysophyta (Not et al. 2008) and Cercozoa (Bass & Cavalier-Smith 2004; Piwosz & Pernthaler 2009). Of the latter three, the cercozoan OTU clustered within the novel clade VI (Bass & Cavalier-Smith 2004; Bass et al. 2009), which to date is only known at a sequence level, allowing us to assign an ecological role for the first time to a member of this clade. Of the potentially enriched taxa, one belonged to the ciliate group Armophorea, and the other two were only distantly related to cultured representatives, and grouped within the euglenozoans and the dinoflagellates.

Other identified OTUs tended to group with sequences obtained from hypoxic environments, such as the Framvaren and Mariager Fjords (Zuendorf et al. 2006; Behnke et al. 2010) and the Black Sea (Coolen & Shtereva 2009; Wylezich & Jürgens 2011); and were dominated by ciliates. One sequenced OTU (Band H) belonged to a recently discovered choanoflagellate species of Baltic Sea pelagic redoxclines, *Codosiga baltica* n. sp. (Wylezich et al., submitted). Among non-enriched protists, the presence of a close relative of *Euplotes raiseta* (97 % sequence identity) was surprising, since in a previous study conducted using [^{13}C]-bicarbonate as substrate, the isotopic signal was transferred to a close relative of this protist, indicative of grazing on chemoautotrophic prokaryotes (Glaubitz et al. 2009). However, the latter study was conducted at a different study site, under higher hydrogen sulphide concentrations, and with a wider prey pool (grazing on

the whole assemblage of [^{13}C]-enriched prokaryotes). Therefore, habitat and prey optimization could play an important role in the different results obtained.

Finally, the identification of flagellate taxa as active grazers on "*Sulfurimonas gotlandica*" strain GD1 is an additional interesting point. A previous study determined that, due to their very low abundance, heterotrophic nanoflagellates (HNF) could not be major bacterivores on the prokaryotic community (Chapter 2). Here we show that flagellates could still have a significant impact on pelagic redoxcline prokaryotic community through selective feeding on highly active members.

3.6. Conclusions

The present study demonstrates, for the first time, that protist grazing can account for the complete cell production of a chemolithoautotrophic key-player of Baltic Sea pelagic redoxclines, the *Sulfurimonas* subgroup GD17, thus constituting a major regulating factor for the growth, and potentially the distribution, of this prokaryotic group. We additionally show a higher grazing impact on this bacteria at depths where it is actively growing, indicating an adaptation of the protist community in these zones to feeding on active *Sulfurimonas* subgroup GD17. RNA stable isotope probing was successfully combined to unamended sea water incubations to identify active grazers on the *Sulfurimonas* subgroup GD17. Through this method, flagellates, which were previously identified as negligible grazers on the total prokaryotic community, were shown to potentially still be having a significant impact on the prokaryotic community through selective grazing on highly active members.

Conclusions and outlook

In the present thesis, two case studies of under-researched fields in protist-bacteria interactions were presented and further explored. The first addressed a species level interaction, assessing how the starvation-survival state alters the vulnerability of bacteria to a given protist grazer. The second addressed an under-explored environment, looking at the ecological relevance of protist grazers in pelagic redoxclines, with two deep Baltic Sea basins as model systems. The results obtained highlight the unexplored potential of `the less travelled roads´ in protist ecology and protist-bacteria interactions, and pin-point interesting new avenues for future research. In the following the principal results obtained and remaining open questions are detailed:

The starvation survival state and its relation to grazing vulnerability

In chapter 1 of the present work I demonstrate through the use predator-prey model systems that the starvation-survival state can confer a lower vulnerability to protist grazing, but the response is highly species-specific. These results cannot be directly extrapolated to the environment, but show for the first time that resistance to protist grazing is possible for carbon-starved bacteria, and set an intriguing base for future studies. Conceptually, the carbon-starved fraction of prokaryotic communities could present three different levels of vulnerability to protist grazers, with different implications for ecosystem productivity (Fig. i):

(1) Most carbon-starved bacteria are resistant to protist grazing (e.g., *V. vulnificus*), implying little or no losses for these cells (Fig. iA). The prey base available to protists is therefore reduced, leading to a lower new protist biomass production and a higher grazing pressure on the edible fraction of the prokaryotic community.

(2) Carbon-starved bacteria have a low vulnerability to protist grazers and experience strongly reduced losses in comparison to the rest of the prokaryotic

community (Fig. iB). This could occur through a predominance of carbon-starved bacteria with lowered food quality, which could e.g., be selected against (e.g., *S. alaskensis*); or through a mix of difference levels of food qualities and vulnerabilities, leading to only part of these the cells being consumed. New protist biomass would be higher than in the previous scenario, allowing a higher productivity.

(3) Resistance to protist grazing is rare, and most carbon-starved bacteria have a good food-quality (e.g., *P. angustum*, or the mixed community tested in González et al. 1993) (Fig. iC). Grazing losses would be high, and would only be reduced in comparison to other members of the prokaryotic community due to smaller cell-size (i.e. lower encounter rates with grazers). This scenario would lead to the highest new protist biomass production and transfer of carbon and energy to higher levels in the food-chain.

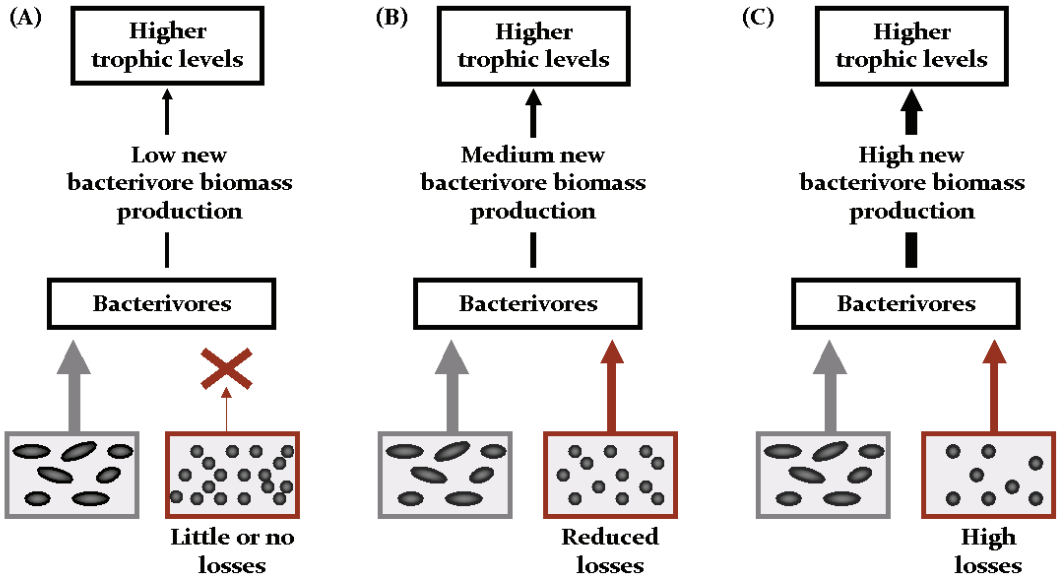

Figure i. *Hypothetical scenarios of how altered vulnerabilities of carbon starved bacteria to protist grazing could affect protist biomass production and energy transfer to higher trophic levels. (A) resistance; (B) low vulnerability; and (C) high vulnerability. Orange boxes and arrows indicate carbon starved bacteria; grey boxes and arrows indicate the remaining prokaryotic community.*

These conceptual models are highly simplified, since the outcome will also be influenced by e.g., the edibility of the other members of the prokaryotic community and other microbial food-web interactions. However, they serve to highlight the potential implications of the vulnerability of carbon-starved bacteria to the productivity of

oligotrophic environments. A full study of interactions between different bacterivores and model prey, and the potential underlying mechanisms to reduced vulnerability or resistance to grazers (e.g., resistance to digestion (King & Shotts 1988; Barker & Brown 1994) or toxin production (Matz et al. 2004, 2005)), are important first steps to unveiling the relevance of these phenomena in oligotrophic environments.

It should also be noted that differing patterns in the vulnerability of carbon-starved bacteria to protist grazers will affect the capacity of these cells to survive for long time periods (from months to years) (Fig. i). From an anthropogenic point of view, this could be especially important for diverse pathogenic bacteria which are known to enter the starvation-survival state (e.g., *Vibrio cholerae* and many virulent enteric bacteria (Kjelleberg 1993; Spector & Kenyon 2012)). In the present study, *Vibrio vulnificus,* a septicaemia causing human pathogen found in marine and brackish systems (Oliver 2006), could completely resist protist grazing. Thus, a need exists for further studies on these particular bacteria, with consideration to its implications for environmental standing stocks of pathogens.

The ecological relevance of protist grazers in pelagic redoxclines

In the present work I successfully optimized existing techniques for the estimation of protist bacterivory rates and the identification of grazers (FLB, predator-exclusion and RNA-SIP) for the application to redoxcline environments. Combined with microscopy analysis, this allowed a first detailed vision of the structuring and ecological relevance of Baltic Sea redoxcline protist communities (chapters 2 and 3). The following principal points were unveiled:

a) Baltic Sea redoxclines host characteristic and highly stratified protist communities, with clear suboxic, O_2 / H_2S interface, and anoxic / sulphidic protist associations.

b) Bacterivory is the major prokaryotic mortality factor at suboxic and O_2 / H_2S interface zones, but decreases strongly in relevance at sulphidic depths.

c) Protist grazing is, for the first time, demonstrated to be capable of controlling the growth of a redoxcline chemolithoautotrophic key-player, indicating the potential regulating role of grazing for biogeochemical cycling (in this case denitrification and H_2S oxidation).

Ciliate morphotypes detected in this work primarily fell within those `typical´ for redoxcline systems (e.g., Fenchel et al. 1995), though the presence of *Metacystis* spp. as a major component in terms of abundance could be characteristic for Baltic Sea redoxclines. For dinoflagellates, the taxonomic resolution of the methods employed was very low. It should be noted that, overall, knowledge on redoxcline dinoflagellate populations is scarce. As seen in the present work, they tend to be abundant when O_2 is still present, but decrease strongly on from the O_2 / H_2S interface (Chapter 2, Fig. 2.2 & 2.4., and, e.g., Fenchel et al. 1990). However, they can still be detected in 18S rRNA based surveys of protist diversity in anoxic systems, indicative of not only of presence but also of activity (Stoeck et al. 2007; Alexander et al. 2009; Wylezich & Jürgens 2011). In the present work, a protist affiliated to dinoflagellates was putatively seen to incorporate labelled *Sulfurimonas* subgroup GD17 (*Epsilonproteobacteria*) biomass at the O_2 / H_2S interface (Chapter 3, Fig. 3.6 & 3.7 (band Q); Supplementary fig. S3.1B). This indicates that they could be active, potentially relevant, bacterivores in this zone. Finally, for heterotrophic nanoflagellates (HNF), most identified species showed a wide distribution throughout the O_2 gradient (Chapter 2, Fig. 2.5), though it remains to be revealed whether these are indeed species with a wide physico-chemical tolerance or are in fact genetically different strains with a highly similar morphology. A recent study of two choanoflagellate strains, isolated respectively from suboxic and sulphidic zones of Baltic Sea redoxclines and identified as two novel *Codosiga* species, showed traits indicative of adaptation to their respective environments, but also the capacity to grow under fully-oxygenated conditions (Wylezich et al., in preparation). Overall, it is likely that fluctuating redoxcline conditions, with small-scale mixing episodes and larger inflow events, favour protist species capable to adapting and even exploiting transiently unfavourably conditions.

Protist grazing impact on the whole prokaryotic community

Protist grazing was revealed in the present work as the major prokaryotic mortality factor for Baltic Sea redoxcline prokaryotic communities at suboxic and O_2 / H_2S interface zones, accounting respectively for an average 80 and 50 % of standing stocks per day (Chapter 2; Table 2.3). Food-vacuole analysis and theoretical considerations showed that ciliates and dinoflagellates will be the main bacterivores in these zones. In contrast to grazing, frequencies of virally infected cells remained low, indicating a low viral impact (Chapter 2; Fig. 2.7). Thus, food web structuring differs from that normally found in oxygenated surface waters, with microzooplankton (ciliates and dinoflagellates) acting as

the major prokaryotic mortality factor, instead of HNF or viral lysis (Pernthaler 2005; Jürgens & Massana 2008; Breitbart 2012) (Fig ii). In sulphidic zones, the relevance of grazing mortality strongly decreased and viral lysis remained low (<5 % of total prokaryotic cells were virally infected) (Chapter 2; Table 2.3 & Fig. 2.7). Therefore, an imbalance occurs between the measured high prokaryotic production rates (Jost et al. 2010) and low mortality rates. The underlying reason/s for this phenomenon (i.e., over- or underestimation of production or mortality rates, and other potential mortality factors) is an important open question for future studies.

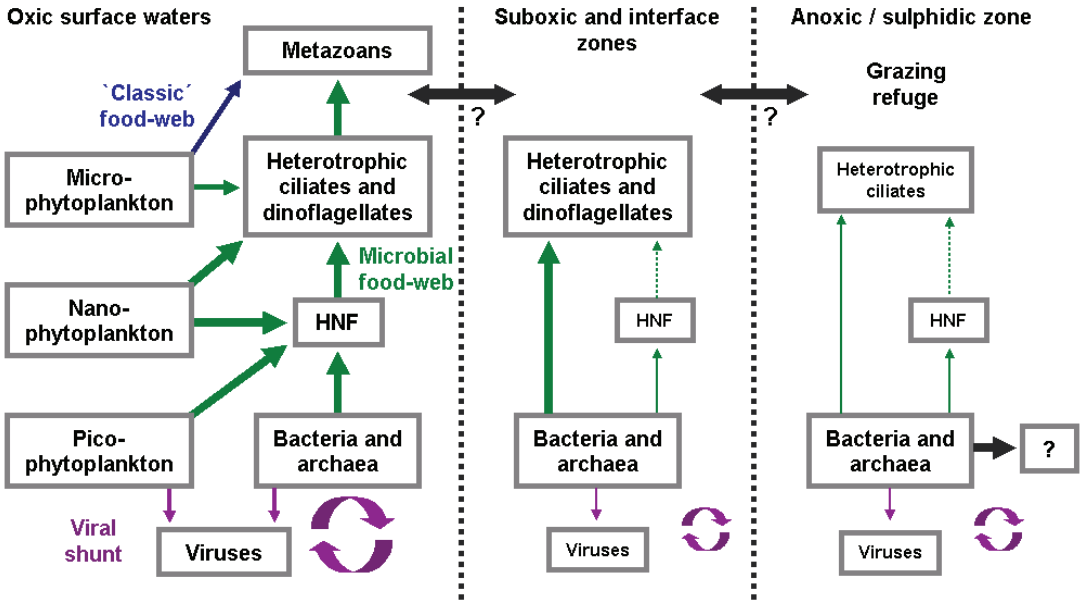

Figure ii. *Comparison of suboxic, O_2 / H_2S interface and anoxic / sulphidic microbial food web structure to a simplified version of the microbial food-web found in oxygenated surface waters (for a more complete version, including mixotrophs, see Fig. I in the introduction).*

The overall effect of these processes is an apparent simplification of redoxcline microbial food webs (Fig. ii). However, this does not necessarily imply a higher amount of carbon and energy reaching higher trophic levels, as would be expected in oxygenated water masses (Sherr et al. 2007), due to lower gross growth efficiencies of protists in these zones. For anaerobic ciliates the efficiency of transforming prokaryotic biomass into new protist biomass was estimated to be 25 % of that achieved by their aerobic counterparts, based on fermentative metabolism (Fenchel & Finlay 1990). However, the *in situ* gross growth efficiencies of free-living anaerobic and microaerophilic protists are largely unknown, and need to be further analysed to properly understand the flow of carbon and

energy in these zones. For this, culturing of environmentally relevant protist species and autoecology studies are necessary. However, the application of culture independent –omics techniques (transcriptomics, genomics and proteomics) could be an invaluable tool for uncovering the bulk metabolic potential of protist in these zones, as has been done for prokaryotes in different water masses (Vieites et al. 2009; Gilbert & Dupont 2011; Morales & Holben 2011; Tomanek 2011; Zehr & Kudela 2011).

Another important point for future studies is the magnitude and relevance of carbon and energy flows between oxic and suboxic to anoxic water masses. Higher organisms have been shown to conduct feeding forays into hypoxic zones, thereafter returning to oxic waters for digestion and growth (e.g., fish (Neuenfeldt et al. 2009), metazoans (De Robertis et al. 2001) or jellyfish (Markulla et al., unpublished data)). They thus effectively act as a shuttle of carbon and energy out of the system. A similar transport system, though acting in both directions, could be protists that conduct long vertical migrations from surface waters to nutrient rich redoxcline zones (Mamaeva 1988). The latter phenomenon could explain the presence of pigment containing *Dinophysis* sp. and *Mesodinium* spp. at respectively suboxic and O_2 / H_2S interface zones (the latter observed live under the microscope), since both species have been previously postulated to conduct large vertical migrations in search of nutrients (Villarino et al. 1995; Olli 1999; Gisselson et al. 2002).

Specific grazing on a prokaryotic key-player for Baltic Sea biogeochemical cycling

In the present work, protist grazing was shown to effectively control the growth and abundance of a chemolithoautotrophic key-player of Baltic Sea redoxclines, the *Sulfurimonas* subgroup GD17 (*Epsilonproteobacteria*), consuming above 100 % of the daily cell production (Chapter 3, Table 3.2). The high growth exhibited by the *Sulfurimonas* subgroup GD17 in absence of grazers at the O_2 / H_2S interface, confirm studies that postulated this as an optimal growth zone for this bacterial group (Grote et al. 2012) (Fig. iii). Further, the high grazing rates obtained serve as a potential explanation as to why the maxima in *Sulfurimonas* subgroup GD17 abundance and chemoautotrophic production are often found deeper in the water column than their theoretical optimal growth zone (Fig. iii), with this bacterial group shifting its vertical distribution into zones where grazing is greatly decreased (refuge zone) (Fig. iii).

The strong grazing impact on the *Sulfurimonas* subgroup GD17 could also have important implications for how the prokaryotic community is structured. The high growth rates exhibited by this bacterial group in absence of grazers at the O_2 / H_2S interface

(doubling times of ~ 1 day), implies that bacterivorous pressure (top down) rather than nutrient availability (bottom-up) regulates its growth in this zone. Thus, the action of protist grazers could indirectly free nutrients for use by other prokaryotic groups that might have otherwise been out-competed by this bacterial group, which appears extremely well adapted to its ecological niche (Grote et al. 2012). In Baltic Sea redoxclines, a second chemolithoautotrophic clade co-occurs at similar depths to the *Sulfurimonas* subgroup GD17, the sulphur-oxidizing *Gammaproteobacteria* (Glaubitz et al. 2009). An interesting focus for future studies will be how the interplay between nutrient availability and protist grazing pressure affects the relative abundance and activity of these and other prokaryotic key-players of Baltic Sea redoxclines, and thus the rates of the biogeochemical processes they conduct.

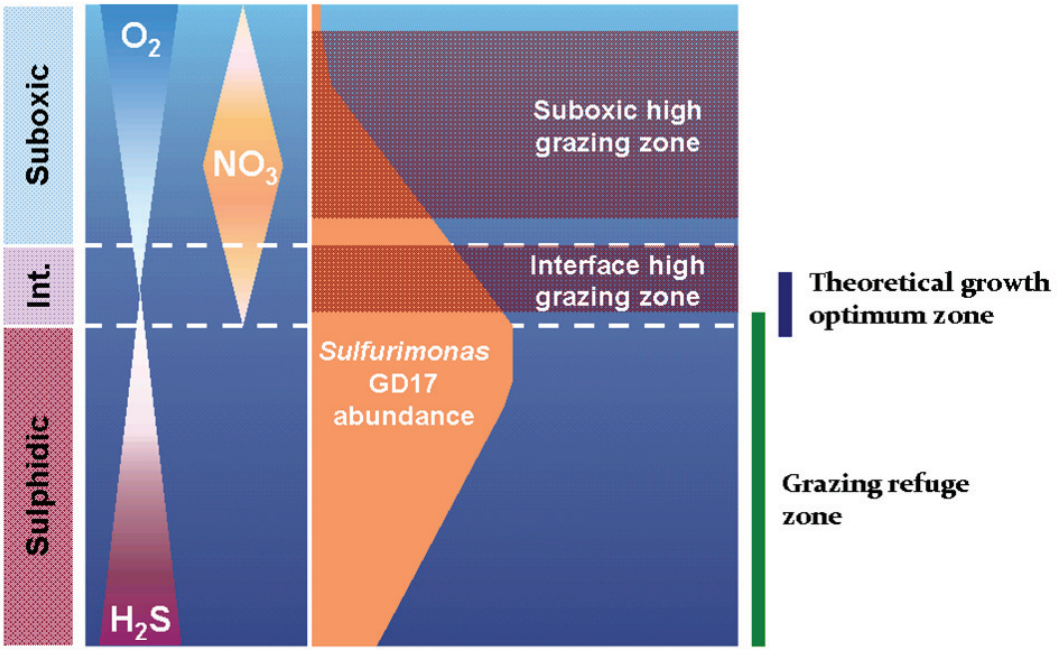

Figure iii. *Schematic representation showing a typical distribution of the Sulfurimonas subgroup GD17 across the oxic-anoxic interface (adapted from Grote et al. 2012), the theoretical optimum zone for growth (Grote et al. 2012, Bruckner et al., submitted), and major grazing pressure zones.*

Studies on the effect of protist grazers on specific key-prokaryotic groups are relatively rare for marine systems, and the present work highlights their importance for understanding ecosystem functioning. The *Sulfurimonas* subgroup GD17 is a highly abundant and active prokaryotic group of Baltic Sea redoxclines, which grows by coupling

dark CO_2 fixation to H_2S oxidation and NO_3 reduction (Grote et al. 2007, 2008, 2012; Glaubitz et al. 2009). As a denitrifier it contributes to Baltic Sea nitrogen losses, and as a H_2S reducer, it effectively acts as a detoxifier of sulphidic water masses (Grote et al. 2012). Both processes could be highly important for the strongly eutrophic Baltic Sea. NO_3 and NO_2 trigger microalgae bloom formation in surface waters, and the degradation of resulting organic matter intensifies suboxic and anoxic conditions in bottom waters. The resulting spread in hypoxic conditions can impact higher organisms and consequently Baltic Sea fisheries. As an example, the cod-fish requires higher salinity (~ 11 ‰) oxygenated waters for successful egg fertilization and development, with spreading hypoxic conditions thus seriously impacting spawning (Rheinheimer 1995; Limburg et al. 2011). H_2S, on the other hand, is highly toxic for aerobic organisms, triggering mass mortality events (Theede 1973; Vaquer-Sunyer & Duarte 2008). Thus, the effect of protist grazers on the abundance and, therefore, the population level activity of the *Sulfurimonas* subgroup GD17 and, potentially, other highly active key-prokaryotic groups involved in the nitrogen and sulphur cycles, could impact the Baltic Sea ecosystem as a whole.

In the present work, a combination of `black-box´ approaches to obtain bulk rates, and techniques that identified prokaryotic prey (CARD-FISH) (Jezbera et al. 2005; Lin et al. 2007; Šimek et al. 2007; Bautista-Reyes & Macek 2012) and protist grazers (RNA-SIP) (Lueders et al. 2006; Frias-Lopez et al. 2009; Moreno et al. 2010) were applied. This allowed not only the determination of the community level bacterivorous impact, but also a first glimpse at possible underlying species-specific interactions, such as selective HNF grazing on the *Sulfurimonas* subgroup GD17. Future studies should continue in this direction, analysing the implications of selective grazing, symbiotic and parasitic relationships and host-specific viral lysis on biogeochemical cycling in suboxic to anoxic water masses. As an example, nano-scale resolution secondary ion mass spectrometry (Nano-SIMS) (Li et al. 2008; Foster et al. 2011; Ploug et al. 2011), and single-cell genomics (Not et al. 2009; Martínez-Garcia et al. 2011; Yoon et al. 2011) could constitute optimal methodologies for observing how protists, prokaryotes and viruses interact to shape the microbial food web. Finally, its worth noting that in a recent study, Edgcom et al. (2011b) revealed that only about 6 % of protists in deep sulphidic waters in the Cariaco Basin hybridized with a universal 18S rRNA FISH probe (Edgcomb et al. 2011b). Thus, a wide range of protist organisms, genetically highly divergent from those known to date, and with unknown ecosystem functions, could still remain to be discovered.

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Conclusions

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List of Abbreviations

A	Adenine
BLAST	Basic Local Alignment Search Tool
BD	Bouyant Density
bp	Base pair
C	Cytosine
CARD	CAtalyzed Reporter Deposition
CI	Confidence Interval
CsTFA	Cesium TriFluoroAcetate
CTD	Conductivity-Temperature-Depth
DAPI	4',6-Diamidino-2-Phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	DeoxyriboNucleic Acid
cDNA	Complementary DeoxyriboNucleic Acid
rDNA	Ribosomal Deoxyribonucleic acid
dNTP	DeoxyNucleoside TriPhosphate
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
EUB	(Eu-)Bacteria
FIC	Frequency of Infected Cells
Fig.	Figure
FISH	Fluorescence In Situ Hybridization
FLB	Fluorescently Labeled Bacteria
G	Guanine
H_2	Hydrogen
H_2S	Hydrogen sulphide
HNF	Heterotrophic Nano-Flagellate
K_s	Half velocity coefficient
LB	Luria Broth
MMM	Marine Minimum Medium

N_2	Molecular nitrogen
NH_4	Ammonium
NO_2	Nitrite
NO_3	Nitrate
O_2	Oxygen
OD	Optical Density
OTU	Operational Taxonomic Unit
PCR	Polymerase Chain Reaction
qPCR	Quantitative Polymerase Chain Reaction
POC	Particulate Organic Carbon
POM	Particulate Organic Matter
R^2	Level of significance
RNA	RiboNucleic Acid
rRNA	Ribosomal RiboNucleic Acid
rpm	Revolutions Per Minute
RT-PCR	Reverse Transcription Polymerase Chain Reaction
R.V.	Research Vessel
SD	Standard deviation
SE	Standard Error
SIP	Stable Isotope Probing
SO_4	Sulphate
SSCP	Single Stranded Conformation Polymorphism
T	Thymine
U	Enzyme unit
UV	Ultraviolet radiation
VPR	Virus to prokaryote ratio

Supplementary material

Chapter 1

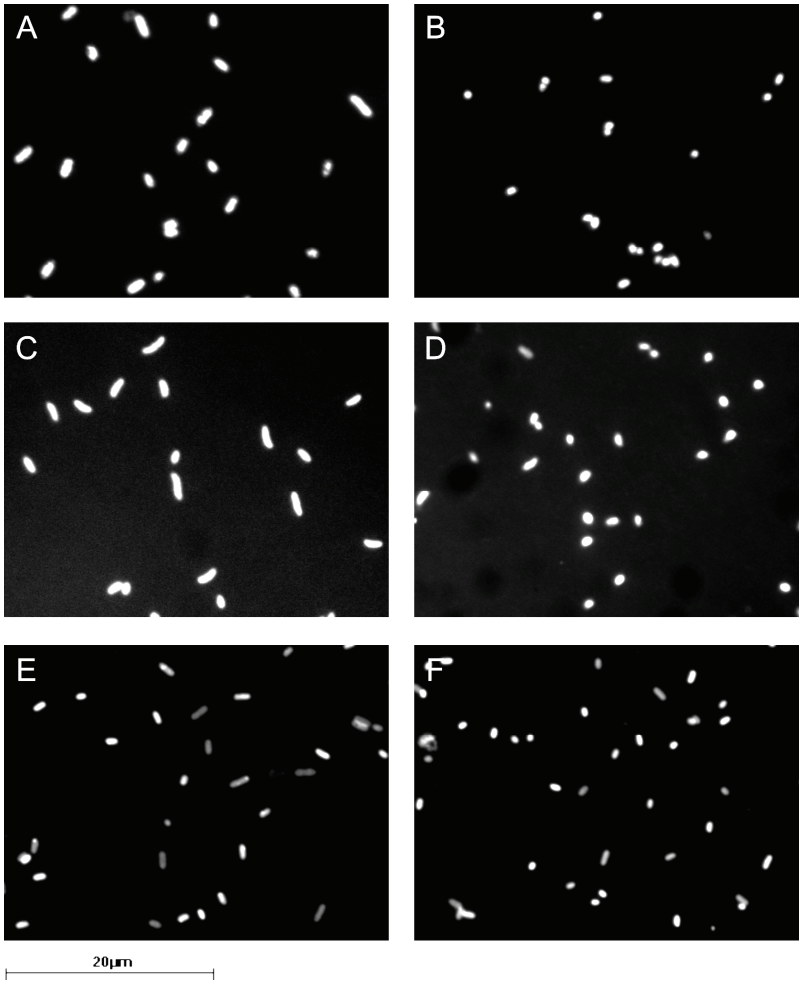

Figure S1.1. Epifluoresence micrographs at 1000 X magnification taken at the start of the experiments with *P. angustum* S14 (A, B), *V. vulnificus* M06-24/O (C, D) and *S. alaskensis* RB2256 (E, F). In all cases, bacteria are shown in their non-starved physiological state on the left (A, C, E), and in their starved state on the right (B, D, F).

Chapter 2

Table S2.1. Comparison of experimentally obtained average grazing rates (standard deviation in parenthesis) with estimated grazing rates obtained through the application of literature clearance rates to average protist biovolume and abundance data from the start of the experiments.

	Clearance rate (nl cell^{-1} h^{-1})	Ref.	% of bacterial standing stocks d^{-1}					
			Gotland Deep			Landsort Deep		
			Sub.	Int.	Sul.	Sub.	Int.	Sul.
Flagellates	5	(Kuuppo Leinikki 1990)	6	2	1	4	5	3
Dinoflagellates								
rounded 10-25 µm in length	1500	(Neuer & Cowles 1995)	2	6	1	5	10	1
elongated 10-30 µm in length	1000	(Neuer & Cowles 1995)	0.01	0.01	0.01	5	5	0.2
Ciliates								
10-20 µm ESD	2000	(Setälä & Kivi 2003)	7	3	0.2	20	14	3
20-30 µm ESD	3000	(Setälä & Kivi 2003)	4	17	20	1	1	10
>30 µm ESD	5000	(Setälä & Kivi 2003)	3	4	3	15	10	7
Total estimated			22	32	25	50	45	24
Total measured			25.4 (22.0)	49.0 (11.1)	14.0 (18.2)	80.8 (28.3)	51.5 (10.9)	38.0 (22.0)

Chapter 3

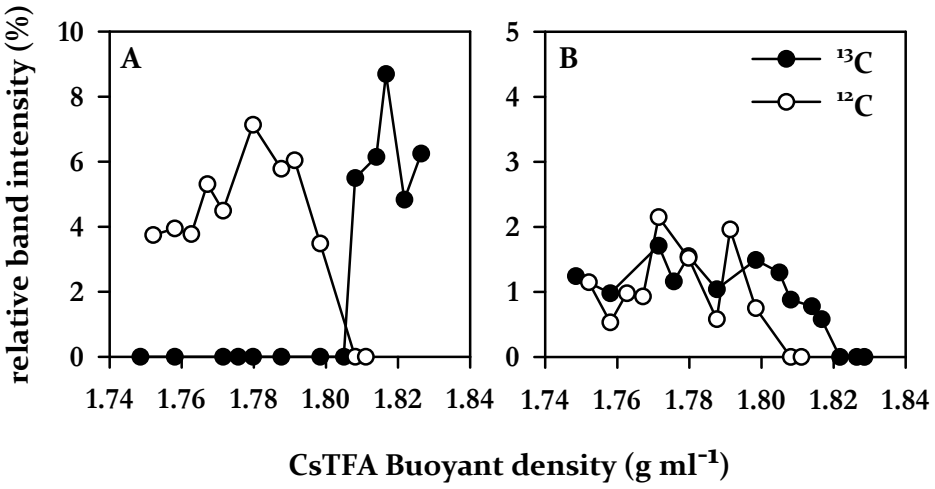

Figure S3.1. Examples of changes in relative band intensity along the density gradient for the [^{13}C]- and [^{12}C]-treatments: (A) clearly enriched band (e.g., band J), and (B) potentially enriched band (e.g., band Q). Comparison of relative band intensity was carried out with both controls, but only the [^{12}C]-treatment is shown here for clarity.

Publications and conferences

The contents of Chapter 1 have already been published, the contents of Chapter 2 have been submitted to a peer-reviewed journal, and the contents of Chapter 3 are ready for submission. The author contributions are indicated in the following:

Publications in peer-reviewed journals

- **R. Anderson**, S. Kjelleberg, D. McDougald and K. Jürgens (2011)
  Species-specific patterns in the vulnerability of carbon starved bacteria to protist grazing. Aquatic Microbial Ecology 64: 105–116 (Feature Article).
  Contributions by authors - practical work: **R.A**.; first edition of the manuscript: **R.A.**; revision of the manuscript: S.K., D.M. and K.J.

Manuscripts under review and in preparation

- **R. Anderson**, A. Mylnikov, C. Winter, W. Foissner and K. Jürgens. *Protist abundance, diversity and importance as predators in hypoxic waters of the central Baltic Sea.* (under review)
  Contributions by authors - practical work: **R.A**. (protist quantification and morphotype identification, bacterivory measurements, food-vacuole content analysis), A.M. (protist quantification and flagellate identification), C.W. (virus quantification and determination of FIC), W.F. (ciliate identification); first edition of the manuscript: **R.A.**; revision of the manuscript: A.M., C.W., W.F. and K.J.

- **R. Anderson**, C. Wylezich, S. Glaubitz and K. Jürgens. *Protist grazing impact on a chemoautotrophic bacterial key-player of Baltic Sea redoxclines, and identification of the main grazers through RNA-stable isotope probing*. (ready for submission)
  Contributions by authors - practical work: **R.A**. (grazing experiments, RNA-SIP), C.W. (probe design), first edition of the manuscript: **R.A.**; revision of the manuscript: C.W., S.G. and K.J.

Oral presentations and posters at conferences

2011: Importance of protist grazing on a chemolitoautotrophic key player (*'Sulfurimonas`* GD17) of Baltic Sea redoxclines.

R. Anderson, C. Winter, C. Wylezich and K. Jürgens. 12th Symposium on Aquatic Microbial Ecology (SAME 12), Warnemünde, Germany. (Poster)

2011: Protist diversity, distribution and bacterivory in Baltic Sea pelagic redoxclines.

R. Anderson, F. Weber, C. Wylezich, W. Foissner, A. Mylnikov and K. Jürgens. 2011 Aquatic Sciences Meeting (ASLO), San Juan, Puerto Rico. (Oral presentation)

2010: Protist bacterivory in two Baltic Sea pelagic redoxclines: a first view of a complex system.

R. Anderson, F. Weber and K. Jürgens. 29th Annual Meeting of the German Society for Protozoology (DGP), Düsseldorf, Germany. (Poster)

2009: Are carbon starved bacteria more resistant to protist predation?

R. Anderson, D. Mc Dougald, S. Kjelleberg and K. Jürgens. 11th Symposium on Aquatic Microbial Ecology (**SAME 11**), 2009, Piran, Slovenia. (Oral presentation)

2009: Baltic Sea Redoxclines: a unique Model System for linking in situ and in vivo studies. C. Bruckner, M. Labrenz, G. Jost, T. Schott, C. Wylezich, **R. Anderson**, J. Feike, S. Glaubitz, J. Grote, F. Weber and K. Jürgens. Gordon Research Conference on Applied and Environmental Microbiology. South Hadley, MA, USA. (Poster)

2009: Protists in Baltic Sea Pelagic Redoxclines.

F. Weber^, **R. Anderson**^, C. Wylezich and K. Jürgens. 28th Annual Meeting of the German Society for Protozoology (DGP), Naumburg, Germany. (Poster)

^ both authors contributed equally to the present work

2008: Differential resistance to flagellate predation of two *Vibrio* species subjected to carbon starvation.

R. Anderson, D. McDougald, S. Kjelleberg and K. Jürgens. 27th Annual Meeting of the German Society for Protozoology (DGP), Warnemünde, Germany. (Poster)

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Statement of Independence

Selbständigkeitserklärung

The opportunity for this doctoral project has not been conveyed to me commercially. In particular, I did not engage an organization that, for a fee, seeks supervisors for the preparation of dissertations. Nor did I engage an organization to, in whole or in part, take care of incumbent obligations with regard to the examinations for me.

I hereby declare that I produced the present thesis independently and without external help. I did not use any auxiliary materials or sources other than the ones indicated and passages used literally or with regards to content have been declared as such.

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30.03.2012 Ruth Anderson

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