WALTER AND ANDRÉE DE NOTTBECK FOUNDATION
SCIENTIFIC REPORTS

No. 34

A systematic-ecological approach to Baltic Sea ice studies of algae and protists

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Academic dissertation in Aquatic Sciences,
to be presented, with the permission of
the Faculty of Biosciences of the University of Helsinki,
for public criticism in the Lecture Hall
of the Tvärminne Zoological Station,
J. A. Palménin tie 260, Hanko,
on September 18th, 2009, at 12 noon.

HELSINKI 2009

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

I Rintala, J.-M., Piiparinen J. & Uusikivi, J. 2009: Drift-ice and under-ice water communities in the Gulf of Bothnia (Baltic Sea). – Polar biology (in press) DOI 10.1007/s00300-009-0695-1.

II Rintala, J.-M., Piiparinen, J., Ehn, J., Autio, R. & Kuosa, H. 2006: Changes in phytoplankton biomass and nutrient quantities in sea ice as responses to light/dark manipulations during different phases of the Baltic winter 2003. – Hydrobiol. 554: 11-24.

III Rintala, J.-M., Spilling, K. & Blomster, J.2007: Temporary cyst enables long term dark survival of *Scrippsiella hangoei* (Dinophyceae). – Marine Biology 152: 57-62.

IV Rintala, J.-M., Hällfors, H., Hällfors, S., Hällfors, G., Majaneva, M. & Blomster, J.: The geographical distribution and seasonal occurrence of a new dinoflagellate in the Baltic Sea: *Heterocapsa arctica* ssp. *frigida*, ssp. nov. (Peridiniales, Dinophyceae). – Journal of Phycology (submitted).

CONTRIBUTIONS	I	II	III	IV
Original idea	JR	JR	JR, KS	GH, SH, JR
Study design and methods	JR	JR	JR, KS, JB	JR, GH, SH, HH, MM
Sea-ice structures	JU	-	-	-
Culturing	-	-	JR	JR
Data gathering	JR	JR, JP	JR, KS	JR, SH, GH, HH
Light microscopic species identification	KK	JR	JR, GH, SH	JR, GH, SH
Electron microscopic species identification	-	-	JR	JR
DNA extraction phylogenetics	-	-	JB	JB, MM
Statistics	JP	JP	-	HH, MM
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Dedicated to

In the memory of
Kirsti Tellervo Rintala
* 4.1.1945
† 3.4.1978

and To my father, Raimo,
in recognition of all single fathers,
who are still always considered to
be less than their female
counterparts

PROLOG

The Microbe is so very small
You cannot make him out at all,
But many sanguine people hope
To see him through a microscope.

His jointed tongue that lies beneath
A hundred curious rows of teeth;
His seven tufted tails with lots
Of lovely pink and purple spots,
On each of which a pattern stands,
Composed of forty separate bands;
His eyebrows of a tender green;
All these have never yet been seen –

But Scientists, who ought to know,
Assure us that they must be so…
Oh! let us never, never doubt
What nobody is sure about!

Hilaire Belloc 1900: “The Microbe”
In: *More Beasts for Worse Children*.

A systematic-ecological approach to Baltic Sea ice studies of algae and protists

JANNE-MARKUS RINTALA

Rintala, J.-M. 2008: A systematic-ecological approach to Baltic Sea ice studies of algae and protists. – W. & A. de Nottbeck Foundation Sci. Rep. 34: 1–48. ISBN 978-952-99673-5-3 (paperback), ISBN 978-952-10-5630-7 (PDF).

The seasonal occurrence of sea ice that annually covers almost half the Baltic Sea area provides a unique habitat for halo- and cold temperature-tolerant extremophiles. Baltic Sea ice biology has more than 100 years of tradition that began with the floristic observation of species by the early pioneers using light microscopic techniques that were the only thing available at the time. Since the discovery of life within sea ice, more technologies have become available for taxonomy. Electron microscopy and genetic evidence have been used to identify sea ice biota revealing increased numbers of taxa. Meanwhile ecologists have used light microscopic cell enumeration in addition to the chemical and physical properties of sea ice in attempts to explain the food web structure of sea ice and its functions.

Thus, during the Baltic winter, the sea ice hosts more abundant and diverse microbial communities than the water column beneath it. These communities are typically dominated by autotrophic diatoms together with a diverse assortment of dinoflagellates, auto- and heterotrophic flagellates, ciliates, metazoan rotifers and bacteria, which are mostly responsible for the recycling of nutrients.

This thesis comprises ecological and systematic studies. In addition to the results of the previous studies carried out on landfast ice, the data presented here provide new insight into the spatial distribution of pelagial sea ice, which has remained largely unexplored. The studies reveal spatial heterogeneity in the pelagial sea ice of the Gulf of Bothnia. There were mismatches in chlorophyll-*a* concentrations and in photosynthetic efficiencies of the communities studied. The temporal succession was followed and experimental studies performed investigating the community responses towards increased or decreased light in landfast ice in the Gulf of Finland. The systematic studies carried out with established dinoflagellate cultures revealed a new resting cyst belonging to common sea ice dinoflagellate, *Scrippsiella hangoei* (Schiller) Larsen 1995. The cyst can be used to explain the overwintering of this species during prolonged periods of darkness.

The dissimilarities and similarities in the material isolated from the sea ice called for description of a new subspecies *Heterocapsa arctica* ssp. *frigida*. The cells obtained in the cultured material were unlike those of the previously described species, necessitating description of ssp. *frigida*. As a result of its own unique habitus, the subspecies had been noted by Finnish taxonomists during the past three decades and thus its annual occurrence and geographical distribution in the Baltic Sea. This illustrates how combining ecology and systematics increases our understanding of organisms.

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CONTENTS

1. INTRODUCTION

The annual phytoplankton succession in the Baltic Sea follows a general pattern, beginning in the spring when increasing light triggers phototrophic growth, a phenomenon also known as the spring bloom. It is first observed as increased diatom and dinoflagellate biomasses in the southern and central Baltic, reaching the northernmost Baltic Sea in early summer (Hällfors et al. 1981). During the spring bloom the diatoms and dinoflagellates rapidly consume the available inorganic nutrients, generally attaining their maximum biomass when the nutrients are bound to phytoplankton. Since there are very few grazers present at this time of the year, most of the phytoplankton biomass sinks out of the water column at the termination of their vegetative life cycles (Kilham & Kilham 1980, Heiskanen 1998). Phytoplankton biomass, species composition and community structure in the Baltic Sea were thoroughly summarized by Hällfors et al. (1981). The monitoring results by HELCOM (2002) led to the conclusion that during recent decades dinoflagellates have gradually become more predominant during the spring bloom at the expense of diatoms (Niemi 1975, Kononen & Niemi 1984, Heiskanen 1993, Wasmund & Uhlig 2003, Tamelander & Heiskanen 2004, Jaanus et al. 2006). Yet, the diatoms still often dominate the spring bloom community until they are gradually replaced by co-occurring common cold-water dinoflagellates, such as the *Peridiniella catenata* (Levander) Balech 1977 and *Scrippsiella hangoei* (Schiller) Larsen 1995. These were considered as the most dominant postspring bloom species until the recent discovery of *Woloszynskia halophila* (Biecheler) Elbrächter & Kremp (Kremp et al. 2005) which previously may have been misidentified as *S. hangoei*, and therefore their reciprocal importance must be re-evaluated. Nevertheless, these peridinoid dinoflagellate species become gradually more abundant during the spring bloom, when thermal stratification develops. After the spring bloom, the cold-water dinoflagellates sink out of the water column (Heiskanen 1998, Kremp 2000), which is seen in the water column becoming more transparent. This stage is characterized by a small phytoplankton biomass that is regulated by the increased zooplankton community (Niemi 1973, 1975, Hällfors et al. 1981) until the diazotrophic cyanobacteria bloom begins (Bianchi et al. 2000, Finni et al. 2001). Typically, the diatoms reappear in the autumn before the Baltic Sea is frozen over (Hällfors et al. 1981, Bianchi et al. 2002).

Winter marine research in the Baltic began more than 100 years ago. It was initiated by Levander (1900), who was astonished to encounter *Rotatoria* in the under ice-water samples collected from the Gulf of Finland and the Archipelago Sea.

Almost 30 years later Häyrén (1929) noted algal growth in tiny water pockets in the surface layer of the sea ice. He was puzzled over two major phenomena: How come there is unfrozen water inside the ice and is it possible to have life in it? His contemporaries made similar observations in the vicinity of Tvärminne on the southwest coast of Finland, a location popular among ice scientists even today. Häyrén considered this to be a common event near coastal regions where the ice scrapes off algae from the littoral zone, thus releasing them to the wind, which is then responsible for transporting the algae out to the ice. He explained that the water found inside the ice was the result of solar heating around the algae. Häyrén showed that the coloration of the inner ice water was of microalgal origin, including mainly the littoral species *Hormidium flaccidum* Kützing

1845, *Chlamydomonas* spp. (Chlorophyceae) and *Ulothrix* spp. (Chlorophyceae). Even though he had no explanation for the diatom cells and colonies encountered other than dispersal via ice floes between locations, he was interested in the survival of algae, just as I am (**I-III**).

The answer to Häyren's principal question: "how come there is unfrozen water in the ice" is explained by the sea-ice properties. The dissolved constituents, such as the salts, are removed from the parent water during the freezing and concentrated in the surrounding water that remains unfrozen, because the higher salinity decreases the freezing point. This highly saline concentrated liquid thus forms small channels and pockets that remain unfrozen between the ice crystals and is commonly referred to as brine (Eicken 2003). Most of the biological matter is trapped in these inclusions during the physical ice formation process (Ackley 1982, Garrison et al. 1983). Any water salinity above 1 practical salinity unit (psu) is enough to cause similar physical processes during ice formation as is described in the Arctic and Antarctic polar regions (Palosuo 1961, Weeks et al. 1990). The Baltic Sea ice formation occurs usually during the autumn. First, ice crystals appear in the water when the temperature is cooled down to -0.2 or -0.5 °C, depending on the water salinity that on the coasts of Finland is between 2 and 6 psu. During low temperatures, the freezing continues with more ice crystals (frazil ice) that appear together in the surface water, forming grease ice or slush, which under wind- and waveless conditions form clear nilas ice. Usually, however, the wind is present, thus making waves that have a shearing effect on the ice, causing it to form round pancake-shaped floes (pancake ice, PCI) that grow in diameter until they are frozen together. They drift on top of the water column or under heavier weather conditions, raft on top of each other. This dynamic ice formation phase is followed by static or thermodynamic growth (Palosuo 1961, Lange et al. 1989, Spindler 1990, Weeks et al. 1990, Melnikov 1995, Thomas & Dieckman 2003).

Mostly the incorporation of organisms into the newly formed sea ice is caused by passive accumulation during the ice formation (Różańska et al. 2008), which could affect community composition in different ice types. At larger scales this would cause variation in the sympagic (inner ice) communities' geographical distribution. The fraction of the sympagic ice community that originates from the colonization of the bottom of the ice cover by the organisms from the underlying water that would become part of the sympagic flora after the thermodynamic ice growth, is considered to be negligible (Palmisano & Sullivan 1983). The structure as well as the functioning of the sympagic assemblages encountered are exposed to temperature-dependent changes in ice porosity, brine channel morphology and brine transport (Gradinger et al. 1992, Krembs et al. 2000, Granskog et al. 2005).

After the world wars interest was turned towards pelagic energy flows and therefore to the microorganisms that consume atmospheric carbon and nitrogen. At that time Hickel (1969) reported pennate diatoms from the bottom of the sea ice in Eckernfördern in the southern Baltic Sea, where sea ice is very rarely formed. Several years later sea-ice diatoms were described at the entrance of the Gulf of Finland (Niemi 1973). Soon a brown coloration was found in the ice and in the water beneath the ice close to Tvärminne Zoological Station. The coloration was caused by a new haptophyte, *Chrysochromulina birgeri* G. Hällfors & Niemi 1974, named after the observor Birger Sjölund, an employee of the Station (Hällfors & Niemi 1974).

Almost two decades later Vørs (1992) focused in particular on cold-water, ice-related and warm-water species of heterotrophic protists. She found in all 95 different taxa with two new genera and eight new species. Several years later Ikävalko and Thomsen (1996, 1997) combined electron microscopy with light microscopic investigations, establishing the diversity of photo- and heterotrophic flagellates within the Baltic Sea ice. At the same time Laamanen (1996) acquired molecular genetic evidence to identify the cold-water and sea-ice cyanoprokaryote diversity in the Gulf of Bothnia. In 2001, I isolated sea-ice dinoflagellates that were later identified with more experienced phytoplankton taxonomists as resembling the cells that were first observed by G. Hällfors in the 1970s. Detailed examination of the cultured material revealed enough differences to warrant classification as a new subspecies (**IV**).

With regard to biomass as well as production, the most important group of organisms within the polar sea ice are generally the algae (Brierley & Thomas 2002). In the Baltic the sympagic flora is most often dominated by pennate diatoms (Huttunen & Niemi 1986, Ikävalko & Thomsen 1997, Haecky et al. 1998, Haecky & Andersson 1999), combined with auto- and heterotrophic chryso-, crypto-, dino-, hapto- and prasinophyte flagellates (Ikävalko & Thomsen 1997).

Modern process-oriented Baltic ice ecology was initiated by Norrman and Andersson (1994), who linked the diminishing chlorophyll-*a* (Chl-*a*) quantity with ciliates and other encountered organisms and concluded that the Baltic Sea ice encloses a complete microbial loop. Kaartokallio (2001) described active microbial nitrogen transformations that were soon combined with experimental evidence concerning nutrient limitation in the Gulf of Finland (Kuosa & Kaartokallio 2006). The dynamics of the ice environment in the Baltic has been associated with the severity of the winter. Temperature affects the porosity that influences the interactions between the physical, chemical and biological properties of the ice and its food webs (Kaartokallio 2004). The succession sequence of sympagic organisms is considered to be similar in the Gulf of Bothnia and Gulf of Finland, with the exception that there is additional ice algal blooming or minor algal biomass maxima in the Gulf of Finland during the low-light period in January (Kaartokallio 2005).

During the winter few phytoplankton cells are present in the water column beneath the ice cover (Edler 1979) compared to what is known from the open water of the Baltic Sea during the ice-free mid winter months (HELCOM 2002). Very little is known about the organisms living in Baltic Sea ice, although scientific tradition can be traced to almost as far back as the beginning of modern phytoplankton ecology in the Baltic. The identification of phytoplankton in the Baltic Sea was initiated by Hensen (1887). The invention of the Utermöhl (1958) method enabled the conversion of these floristic observations into accurate cell numbers, volumes and biomass estimates (Melvasalo et al. 1973, Smetacek 1975), after which more accurate primary production estimates became possible, using the ^{14}C method that had been invented earlier by Steemann-Nielsen (1952). Even though the ^{14}C method has been widely used throughout the Baltic Sea area since 1972 (Lassig et al. 1978), measurements are still lacking from the pelagial sea ice and from the water column beneath the ice cover, mostly due to the prevailing view of negligible winter production in the water column beneath the ice (Hällfors et al. 1981). The lack of knowledge is most probably also caused by logistical obstacles present with sea-ice sampling that could only be overcome with use of

an icebreaker. Therefore, primary production measurements previously performed in the Baltic Sea have been carried out only within the vicinity of the coastal stations, focusing on the annual succession of algae in the landfast ice in the Bothnia Sea near Umeå, Sweden (Norrman & Andersson 1994, Haecky & Andersson 1999) and in the Gulf of Finland near Tvärminne, Finland (Kaartokallio et al. 2007). The ice algal contribution to the total sympagic production is estimated to be 10 % (Haecky & Andersson 1999).

During the past decade increasing numbers of organisms that have earlier been regarded as solely photosynthetic due to their photosynthetic pigments are now known to be mixotrophic. Mixotrophic organisms are able to combine photosynthesis with heterotophy or osmotrophy (Stoecker 1999 and references therein). One mode of mixotrophy is enzymatic decomposition of naturally synthesized macromolecules, which is common in dinoflagellates (e.g. Mullholland et al. 2002, Stoecker & Gustafsson 2003). Investigations of the enzymatic activity and hydrolysis of organic substrates in the water column usually ascribe the activity to heterotrophic bacteria and ignore the contribution of larger organisms applying osmotrophy, since it was believed to be minor (Rosso & Azam 1987, Sinsabaugh et al. 1997). Enzymes that hydrolyse organic substrates in the water column may be extracellular (exoenzymes) that occur free in the water or ectoenzymes on the surfaces of cells or in the periplasmic space of the cell (Chróst 1991).

Baltic Sea phytoplankton are also capable of excreting at least two different extracellular enzymes: leucine aminopeptidase (LAP) and alkaline phosphatase (AP) (Stoecker et al. 2005, Vahtera et al. 2007). LAP is commonly used as a measure of exo- and ectoproteolytic activity in plankton (Hoppe 1983, 2003, Rosso & Azam 1987, Crottereau & Delmas 1998, Hoppe et al. 1998, Patel et al. 2000). LAP hydrolyses a broad spectrum of substrates, including polypeptides with a free amino group, but has a preference for N-terminal leucine and related amino acids (Mahler & Cordes 1966). After the hydrolysis of macromolecules, the amino acids and peptide products may be assimilated by the phytoplankton as a source of carbon (C) and nitrogen (N) (Mulholland et al. 2002). AP releases inorganic phosphate from organic phosphorus (P) compounds (Perry 1972, Ammerman 1990, 1993) and it is often used as an indicator of P limitation (Perry 1972, Ammerman 1990, 1993, Dyhrman & Palenik 2001), which is commonly encountered in the Gulf of Finland in late summer (Grönlund et al. 1996). Yet the AP activity and the availability of inorganic P are often unlinked, perhaps due to variations in the availability of dissolved organic P (Boström et al. 1988, Kononen & Nõmmann 1992, Grönlund et al. 1996). LAP activity is advantageous for both N and C acquisition and AP for nitrogen-fixing cyanobacteria, since their growth is often P-limited (Vahtera et al. 2007). These enzymes could be similarly beneficial to the metabolically active sympagic communities living on limited resources in brine channels (Gleitz & Thomas 1992; Gradinger & Ikävalko 1998).

Only a minority of the most common members typically present in the sympagic community, i.e. the diatoms or dinoflagellates, have been observed to form resting spores or to use resting cysts and thus use the ice as an overwintering platform (Garrison & Buck 1985). Cyst formation is well known in Antarctic regions (Garrison & Buck 1989, Buck et al. 1992, Stoecker et al. 1992, 1993, 1997, Montresor et al. 1999), but records from the Arctic are very scarce (Ikävalko & Gradinger 1997, Okolodkov 1998).

In a recent review Granskog et al. (2006)

stated that during recent decades ice research has been process-oriented, but both the spatial and temporal data needed to estimate the importance of ice biota to the overall productivity, nutrient and carbon cycles in the Baltic Sea is still missing. The spatial variability, both vertical and horizontal, is still an understudied aspect.

2. OUTLINE OF THE THESIS

My aim here was to show the spatial (**I**) and temporal (**II**) distributions of the sympagic eukaryotic communities, explain the autotrophic organisms' survival within the sea ice (**III**) and to be able to name the organisms encountered (**IV**). The communities consisted mostly of unicellular or chain-forming eukaryotic organisms that influence the parameters measured: possessing the photosynthetic pigment chl-*a* and thus affecting its concentrations. These organisms also utilize the available nutrients, thus also affecting the measured nutrient concentrations (**I**, **II**), but only if they are photosynthetically active. The latter is shown in the photosynthetic efficiency (P-E) measurements (**I**). On two occasions identification was more demanding, leading to recognition of a new dinoflagellate resting cyst (**III**) and the description of a new algal subspecies (**IV**).

I tried to increase the level of knowledge of sympagic community spatial distribution on a whole- sea area scale (**I**). The logistical obstacles have kept scientists close to the necessary scientific infrastructure provided by various field stations. In previous studies microbial organisms were found within the Baltic sea-ice (Ikävalko & Thomsen 1995, 1997, Laamanen 1996, Ikävalko 1997, 1998). The P-E of these communities is shown (**I**).

During winter Baltic Sea sea ice is often snow-covered. The light environment inside the ice is affected by the presence or absence of snow cover (**II**). Both situations cause successional changes in the sympagic communities. These changes were studied *in situ*, *in vivo* and *in vitro*, while simultaneously following the development of the natural succession. In addition to the results already published and discussed (**II**), the community response to rapid and prolonged increase or decrease of light was also assessed by measuring changes in their extracellular LAP and AP enzyme activities.

In contrast to the newly formed habitats that were formed after the last ice age 10 000 years ago, the organisms living in sea ice have had sufficient time for evolutional adaptation. An example of such adaptations is (**III, IV**). Single cells were isolated from melted sea ice and introduced into monocultures, which were studied live under laboratory conditions. The experiment investigated the dark survival of one of the sea-ice-thriving unicellular algae, revealing the formation of a new pellicular resting cyst.

Due to possible risks for human health and ecosystem effects, much of the research resources as well as efforts dealing with phytoplankton are currently allocated to bloom-forming species, such as cyanobacteria in the Baltic Sea and dinoflagellates in the oceans (Hallegraeff 2003). An example is shown of how less abundant species can exist unrecognized, unidentified and undiscribed, except on a local scale as a unique taxa for decades; without proper descriptions they remain globally unidentifiable (**IV**). Thus, the compilation of data cannot begin, because without a name there is nothing, no species to link the ecological data. This linkage is needed to evaluate the ecological importance of any species.

3. STUDY AREA

3.1. Characteristics of the Baltic Sea

The Baltic Sea is one of the world's largest brackish water areas, covering 422 000 km^2 of the earth's surface. It contains 21 000 km^3 of brackish water, which is a mixture of saline water from the Atlantic and fresh water supplied by numerous rivers (Voipio 1981). The freshwater influence in the Baltic Sea results in oligohaline (below 3 psu) to euryhaline (20-30 psu) salinity gradients. Salinity decreases horizontally from the Danish Straits towards the Gulfs of Finland and Bothnia as well as vertically from bottom to surface (Voipio 1981, Stigebrandt 2001).

The entire Baltic Sea is on the Eurasian plate, which makes it relatively shallow. The mean depth is only 55 m in the Gulf of Finland and 40 m in the Bothnian Bay (Voipio 1981). The regional differences are most obvious in winter when the brackish water freezes, forming sea-ice similar to that in the Arctic and Antarctic. The duration of the ice-covered period in the Baltic Sea is longest in the northern Bothnian Bay, where it lasts an average of 6 months each year, whilst in the northern Baltic Proper the mean length is only 20 days (Seinä & Peltonen 1991). The annual ice coverage varies interannually from 10 % to 100 % of the Baltic Sea area (Haapala & Leppäranta 1997).

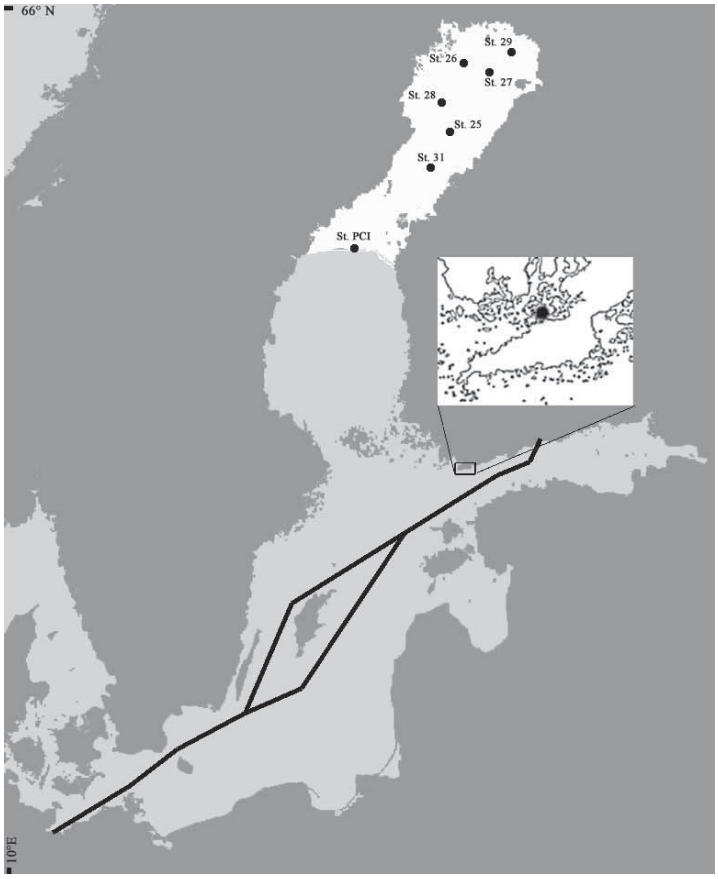

Fig. 1. Map of the study area showing the pelagial sampling sites. The northern Bothnian Sea and the Gulf of Bothnia are painted white to the extent of ice cover during sampling **(I)**. The pancake ice (PCI) samples where obtained from the narrowest location between Finland and Sweden, known as the Quark area.

The insert shows the coastal sea-ice sampling site (Santala Bay), which was used for the *in situ* experiments **(II)** and as the isolation site for the *Scrippsiella hangoei* and *Heterocapsa arctica* ssp. *frigida* monocultures **(III, IV)**.

The lines indicate the ship routes used by ships of opportunity equipped with automated sampling devices used by the Finnish monitoring programme Algaline **(IV)**.

3.2. Characteristics of the study sites

All the work presented in this thesis was carried out in the Baltic Sea (Fig. 1). Samples from PCI, fast ice as well as drift ice were collected from the Gulf of Bothnia (Paper **I**) onboard RV Maria S. Merian in March 2006. In the beginning of the cruise the ice edge was located south of the Quark area and the Bothnian Bay was almost completely ice-covered. Satellite images, however, revealed that northeasterly winds caused large openings, such as cracks and leads in the ice cover in the southeastern Bothnian Bay, north of the Quark. Due to low temperatures the entire Bothnian Bay was ice-covered at the end of the study period.

The *in situ* experiments were conducted in Santala Bay, SW Finland, which has strong water exchange with adjacent sea areas and a mean water depth of approximately 6 m (**II**). The Bay has no significant freshwater inputs, hence no under-ice freshwater lenses have been detected. Due to its sheltered location between the Hanko Peninsula and several small islands, it also freezes over during mild winters (Granskog et al. 2004). These characteristics make the Bay ideal for experimental sea-ice research. The established sea-ice organism monocultures were also isolated from the Santala Bay ice (**III**, **IV**). The seasonal and geographical distribution of the cultured *Heterocapsa arctica* ssp. *frigida* Rintala & G. Hällfors ssp. nov. (**IV**) in the surface waters of the Baltic Sea was studied, using long-term data collected in 1993-2005 as part of the routine phytoplankton monitoring project Algaline (Finnish Institute of Marine Research, FIMR). The samples were obtained using automated water samplers installed aboard commercial vessels (GTS Finnjet, m/s Finnpartner) travelling between Germany and Finland (Fig. 1).

4. MATERIAL AND METHODS

4.1. Field sampling (I, II)

The sea-ice samples (excluding PCI floes in **I**) were obtained using a motorized CRREL-type ice-coring auger (9 cm inner diameter; Kovacs Enterprises). The cores were cut into approximately 10 cm sections and enclosed in one litre plastic containers. The under-ice water samples (< 1 m depth) were taken into 1 litre plastic bottles by lowering them into the water through the drill hole.

To avoid organism loss due to cell lysis caused by too rapid changes in salinity during melting (Garrison & Buck 1986, Kottmeier & Sullivan 1988), sea-ice core sections used for cell counts, chl-*a* determinations or metabolic activity measurements were submerged in 0.2-µm-filtered seawater (FSW) of the same salinity as the under-ice water at the sampling site. The volume of the added FSW was always measured and dilution by it corrected for chl-*a*, biomass and P-E calculations. The ice cores used for the nutrient measurements were melted without FSW addition. All the ice samples were kept in the dark and at +4 °C during melting for approximately 24 hours.

4.2 Experimental setup (II)

Transparent plexiglass tubes (mimicking no-snow situations) and completely darkened plexiglass tubes (mimicking heavy snow cover) were used for the *in situ* light manipulation experiments (**II**). Complete ice cores were placed in the tubes together with 2 litres of autoclaved FSW, after which they were placed in the core holes for 1 to 2 week incubation. Information on the ambient inner ice light milieu was obtained by letting photosynthetically active radiation (PAR) sensors with individual data loggers to freeze into the sea-ice field one week before the beginning of the experiment.

4.3 Cultures, culture manipulation experiments and investigations (III, IV)

Motile dinoflagellate cells were isolated from sea ice melted in FSW. The isolation was done under a Leica MZ7.5 preparation microscope (Leica Microsystems, Wetzlar, Germany) using autoclaved glass Pasteur pipettes. Each cell was put into a 50-ml Cellstar® tissue culture flask (Greiner Bio-One, Kremsmünster, Austria) filled with f/2 (-Si) culture medium (Guillard 1975) made from aged and autoclaved Baltic Sea water of 6 psu salinity. The flasks were kept at +4 °C under 40-80 µE m^{-2} s^{-1} with a daily light/dark cycle of 8/16 hours, which is close to their natural conditions. The established monocultures were used in the laboratory experiments investigating the dark survival of cells (**III**) and in the detailed species identification of *Heterocapsa arctica* ssp. *frigida* (**IV**).

4.4. Measurements (I-IV)

Salinity as well as the inorganic nutrient concentrations (PO_4-P, NH_4-N, NO_3-N, NO_2-N, SiO_4-Si) were measured from thawed samples, using standard seawater protocols as described in detail by Hansen and Koroleff (1999). The chl-*a* concentration was determined from ice samples melted with added FSW and from under-ice water samples by filtering either two 50 ml or 100 ml aliquots onto Whatman GF/F filters (Whatman (GE Healthcare), Maidstone,

Kent, UK). The filters were put into 15 ml plastic test tubes and stored frozen until further processing in the laboratory. The analysis was continued by additions of 10 ml 96 % v/v ethanol and extraction at room temperature in the dark for 24 h. The extract was filtered again through Whatman GF/F filters and the fluorescence was measured with a Jasco FP-750 spectrofluorometer (Jasco International Co. Ltd., Tokyo, Japan) calibrated with pure chl-*a* (Sigma-Aldrich). The chl-*a* concentrations were calculated according to HELCOM (1988) and corrected for the dilution caused by the volume of FSW added prior to the melting.

The P-E of the sympagic communities was measured as ^{14}C-CO_3 incorporation (for detailed description, see Setälä et al. 2005) in melted sea-ice and under-ice water. Duplicate 3 ml samples with added $NaH^{14}CO_3^-$ (50 µl, final conc. 0.33 µCi ml^{-1}) (Carbon 14 Centralen, International Agency for ^{14}C Determination, Hørsholm, Denmark) were incubated for two hours in incubators at different light levels, ranging from 0 to 505 µE m^{-2} s^{-1}. The incubators were cooled to +4 °C by circulating ice-cold water through the incubators with a peristaltic pump. The incubation was stopped by adding 100 µl formaldehyde (final conc. 1.23 %), after which the samples were acidified with 1 N HCl for 48 h to remove the unincorporated $NaH^{14}CO_3^-$. An Insta-Gel Plus (PerkinElmer, Turku, Finland) scintillation cocktail was added to the acidified samples and the activity was measured with a Wallac WinSpectral 1414 liquid scintillation counter (Wallac Oy, Turku, Finland) using external standards. The P-E values were normalized to chl-*a* (µg C (µg Chl-*a*)$^{-1}$ h^{-1}) instead of presenting daily *in situ* production (µg C l^{-1} d^{-1}), because these short-term laboratory measurements are suitable for measuring physiological responses instead of daily production (Henley 1993).

The O_2 consumption was used for verifying the metabolic activity of the cells **(III)**. Respiration was measured as O_2 net change **(III)** and determined by an automated Winkler titration (Williams & Jenkinson 1982).

The LAP measurements were carried out with the modified method described by Stoecker and Gustafson (2003) using L-leucine 7-amido-4-methyl-coumarin (Leu-AMC, Sigma Chemicals) as a substrate (98 µM final concentration Leu-AMC) (Sarath et al. 1989). The LAP enzyme assays were conducted with three replicates collected into disposable semi-ultraviolet (UV) cuvettes. The samples were incubated in the dark at +3 °C up to four hours. The reaction was terminated with 10 % dodecyl sulphate after which the fluorescence was determined with a Jasco FP-750 spectrofluorometer. The standard curve was determined for concentrations 0-2.5 µM of 7-amino-4-methyl-coumarin (AMC) (Sigma Chemicals) in sterile water. A culture without added substrate was used as a control of the background fluorescence, and 2-µm-filtered culture (polycarbonate filters, Osmonics Inc., Minnetonka, MN, USA) was used to separate the bacterial enzyme activity from the activity of the eukaryotic community.

The total AP activity was measured according to Pettersson (1980), with modifications (Kononen et al. 1993, Grönlund et al. 1996). Duplicate 8 ml subsamples of the total and < 2-µm-filtered water were collected in acid-washed glass test tubes and put into a water bath with circulating ice-cooled water of approximately +4 °C. The substrate 4-methylumbelliferyl-phosphate (MUP) (Sigma Chemicals) was added to obtain a final concentration of 0.11 mM. The zero reading was obtained immediately after addition of the substrate with a Jasco FP-750 spectrofluorometer equipped with a sipper Jasco SHP 292, using excitation at

365 nm with a 10 nm slit and a 460 nm emission filter with a 5 nm slit. The reaction was followed throughout the 75 min incubation, with measurements every 15 min. The fluorescence units were calibrated with standard solutions of 4-methylumbelliferone (MUF) (Sigma Chemicals) over the range of 0.01 to 1 μM and the fluorescence intensity increase in the samples was used to calculate the APA (alkaline phosphatase activity) given as nmol MUF hydrolyzed h^{-1}.

4.5 Species identification and cell enumeration (I-IV)

Samples for species identification as well as cell enumeration were preserved with glutaraldehyde (1.25 % final concentration) and stored in the dark at +4 °C. A 50 ml subsample was settled according to Utermöhl (1958) for 24 h and examined with an inverted light microscope (equipped with 12.5× oculars and 10×, 20×, 40× objectives). The microscopic examination was carried out using a Leitz DM IRB microscope (Leitz Microscope Co., Wetzlar, Germany) equipped with a Polaroid® digital microscope camera (DMC 1, Polaroid® Corporation, Cambridge, MA, USA) or a Leica DMIL with a digital camera (Leica DC300F).

The bacteria were stained, using acridine orange, and counted under a Leica Aristoplan epifluorescence microscope using a 100× immersion objective (**II**). The bacterial biomass was calculated, using cell numbers, volume estimates and a volume-to-carbon conversion factor of 0.154 pg C μm^{-3} (Scavia & Laird 1987).

For identification of unknown dinoflagellates (**IV**), the cultured dinoflagellate thecal plate patterns were visualized according to Fritz and Triemer (1985) with the following modifications. The cells were fixed in glutaraldehyde (2 % final concentration) and collected on 10 μm polycarbonate filters (Nuclepore®, Cambridge, MA, USA) using a 20 ml glass filtration funnel. The outer cell coverings of the amphiesma were removed from the thecae in a 9.6 % sodium hypochlorite solution, followed by a rinse with 5 ml of FSW. The autofluorescense caused by chl-*a* was extracted into 96 % ethanol and rinsed with 5 ml of FSW. The thecae were then stained for 5 min in 1 ml of Tinopal UNPA-GX fluorescent brightener (Sigma® Chemicals) solution. The excess stain was removed with FSW. The filter was placed on a glass slide where 40 μl of SlowFade® Gold antifade reagent (Molecular Probes (Invitrogen Corp.,) Eugene, OR, USA) was added before placing the cover glass on top. The slides were kept at room temperature and in the dark overnight to allow the cells to slightly withdraw. The tabulation was observed with a Leica Aristoplan epifluorescence microscope fitted with a filter cube A and documented using an attached digital camera (Leica DC300F).

Samples for scanning electron microscopy (SEM) were preserved with glutaraldehyde (2 % final concentration) and dehydrated in a series of alcohol up to 96 %. The SEM samples were treated according to Hansen (1995), with the exception that *Scrippsiella hangoei* cells were plated with colloid platinum after critical point drying with Bal-Tec CPD 030 critical point dryer (Bal-Tec AG, Balzers, Liechtenstein). The samples were examined with a Zeiss DSM 962 scanning electron microscope (Carl Zeiss, Oberkochen, Germany).

Shadow-cast whole mounts were prepared according to Iwataki et al. (2002) and the scales were visualized with a JEOL JEM-1010 transmission electron microscope (Jeol Ltd., Tokyo, Japan) using 90 kV acceleration voltage. Another part of the fixed sample was

concentrated, using centrifugation (7 min 3000 rpm [998 × G]), and dehydrated in a series of alcohol up to 96 %. The cells were then embedded in Spurr resin and processed according to Jensen and Moestrup (1999). A Leica ultracut UCT ultramicrotome was used for thin sectioning. The thin sections were collected on copper grids and examined with a JEOL JEM-1200EX transmission electron microscope, using 60 kV tension.

The cultured material was used for phylogenetic analysis. Live cells were collected with low centrifugation (1 min, 5000 rpm) to the bottom of an Eppendorf tube. The procedure was repeated 10 times to obtain enough material for DNA extraction. DNA was extracted using the phenol-chloroform method (Blomster et al. 1999). Using existing sequence data available in GenBank species-specific primers were designed for the amplification of the ITS1, 5.8S and ITS2 sequences as explained (**IV**). Phylip v. 3.6 was used to analyse the sequences obtained after they were aligned with ClustalW.

4.6. Monitoring data (IV)

The seasonal and geographical distribution of the *Heterocapsa arctica* ssp. *frigida* (**IV**) was studied using the long-term data collected in 1993-2005 as part of the Algaline project (Finnish Institute of Marine Research, FIMR). The samples were obtained using automated water samplers installed aboard commercial vessels (GTS Finnjet, m/s Finnpartner) travelling between Germany and Finland (Fig. 1). The sampling covered six Baltic Sea subareas. The sampling locations varied within the study period: during 1993-1995 sampling was scheduled to take place at specific times, from 1996 onwards sampling occurred at certain longitudinal positions, but allowing for latitudinal variation; furthermore, the ships passed Gotland Island either from the east or west (Fig.1). Particularly during the later years, the sampling was somewhat biased toward the northern part of the study area.

Parameters recorded onboard included *in situ* water temperature and salinity. A maximum of 24 one litre water samples were collected on the return journey to Helsinki and stored refrigerated under unlit conditions until analysis ashore at FIMR. During the study period the models and makes of some of the apparatus varied but the methods used were essentially the same. For a detailed description of the methods, see e.g. Rantajärvi and Leppänen (1994) and Rantajärvi (2003).

5. RESULTS AND DISCUSSION

5.1 Spatial distribution and activity of the sea-ice microbial communities in the Bothnian Bay

The taxonomy, distribution and physiology of ice algae are relatively well studied in other ice-covered areas, e.g. the Antarctic (Thomas & Dieckmann 2003, El-Sayed 2005, Taylor & Marchant 2006), Arctic (Gosselin et al. 1997, Melnikov et al. 2002), Barents Sea (Hegseth 1998), as well as the Okhotsk Sea (McMinn et al. 2008), whereas according to recent literature "the spatial and temporal data needed to estimate the ice biotas' importance to the overall productivity, nutrient and carbon cycles is missing in the Baltic Sea, especially from outside the near shore land fast ice" (Granskog et al. 2006).

The data gathered from the Bothnian Bay (Figs. 2-4 and **I)** show the spatial distribution with considerable heterogeneity and a clear succession of sea-ice biota within the Gulf of Bothnia. The flagellates together with diatoms were found to dominate the pancake ice floes (PCI) (Fig. 2). Diatoms, *Achnanthes taeniata* Cleve 1880, *Chaetoceros wighamii* Brightwell 1856, *Melosira arctica* (Ehrenberg) Dickie ex Ralfs 1861, *Nitzschia frigida* Grunow 1880 and *Skeletonema costatum* (Greville) Cleve 1878 dominated the algal biomass in the sea-ice except at stations 28 and 31 where small unidentified flagellates (< 10 μm) formed the highest biomass. The main under-ice water biomass also consisted of these flagellates (Table 1).

The organisms encountered (Table 1) and their biomasses (Fig. 2) support the prevalent understanding that during the winter more abundant microbial communities are found in the sea-ice than in the water column underneath the ice (e.g. Ikävalko 1997, Kaartokallio 2005, Granskog et al. 2006 and the references therein). Cota et al. (1991) have explained this with the more stable habitat within the ice than in the planktonic environment, since the cells are not subject to large vertical displacements in the irradiance field. Yet the snow cover may cause rapid changes in the inner ice light milieu, while the temperature and salinity are relatively constant over most of the growth period (Fig. 5 and **II**). The sea-ice algae in the Arctic have been found to be highly shade-adapted or obligate shade flora, with low photoadaptive indices and high photosynthetic efficiencies (Cota 1985, Smith et al. 1987, 1988, Cota & Horne 1989). Light microscope examination of the fixed material collected from the Bothnian Bay showed that the vertical distribution of algal biomass was not in accordance with chl-*a* distribution at stations 26 and 27 (Figs. 2 and 3) showing that the cells encountered had low chl-*a* contents at the surface or near the surface of the ice. This can be explained by photoacclimation, the fact that the photosynthetic apparatus is adjusted according to ambient light (Moore et al. 2006). Under dim light conditions (i.e. in the deeper layers of the ice) there is a higher demand for light-harvesting pigments and thus, the cells can increase their pigment contents (Falkowski & Raven 1997 and references therein). In general, the biomasses were higher in the sea-ice than in the underlying water, with two exceptions at stations 28 and 31, and within the sea-ice the highest chl-*a* concentrations were always found in the bottommost layer (Fig. 3). Stations 26 and 29 showed similar distribution patterns, with higher chl-*a* concentrations in the middle layers. The lowest value (≤ 0.5 μg Chl-*a* l^{-1}) was measured in the new ice obtained from the refrozen ship route at station 28. The PCI floes had similar concentrations, averaging 1.6 μg Chl-*a* l^{-1}. Some protists included in the zooplankton are symbiotic, e.g. the ciliate *Mesodinium*

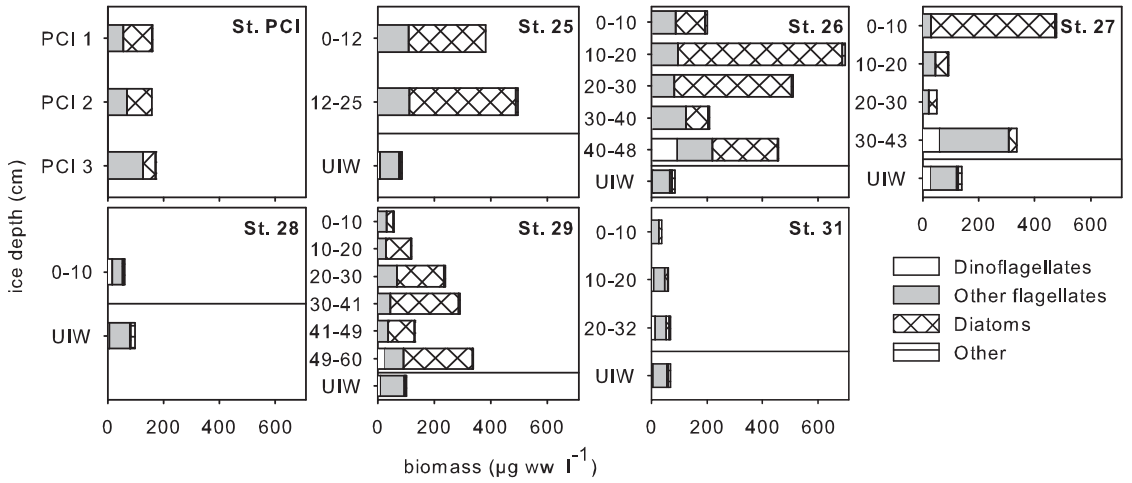

Fig. 2. The biomass (µg ww l^{-1}) of phytoplankton groups encountered in the pancake ice (PCI) floes, sea-ice and under-ice water (UIW) at the sampling sites in the Bothnian Bay. The horizontal line indicates the ice-water interface. Redrawn from **I**.

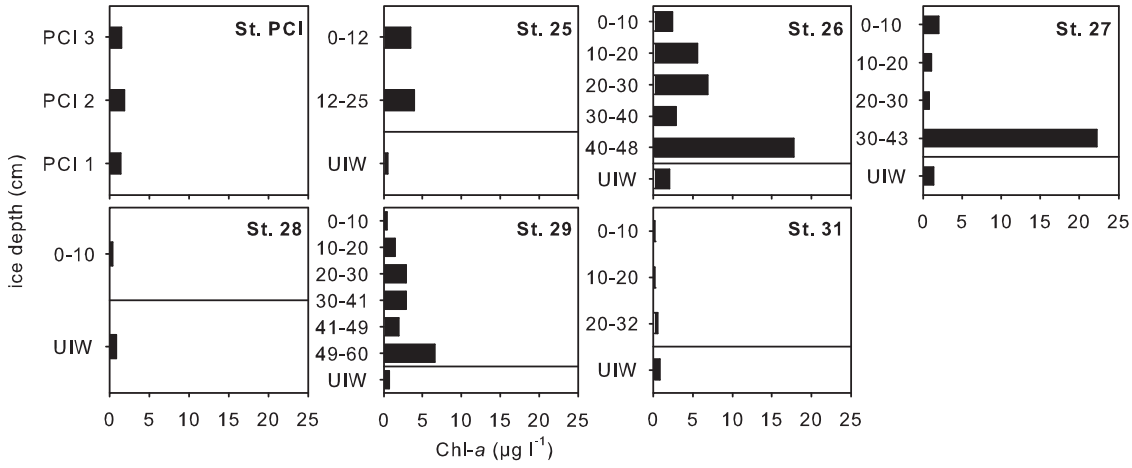

Fig. 3. Chlorophyll-*a* (Chl-*a*) concentration in pancake ice (PCI), sea ice and under-ice water (UIW) at sampling sites. The horizontal line indicates the ice-water interface. Redrawn from **I**.

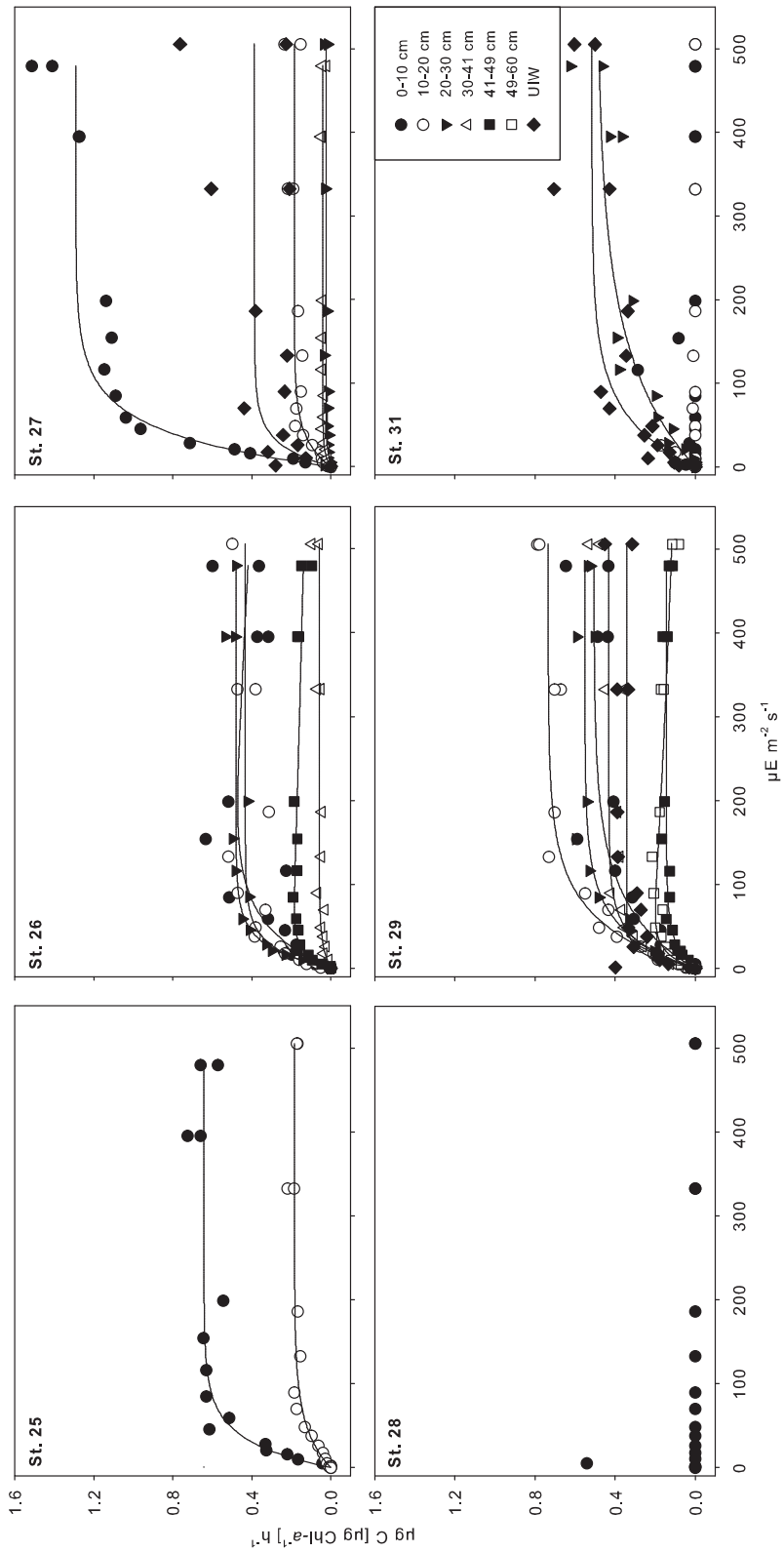

Figure. 4. Photosynthetic efficiencies of the sea-ice communities in the Bothnian Bay Redrawn from **I**.

Table 1. List of encountered algal, protozoan and metazoan species encountered in Bothnian Bay. Classification is based on Integrated Taxonomic Information System (http://www.itis.gov/)

ALGAE		PROTOZOA
Cyanophyceae	Coscinodiscophyceae	Ciliophora
Anabaena sp.	*Chaetoceros danicus*	unid. ciliate spp.
Aphanizomenon flos-aquae	*C. wighamii*	
Gomphosphaeria sp.	*Melosira* cf. *arctica*	Holotrichia
Microcystis sp.	*Melosira* spp.	*Lacrymaria rostrata*
Nodularia spumigena	*Skeletonema costatum*	
Oscillatoria sp.	*Thalassiosira baltica*	Spirotricha
		Bursaria sp.
Dinophyta	Fragilariophyceae	*Euplotes* sp.
Dinophyceae	*Synedra* spp.	*Strombidium* spp.
Dinophysis acuminata		*Tintinnidium fluviatile*
Peridiniella catenata	unid. centric spp.	*Tintinnopsis lobiancoi*
Peridinium sp.	unid. pennate spp.	
Scrippsiella hangoei		Rhapdophorina
unid. athecate spp.	Chlorophyta	*Mesodinium rubrum*
unid. thecate spp.	Chlorophyceae	*Spathidium* sp.
	Monoraphidium contortum	
Bacillariophyta	*Planktonema lauterbornii*	METAZOA
Bacillariophyceae	*Scenedesmus* spp.	
Achnanthes taeniata		Rotifera
Amphiprora sp.	Protista incertae sedis	*Brachionus* spp.
Cylindrotheca closterium	unid. flagellates	*Synchaeta littoralis*
Navicula vanhoeffenii		
Nitzschia closterium		Copepoda
Nitzschia frigida		*Limnocalanus* sp.
Surirella sp.		nauplii spp.

rubrum Leegaard 1915, and they also contain chl-*a* and therefore microscopic enumeration of the zooplankton community was needed.

A total of 16 different zooplankton species were identified from the sea-ice and water samples (Table 1). The zooplankton biomass was higher in sea-ice than in the under-ice water but the ice community was less diverse than the communities in the under-ice water. Copepods were abundant in two of the three PCI floes (averaging 40 µg wet weight (ww) l^{-1}), but were absent from all the other ice and under-ice water samples. Only two species (*Synchaeta littoralis* Rousselet 1902 and *Brachionus* spp.) of rotatoria were present but they dominated the sea-ice communi-

ties in the Bothnian Bay. In the under-ice water the zooplankton biomass consisted of ciliates, station 29 being an exception with rotatoria also present. The ciliates included mostly *Bursaria* sp. and tintinnids. *Mesodinium rubrum* was observed in the under-ice water at station 31 and its abundance could have influenced the measured chl-*a* concentrations and P-E levels. Since the abundance of *M. rubrum* was, however, low, this species' influence on the results is considered negligible.

The P-E curves from the incubations for each sampled ice section are presented in Figure 4. In general the activities were at the lower part of the range reported for ice algae (Lizotte & Sullivan 1991, Robinson et al. 1997). Robinson et al. (1997) speculated that the reason for the low P-E observed in polar ice could be limitation by temperature, but the mechanisms are not well understood. In accordance with the chl-*a* measurements, no photosynthetic activity could be detected in the newly formed ice at station 28, indicating an adaptation period of algae to the new environment.

The highest photosynthetic rate was associated with the ice surface community collected from station 27. The P-E of the community collected from the refrozen ship route at station 28 was below the detection limit of our technique. The under ice water community also had practically no P-E. The sympagic sea-ice and plankton communities of the few-days-old 'new ice' obtained from the refrozen ship route at station 28 (Figs. 2-4) further verify the present understanding that the incorporation of organisms into the sea ice is caused by passive accumulation by physical processing rather than biological activity (Ackley et al. 1979). For this reason the sympagic community composition reflects the community that has been present in the water column during freezing (Figs. 2-4) (Cota et al. 1991, Lizotte 2003).

Diatoms (*Achnanthes taeniata* and *Melosira arctica*) dominated the sympagic surface assemblage at station 27, whereas the bottommost communities at station 31 were dominated by flagellates. The communities had lower P-E levels at other stations, which agrees with the results shown from near shore landfast ice by Haecky and Andersson (1999) during the same time of year or what was measured in other seas (see Table 5.1 in Thomas & Dieckmann 2003).

A clear succession in P-E could be observed from the results. In the newly formed ice (station 26) the P-E was practically zero, increasing with the age and thickness of the ice (Fig. 4), with a clear downwards decreasing trend within the ice (Table 4 in **I**). Otherwise, no or very little activity would indicate that the chl-*a* obtained from the melted sea ice would have been stored frozen inside the ice. This would be possible if the ice were formed of freshwater and had thus no brine channels. Although larger ice crystals were found at stations 25, 26 and 28, similar types of sea-ice crystal structure are also formed under special growth conditions in the brackish water of the Bothnian Bay (Granskog et al. 2003). Hence, the ice was determined to structurally be of brackish water origin (**I**); therefore it is also unlikely that the chl-*a* would have been preserved as frozen.

The fact that higher activities were found in the ice samples with higher nutrient concentrations indicates nutrient limitation in sea ice (**I**). This is in concordance with previous studies carried out in the same area that have shown similar nutrient quantities as encountered during this study and considered them to be growth-limiting (Haecky et al. 1998, Haecky & Andersson 1999, Kaartokallio 2001). If these sea-ice communities were nutrient-limited, the measured chl-*a* would have been inside nutrient-starved cells, either

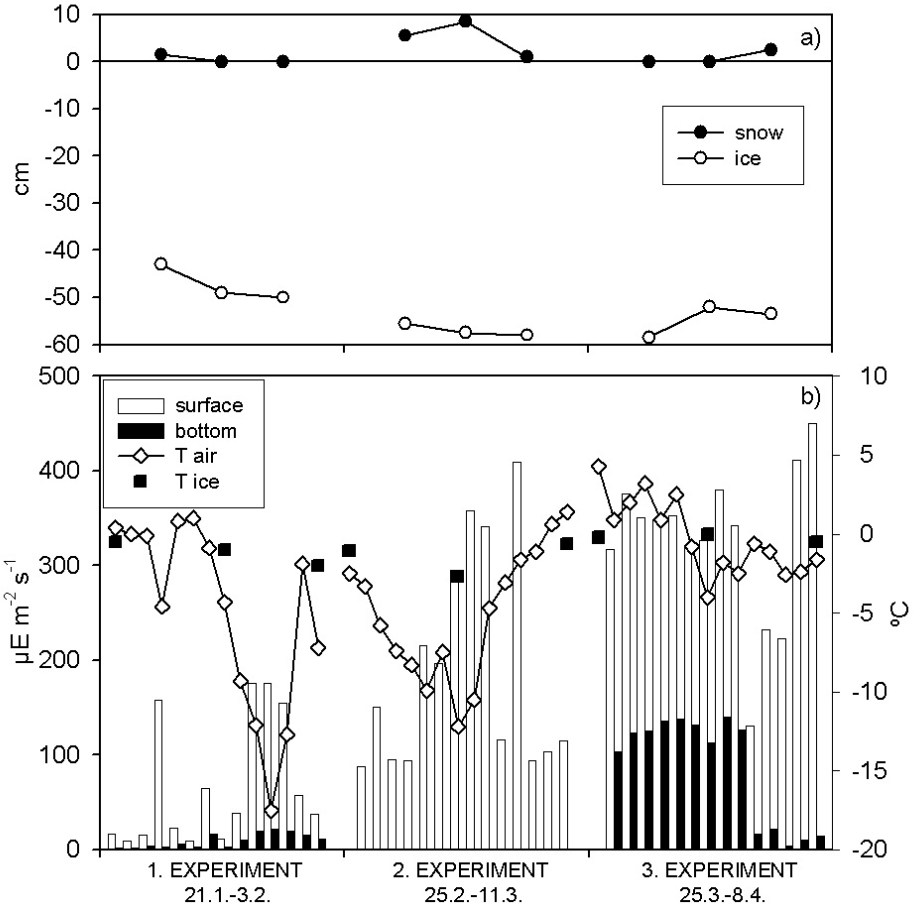

Figure 5. (a) Ice and snow depths (cm) from late January to early April. (b) Light intensity (µE m^{-2} s^{-1}) at the surface and bottom of the ice with air and ice (mean) temperature (°C). Air temperature data provided by the Finnish Meteorological Institute. Redrawn from **II**.

hibernating inside the ice as resting stages (**III**) or alternatively relying on complementary sources of nutrition, i.e. mixotrophy.

5.2. Controlled field experiments: responses of sympagic assemblages to increased light and complete darkness

The inner ice light milieu and nutrient quantities were studied in three subsequent *in situ* experiments carried out in Santala Bay, SW coast of Finland, in 2003 (**II**). The ambient sea-ice light milieu in Santala Bay shows the amount of PAR at the sea-ice surface and at the bottom of the ice during the sympagic growth period. Incoming solar radiation penetration of sea-ice was restricted during most of the ice-covered period (Fig. 5). Phototrophic algae living in such a low-light environment must develop mechanisms to compensate for the lack of light. One common response is photoacclimation, meaning that the photosynthetic apparatus

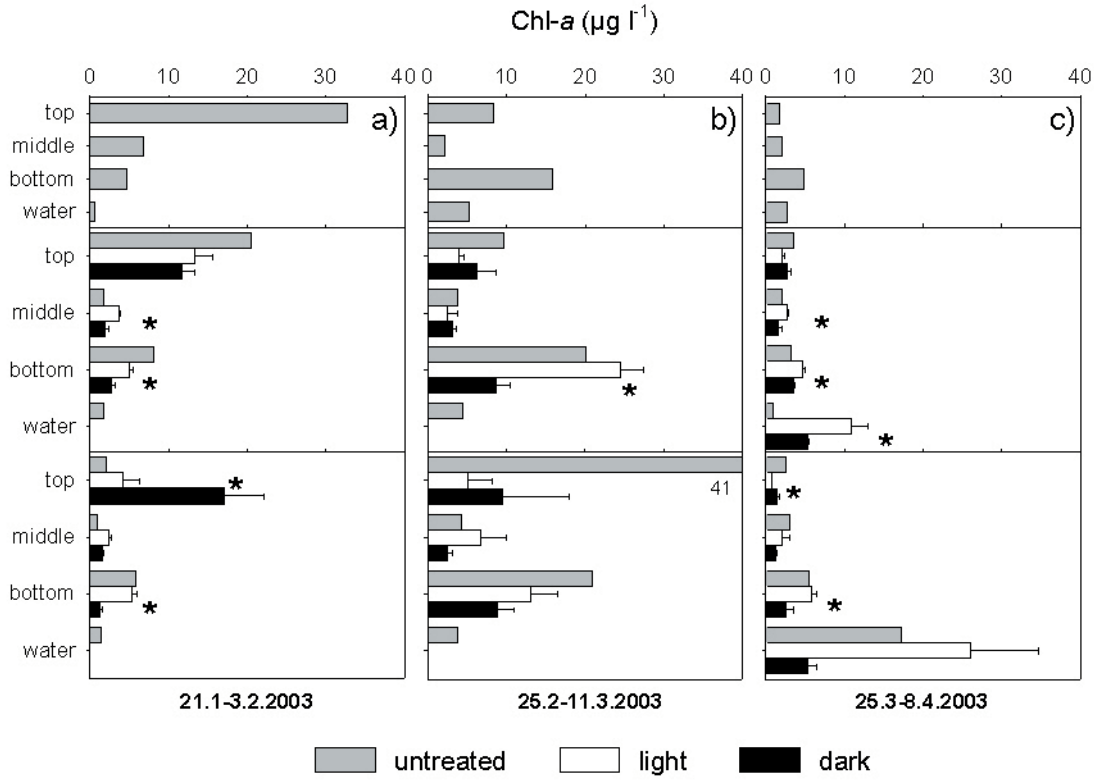

Figure 6. Mean chlorophyll-*a* (Chl-*a*) concentration (µg l^{-1}) with standard deviations in the untreated, light-treated and dark-treated cores during the (a) first, (b) second and (c) third experiments. The concentrations measured from the water inside the incubators in the third experiment exclude the added 0.2-µm-filtered seawater (FSW). Rows from the top: start, 1 week, 2 week. The significant differences are marked with asterisks*. Redrawn from **II**.

is constantly adjusted according to the ambient light (Falkowski & Raven 1997, Moore et al. 2006 and references therein). Light starvation may cause an increase in the amount of light-harvesting pigments. In contrast, the darkening of the ice during the *in situ* light vs. dark manipulation caused cessation in the followed growth, observed as the measured chl-*a* concentration (Fig. 6). During winter, however, the organisms have repeatedly been found to be more abundant within the sea-ice than in the water column (i.e. Figs. 2, 3, 6), which shows that ice provides a platform for growth. Based on the results shown in Figures 5 and 6, there is indirect evidence that photosynthesis cannot, in all cases, be used to explain the increase in biomass (Fig. 6). Therefore, the growth observed was facilitated with some alternative mechanism used for carbon acquisition, namely mixotrophy, which would be an advantageous ability within the sea ice during frequent prolonged periods of darkness (Fig. 5, Ehn et al. 2004). To assess mixotrophy the activities of AP and LAP were also measured to complement the chl-a data collected during the *in situ* light vs. dark experiments. Extracellular enzyme activities

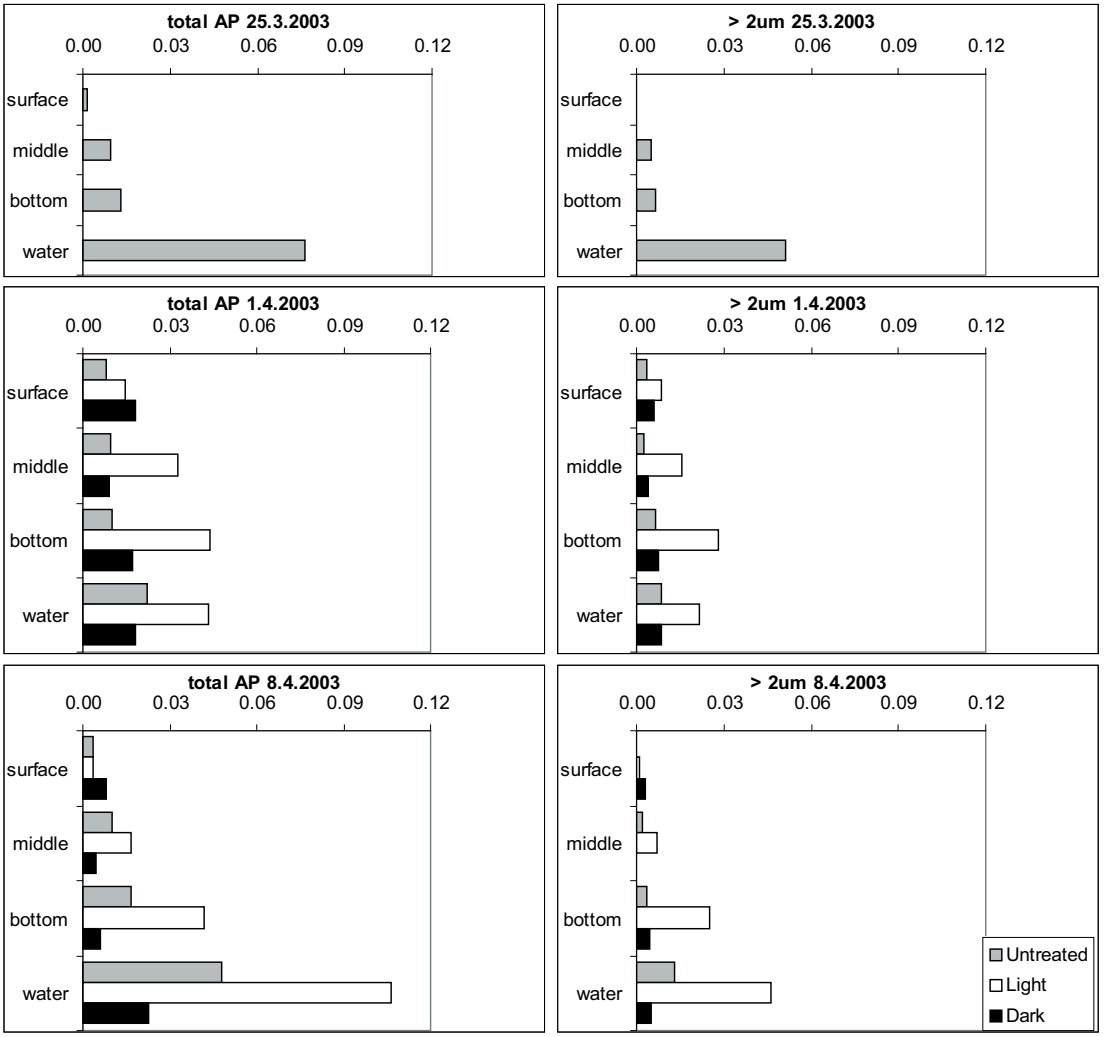

Fig. 7. Concentrations of alkaline phosphatase (AP) (nmol MUF h^{-1}) measured from the melted ice or water (left column) and AP measured from > 2-µm fraction only (right column). Rintala & Piiparinen unpublished data measured from the same ice samples used for the measurements presented in **II**.

have been used as physiological indicators of mixotrophy (Martinez & Azam 1993, Langheinrich 1995, Berg et al. 2002, Stoecker & Gustafson 2003, Stoecker et al. 2005, Vahtera et al. 2007). The enzymes measured were excluded from the free, soluble enzyme pools and interlinked with organisms other than bacteria with fractionation (> 2 µm). If the community had been able to survive with its heterotrophic capabilities alone, the enzyme activity should have increased in the dark-manipulated ice cores. It was, however, mostly favoured by the increase in light (Figs. 7 and 8), showing the importance of photosynthesis to the enzymes produced.

There may be several reasons why phytoplankton would excrete enzymes for processes varying from signalling between in-

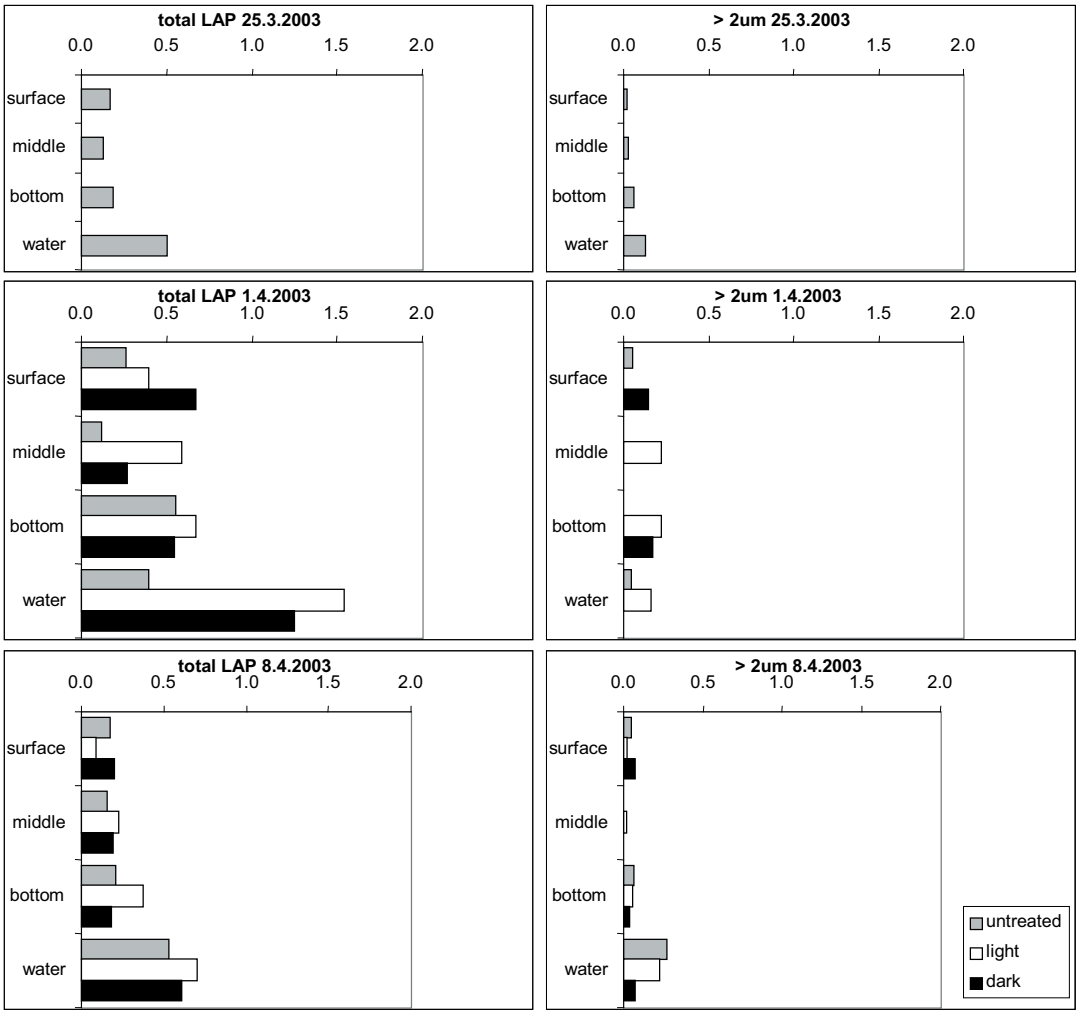

Fig. 8. Concentrations of leucine aminopeptidase (LAP) (nmol AMC h^{-1}) measured from the melted ice or water (left column) showing the amount of LAP measured from the > 2-µm fraction only (right column). Rintala & Piiparinen unpublished data measured from the same ice samples used for the measurements presented in **II**.

dividual cells to being secondary exudates (Matsuda et al. 1994). Yet, improving their nutritional status is the most probable. It is especially useful when living within the sea-ice, where the inner ice light climate is expected to change rapidly and periods of darkness are typical, severely affecting the photoadaptive performance of the sea-ice communities (Kottmeier & Sullivan 1988, Cota & Smith 1991, Falkowski & Raven 1997). Some cyanobacteria can grow in the dark on organic substrates (Vonshak et al. 2000), suggesting that organic compounds may be utilized as supplemental sources of carbon as well as, under some circumstances, nitrogen. The results shown in Figures 7 and

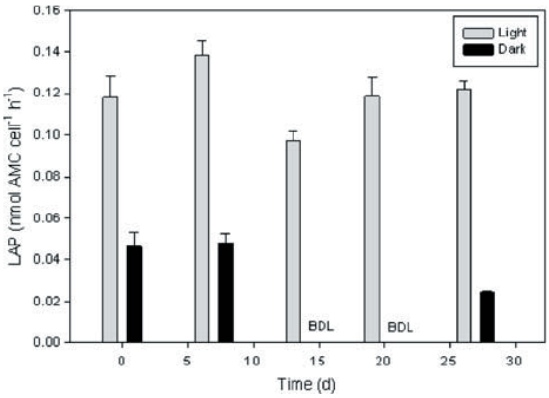

Fig. 9. Cell-specific leucine aminopeptidase (LAP) activity of *Scrippsiella hangoei* in light and dark. LAP measurements were below detection limit (BDL) of the method for the dark-stored *S. hangoei* on days 14 and 20. Measurements were stopped after 27 days. Error bars represent SD of three subsamples. Redrawn from **III**.

8 indicate the importance of mixotrophy in sea-ice microbial food web dynamics. Darkness increased both AP and LAP enzyme production in the ice surface after one week of incubation, the most increase seen in total production. This indicates that the algal community actively utilized organic matter as nutrient sources, which suggests that mixotrophy is used as a mode of nutrient acquisition in the ice. In the deeper ice layers the enzyme production was favoured by the increased amount of light, suggesting that it is not used as a prolonged dark-survival strategy. The surface communities' LAP production showed that this surface community was able to shift from phototrophy towards osmotrophy, perhaps due to better availability of prey, dissolved organic carbon (DOC) or dissolved organic matter (DOM), while enzymes in the middle and bottommost assemblages could not be produced without photosynthesis also occurring. The increase was more apparent in AP than in LAP and agrees with the inorganic nutrient quantities measured (**II**), since more nitrate was available than phosphate. Therefore, the phytoplankton community would have benefited from increasing its phosphate acquisition through AP activity.

Both treatments (increased light and complete darkness) increased the amount of LAP after the first week, but after two weeks it decreased. The decrease may have been caused by increasing P limitation, also seen in increased AP production after two weeks of incubation, or N limitation.

There was also an increase in enzyme production in the water beneath the ice. It was the sterile FSW that was in the beginning of the experiment put into the incubators. Therefore cells of ice origin could only have excreted these enzymes, which is showing that the sympagic community could survive in the water column as well. This may be an indication of the sympagic community acting as a potential seed population for the coming spring phytoplankton bloom, especially if there are no grazers present. Here again there is a temporal change between these measured enzymes, in that the LAP increased after the first week of incubation while the increase in AP amount is seen after the second week, which is another indication of P limitation.

In general the increase in light was more favourable to enzyme production than complete darkness, which completely shuts down photosynthesis. This indicates mixotrophy, which demands light to increase the hydrolytic potential of the community. Thus, the phototrophic mode of these sympagic communities appears obligatory, but since it includes these osmotrophic capabilities that are

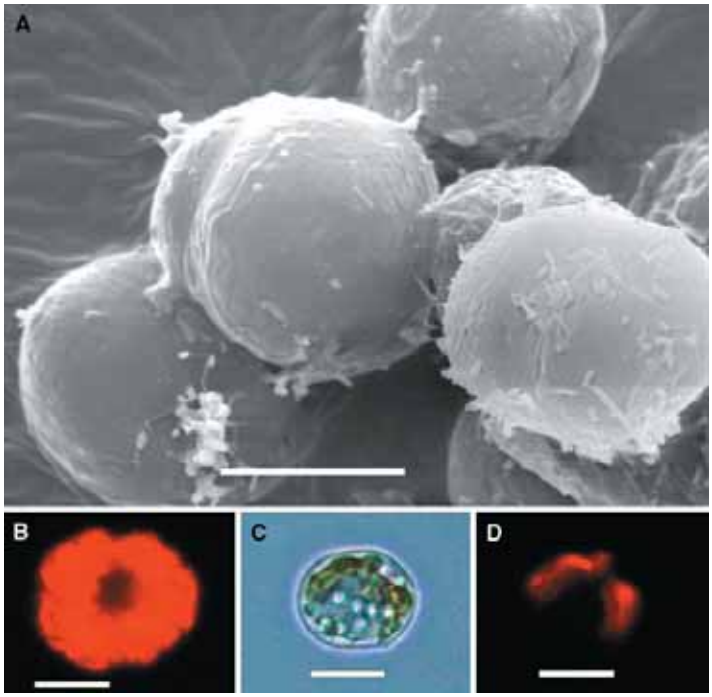

Fig. 10. Scanning electronmicrograph of the immotile stage of *Scrippsiella hangoei* (A), epifluorescence micrograph of the vegetative cell (B), light micrograph of the immotile stage (C) and epifluorescence micrograph of the immotile stage (D). Figures C and D show the same cell that has been in the dark for 3 months. Scale bar is 10 μm. Redrawn from **III**.

used to complement their nutritional status, it makes them mixotrophic. These obligatory phototrophs would probably act in a manner similar to that of the primarily phototrophic species described in the theoretical models of mixotrophy in Stoecker (1998).

5.3 From community to a species-specific survival strategy of sympagic algae: a case study with cultured *Scrippsiella hangoei*

Mixotrophy with its many possible variations (reviewed by Stoecker 1999) is very difficult to detect without being able to study the species in cultures (e.g. **III**).

Scrippsiella hangoei is one of the most common sympagic dinoflagellates within the Baltic Sea ice. To study specific strategies of individual sympagic algae for coping with low light in the ice, experiments with a unialgal isolate of *S. hangoei* were conducted. An indication of mixotrophy was encountered when the number of bacteria remained very low in the vegetative cultures of *S. hangoei*, which also excreted extracellular LAP (**III)**. Similar to the results shown for natural communities (Fig. 8), the rate of LAP activity in the *S. hangoei* culture was lower in darkness than in light, indicating that *S. hangoei* is not able to make a complete shift to osmotrophy. Thus the ability to utilize osmotrophy could be supplementary to photosynthesis, but is not part of the dark-survival strategy of *S. hangoei* (Fig. 9), because the measured enzyme activity was not enough to sustain growth during a prolonged period of darkness. Therefore, measurements of LAP in dark- and light-exposed cultures did not confirm our hypothesis that mixotrophy could be a strategy of the species to survive periods of darkness.

The vegetative cells of *S. hangoei* had a diameter of 20-30 µm. Interestingly, when exposed to darkness, many *S. hangoei* cells transformed into a temporary cyst: the *S. hangoei* cells shed the flagella and theca and the cell size was reduced to 10-15 µm (Fig. 10). Additionally, the chloroplasts gradually decreased in size, but were still fluorescent after 3 months in darkness (Fig. 10). The immotile stage of *S. hangoei* had a very thin cell wall, i.e. the pellicle, and SEM micrographs revealed a smooth surface with no apparent structure. Unlike typical dinoflagellate resting cysts that are usually surrounded by thick walls with ornamentation, these cysts were naked, thus making their light and electron microscopic identification extremely difficult or even impossible without genetic evidence that could be used for verification.

The formation of the temporary cysts occurred as a response to prolonged long-term darkness and involved high metabolic activ-

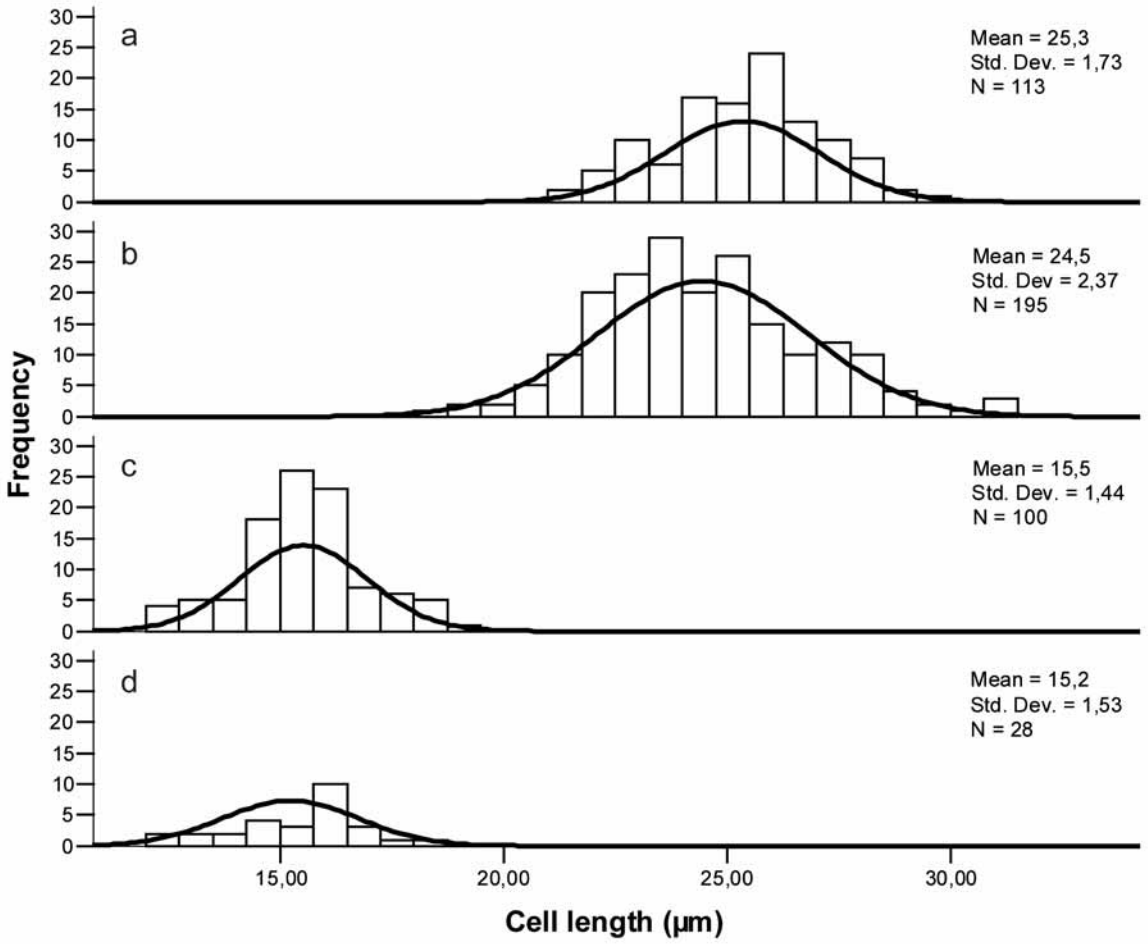

Fig. 11. Cell length of a) *H. arctica* (CCMP 445) ssp. *arctica* in CCMP culture medium (35 psu), b) *H. arctica* ssp. *arctica* in F/2 culture medium in aged Baltic Sea water (6 psu), c) *H. arctica* ssp. *frigida* in f/2 (-Si) medium in aged Baltic Sea water (6 psu) and d) *H. arctica* ssp. *frigida* measured from natural samples. Redrawn from **IV**.

ity as revealed by measured O_2 net change (**III**). Ecologically, it would not be a good strategy to initiate temporary cyst production at high metabolic cost after a short time in darkness, because the ice habitat *S. hangoei* occupies is characterized by low daily irradiance, and short periods of darkness are frequent (Ehn et al. 2004). The transition back to a vegetative cell was relatively rapid after the temporary cysts were introduced to light, enabling *S. hangoei* to initiate growth after improvement in the light milieu. Usually the ice is snow-covered. But after the snowmelt or heavy wind the ice habitat becomes illuminated, which would simultaneously trigger growth in the encysted *S. hangoei* cells before the ice melt. Different triggers may separate between different purposes of the resting stages, e.g. the temperature trigger ensures that the thick-walled cyst is formed in spring as the benthic survival stage. The temporary cyst is probably less useful for survival in the sediment from one winter season to the next than the thick-walled *S. hangoei* cysts (Kremp & Parrow 2006), which are more resistant towards grazing and other loss processes that occur in the sediment (Persson & Rosenberg 2003). All of the above and the results presented in **III** suggest that the temporary resting stage has a function in nature that could be an important survival mode in ice during long dark periods. In the ice, grazing pressure is low and protection provided by a thick-walled cyst may not be important, whereas capability of rapid germination may be better suited for the ice environment.

The formation of resting spores has been suggested as an overwintering strategy for sea-ice diatoms in the Antarctic (Palmisano & Sullivan 1985). However, resting spores have not been considered to play an important role in the winter survival of Arctic ice algae, where facultative heterotrophy and energy storage have been suggested as the main processes enabling winter survival in the Arctic

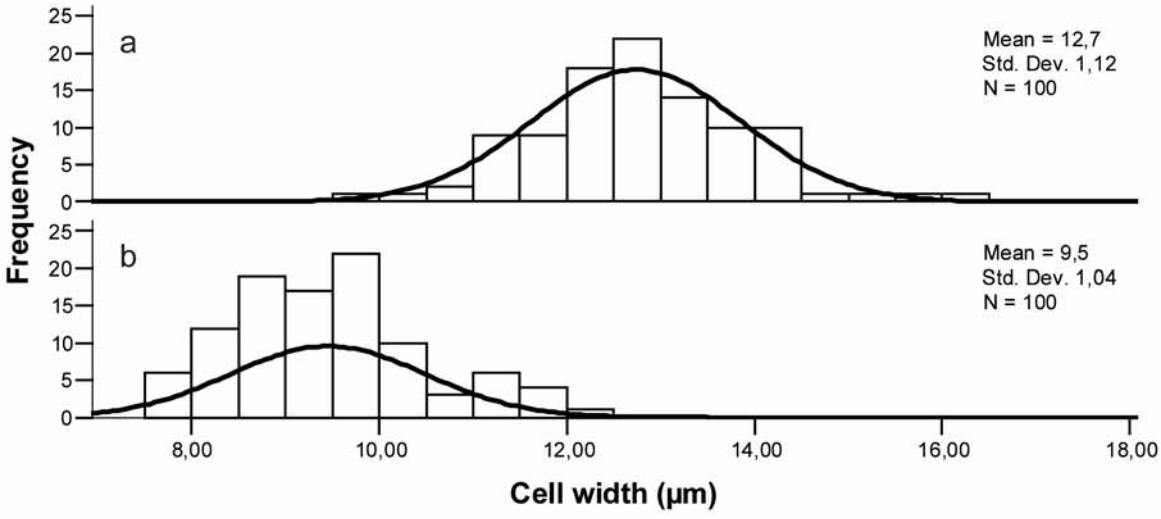

Fig. 12. Cell width of a) *H. arctica* (CCMP 445) ssp. *arctica* and b) *H. arctica* ssp. *frigida* both in f/2 (-Si) medium in aged Baltic Sea water (6 psu). Redrawn from **IV**.

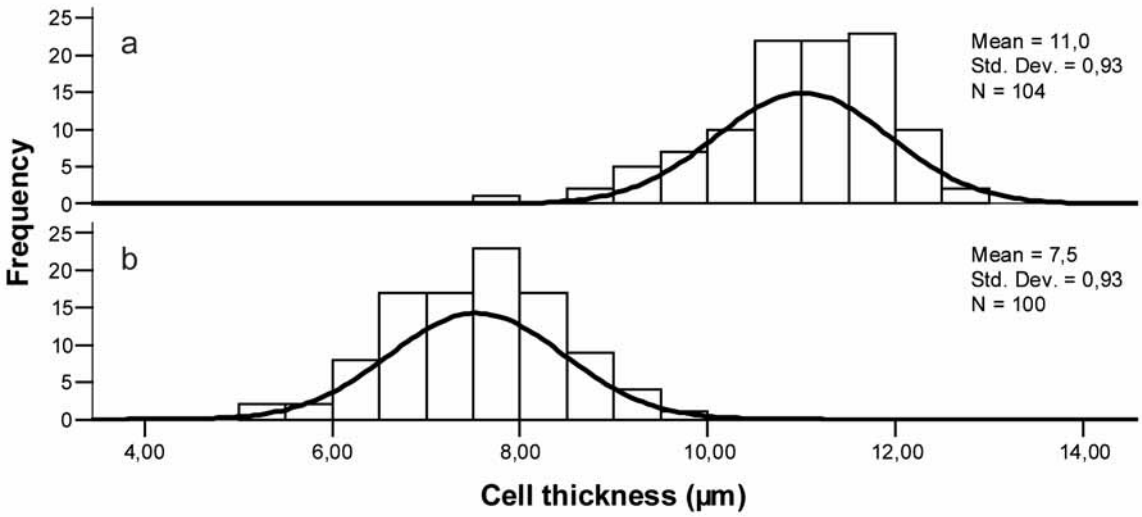

Fig. 13. Cell thickness of a) *H. arctica* (CCMP 445) and b) *H. arctica* ssp. *frigida* ssp. nov., both in f/2 (-Si) medium in aged Baltic Sea water (6 psu). Redrawn from **IV**.

sea ice (Syvertsen 1991, Zhang et al. 2003).

However, these cysts of *Scrippsiella hangoei* are similar to some ice-associated diatoms, e.g. *Cylindrotheca closterium* (Ehrenberg) Reimann & Lewin 1964, that reduce their metabolic rate under unfavourable conditions (Palmisano & Sullivan 1982). Cysts may function as survival stages in ice (Garrison & Buck 1985) and they could also provide an explanation for why the P-E levels encountered were so low in the Bothnian Bay (**I**) during the study period in winter 2006. Theoretically the cysts of *Scrippsiella hangoei* would also contain chl-*a* (Fig. 10) that would be found inactive in the snow-covered sea ice (**I**). The species abundances shown in **I** include flagellates up to 10 µm, which is a very broad description of very little of ecological importance or taxonomic value, because the flagellates include both auto-, hetero- and mixotrophic species belonging to different taxonomic groups, such as coccolithophorids, chlorophytes, dinoflagellates, euglenophytes, haptophytes, prasinophytes and prymnesiophytes (Tomas 1997). It is only an implication of the presence of one or more flagella, thus clearly excluding only diatoms. Yet grouping by size is the only correct way to address cells that cannot be identified or species without a name, such as *Heterocapsa arctica* ssp. *frigida* (described in **IV**), that also exist within the sea ice, without further knowledge of their ecological importance.

5.4 Understanding of community and experimental results is based on the knowledge of individual species

Communities are formed by group of single species occurring together. Ecological studies often include a group of unidentified thecate or nonthecate dinoflagellates (e.g. Table 1). The classification to unidentified thecate or nonthecate dinoflagellates is the correct way to entitle cells, which have characteristic shapes and sizes that do not completely match

any of the earlier descriptions found in the scientific literature for known species. The morphological traits and biogeographical distributions of organisms still continue to be considered as the primary means for traditional species classification. The morphology of an organism is the complex expression of its genotype, subject to phenotypical change due to the environment, life-cycle transformations and other influences (Hallegraeff 2003). An example of cells that have repeatedly been grouped among unidentified thecate or nonthecate dinoflagellates is presented in **IV**.

When examining the sympagic algal communities, a small thecate dinoflagellate resembling a hitherto undescribed species, previously assigned as *Heterocapsa frigida* (Huttunen & Niemi 1986). This dinoflagellate was isolated from an ice sample collected in Santala Bay and its morphology, ultrastructure and molecular genetics were examined to confirm its identity.

The isolated and cultured ice dinoflagellate had morphological features that agreed with the polar *Heterocapsa arctica* Horiguchi: the plate pattern of Po, cp, 5', 3a, 6-7'', 6-7c, 5s, 5''', 2'''' with some variation in the number of precingular and cingular plates. The intracellular ultrastructure was also similar with the only exception being the presence or absence of lipids. The extracellular body scales covering the outer membrane of the amphiesma, which are considered one of the most conservative characteristics in the genus *Heterocapsa* that separate the different species, were discovered to be slightly different: less robust with only a vaguely vein-reticulated basal plate. The ITS sequence of the isolate was almost identical to that of *Heterocapsa arctica*. Thus, based on the results gained, we consider our Baltic isolate (**IV**) to be conspecific with the Canadian *Heterocapsa arctica*, which has not been reported from the Baltic Sea (Hällfors 2004). However, the clear difference in cell size (Figs. 11-13) and the separation in geographical distribution distinguish the two dinoflagellates into regional variants. Thus we propose that the taxon described by Horiguchi (1997) be designated the name *Heterocapsa arctica* ssp. *arctica* once the description of our Baltic subspecies *frigida* is accepted (**IV**). Both of these subspecies identifications are, however, based on single monoclonal cultures and some variation in natural populations could occur. According to Hallegraeff (2003) the cultured cells can have more variable morphology than field material and considerable care should be exercised in basing new species descriptions exclusively on cultured material. For example, in armoured dinoflagellates the thecal plate patterns are considered as the most diagnostic, with hypothecal characteristics being more conservative than epithecal ones (Taylor et al. 2003). For lessornate or unarmoured dinoflagellates as well as the nanoplanktonic and picoplanktonic taxa the ultrastructure of chlorophlasts, pyrenoids, flagellar roots, etc. have become indispensable morphological features (Hallegraeff 2003). In addition, nonmorphological characteristics such as lipids, pigments, toxin biochemistry, immunocytological traits, chromosome number, nuclear or plastic DNA sequences have become increasingly used in species recognitions (e.g. Daugbjerg et al. 2000 in *Gymnodinium* / *Gyrodinium*).

When observed in the light microscope, *Heterocapsa arctica* ssp. *frigida* displays a general morphology that differs from *H. arctica* ssp. *arctica*. The most distinguishing characteristic is the size and shape of the cells, the latter being considerably larger and more elongated in shape (Figs. 11-13). This makes it so distinct in habitus that it has been repeatedly observed as a distinct

morphotype in the waters off the SW coast of Finland and its occurrence has been recorded in phytoplankton monitoring since 1993. Due to the lack of biological sea-ice data already mentioned by Granskog et al. (2006), the established seasonal distribution pattern (**IV**) is based on material collected from open water instead of sea ice, which is the isolation habitat of the ssp. *frigida*. The automatic sampling area covered by the ships of opportunity operating between Finland and Germany is shown in Figure 1. In all, 2764 phytoplankton samples were analysed, and *Heterocapsa arctica* ssp. *frigida* was present in a total of 418 samples, that is 15 % of all of the samples analysed. A total of 98 % of these 418 *H. arctica* ssp. *frigida* observations were made during the cold-water period in the spring during February - May and within the sampling period 1993-2005, it was annually most commonly encountered in April. The species was not observed during late summer and early autumn, and only seldom encountered during late autumn. Although it preferred cold water, covering all of the Baltic sea areas investigated, it was not detected in the water during the winter months of December and January, which could be an artefact caused by infrequent sampling of the water column as well as the sea ice during these winter months (**IV**).

Species are constantly evolving biological units. The Biological Species Concept states they are supposed to be able to interbreed and produce viable offspring (Taylor 1993), which is an often infrequent or completely absent characteristic within the marine microalgae and protists. Their most common mode of proliferation is asexual fission, while sexual recombination is often reserved for special purposes in their complex life cycles (i.e. cyst formation and survival in dinoflagellates). For permanently asexual organisms, only discontinuities in morphological or biochemical characteristics can be used to constitute species boundaries (Hallegraeff 2003). Thus, as Taylor (1993) states: "Looking alike does not necessarily mean genetically identical and looking different does not mean genetically isolated". Therefore, morphospecies as well as genetic evidence can be of limited use for ecological purposes or can easily lead to wrong conclusions.

Usually organisms with identical or almost identical ITS ribosomal (rDNA) sequences are commonly considered to belong to the same species (Montresor et al. 2003). However, Logares et al. (2007) showed that identical ITS rDNA sequences do not necessarily mean the same microbial species. Despite the genetic similarity discovered between *Scrippsiella hangoei* and *Peridinium aciculiferum* Lemmermann 1910, Logares et al. (2007) explained the speciation with different salinities: marine-brackish *S. hangoei* vs. lacustrine *P. aciculiferum*. These species differ in their general appearance as does *H. arctica* ssp. *actica* and *H. arctica* ssp. *frigida*. Thus it is possible that similar diversification is currently taking place in *H. arctica* in the Baltic Sea, as was found in *Scrippsiella hangoei* and *Peridinium aciculiferum* (Logares et al. 2007). Logares et al. (2007) did not discuss the implications that their findings have on the taxonomical nomenclature of the two taxa; neither did they speculate on the possibility of considering them regional variants (= subspecies), instead of different species. While acknowledging the findings of Logares et al. (2007), a more conservative view is well justified in the case of *H. arctica* that has not yet evolved beyond the subspecies state. The two subspecies of *Heterocapsa arctica* have their own separate geographical distributions, that have kept them isolated from each other since the last glaciation approx. 10 000 years ago: *Het-*

erocapsa arctica ssp. *arctica* inhabits the marine waters and sea ice in the Arctic and *Heterocapsa arctica* ssp. *frigida* lives in the brackish water and sea ice of the Baltic Sea.

The fact that *H. arctica* ssp. *frigida* was recognized and counted as a separate unit for decades while it was simultaneously left without taxonomic description reveals how research interest is currently focusing on the occurrence and ecology of the more abundant, usually bloom-forming species. The less abundant species could yet prove to be essential elements in explaining occurrence and abundance in other species and therefore be the key to understanding any species' ecological importance in the food web.

6. CONCLUSIONS

The results show that during winter more abundant microbial communities exist in the sea-ice than in the water beneath the ice. Most of the sea-ice communities encountered contained the photosynthetic pigment chl-*a* and the encountered mismatch between low P-E levels and high chl-*a* concentrations was explained by nutrient limitation. Depleting nutrient values also increased the sea-ice communities' extracellular enzyme production, which indicates their capability for improving their nutritional status to satisfy their nutritional needs via osmotrophy and therefore they do not rely on photosynthesis alone. Therefore, these communities were considered as mixotrophic, even though they could not make a complete shift towards osmotrotrophy and continue their growth using their enzymatic capability alone when photosynthesis was made impossible in prolonged darkness. Darkness caused cessation of growth of the sea-ice communities, which indirectly demonstrated their demand for light. Light also increased enzyme activity, indicating mixotrophy.

Since the number of sympagic organisms encountered increased with age of the ice, it is highly unlikely that the chl-*a* measured would have remained within the sea ice either in single chloroplasts or that the whole cells would have been trapped and stored deep-frozen inside the sea ice and thus maintained their appearance as fresh and alive. Providing some evidence for this activity could help us to compile information and to estimate the importance of sea-ice primary production. That the cultures used (**III**, **IV**) were also isolated from melted sea ice samples provides further evidence that the organisms encountered were alive. On the other hand, dormancy could also be used to explain the low P-E levels encountered (**I**). After all, the new cyst of *Scrippsiella hangoei* also contains chl-*a* (**III**).

Often, as in **I** and **II**, the difficult morphotypes that lack scientific description are only identified as unidentified flagellates or unidentified dinoflagellates, which nevertheless is the correct way to address cells without names. This leaves nutrient and chl-*a* concentrations as the only accurately determined parameters. The changes found in the measured quantities are explained with statistical means without being able to identify the producers responsible or separate them from potential consumers. Or in other cases, a very detailed taxonomic identification is documented without any knowledge of ecology, which would be necessary in recognizing the environmental situations that they prefer, or the ecological situations mostly governed by mixotrophy.

The example of *S. hangoei* in **III** shows that individual species may have very specific ecophysiological strategies to cope with life in the ice. We can expect that many of the different coccoloithophorids, chlorophytes, dinoflagellates, diatoms, euglenophytes, haptophytes, prasinophytes and prymnesiophytes have their ways of surviving and growing in the ice. This emphasizes the importance of species identities in the ice algal assemblages. Treating the algae as a bulk property might not provide the right answers to our questions.

The classification commonly used for unidentified flagellates or even unidentified dinoflagellates, which was also used in **I** and **II,** contains very little information for interlinking the carefully registered ecological information. This information should be produced and linked to as detailed a species-specific level as possible to compile information that could lead towards understanding of the annual occurrence, succession and geographic distribution of species, as exemplified by

Heterocapsa arctica ssp. *frigida* (Paper **IV**). In comparison to grouping by size, identification to species is the only way to ensure that the reason behind the ecological parameter measured has the right name and the conclusions gained a correct meaning.

Genetic evidence alone is not enough to cover the degree of biodiversity that exists in the Baltic Sea, because evidently there are cases when commonly used genetic markers are unable to separate the Baltic taxa from more marine or lacustrine taxa. For example, *Heterocapsa arctica* ssp. *frigida* and *Scrippsiella hangoei* are genetically identical to the marine-arctic *H. arctica* ssp. *arctica* and lacustrine *Peridinium aciculiferum* respectively, but both of them have a unique and easily distinguishable habitus of their own and a more isolated geographical distribution in the brackish Baltic Sea that separates them from the other taxa.

7. ACKNOWLEDGEMENTS

This thesis could not have been produced without loyal financial support from the Walter and Andrée de Nottbeck Foundation. During the years 2003-2005 these studies were partialy funded by the Academy of Finland as part of their Baltic Sea Research Programme BIREME. Tvärminne Zoological Station and the former Finnish Institute of Marine Research provided the necessary office and laboratory infrastructures. Thus the skilled staff and personnel as well as all of the scientists onboard are acknowledged for their help, assistance and enthusiasms towards my work.

The work presented in here was initiated in the mixotrophy research project, led by Riitta Autio, who convinced me that I should give it a try and persuaded me to continue right after I had gained my Master's degree. I want to thank this unique team, because I learned most of the things that I know now and that I am still interested in while doing both field and laboratory experiments together with the other members working in this project: Outi Setälä and Prof. Diane Stoecker.

After my learning period, playing with the open water, I wanted to apply everything to the sea ice. It became possible with the ICEMAT team and I wish to thank the members and collaborators from that era: Jorma Kuparinen, David Thomas, Hermanni Kaartokallio, Elina Leskinen, Mats Granskog, Kristian Spilling, Kimmo Karell, Jens Ehn and Jari Uusikivi. Due to the great enthusiasm shown at that time all ideas appeared to be very easy to do, especially when they existed as thoughts in my head. I have often been frustrated when they did not appear as clear to everyone else. Thus, it must have been difficult to tolerate my scrutiny, first freezing in the ice followed by continuous prolonged days, week after week, that changed to early mornings spent at the lab, together with my closest collegue, Jonna Piiparinen. Thank you, Jonna, for not leaving me alone, for taking care of the sampling gear, many times probably even rescuing them, when I was too excited and thus in a great hurry to continue to squeeze in just one more thing to measure. I also feel the need to thank you and Riitta for not allowing me to get too carried away. Your help also with the calculations, recalculations, corrections, checking, rechecking, statistics, etc. was essential after the heavy-duty fieldwork was done.

In looking back, I was allowed to collect an impressive body of data. Unfortunately, most of it will probably be left unpublished. With these piles of data, I have to accept the fact that ecological data alone are simply not enough to explain the diverse processes necessary before being capable of understanding, e.g. the pelagic productivity measured from sea ice or in the water column and with earlier words of Smetacek et al. (2002) in mind: "at its' best the ecological data could provide hind casts; statistical predictions based on cell abundances and measured concentrations. But, still being unable to accurately identify the key members or separate the producers from consumers. Maintaining ignorance of the interplay of forces (intrinsic or extrinsic, triggers and stops) that drive species life cycles or succession. Leaving a major lack where there should be more comprehension between these two persistently separate categories, those devoted to system-oriented biology and those dealing with properties of organisms (taxonomy, phylogeny, morphology, physiology)". This was just begging for a systematic-ecological attitude; similar to what I was taught at the very beginning of my university studies in botany by Irma Järvinen. Therefore, I simply

had to go in for the identification of species as well. Luckily, I did not have to do it all by myself. Therefore, I sincerely want to thank Heidi Hällfors, Markus Majaneva and Jaanika Blomster, who have become something that I really feel are my own team, where each person has his/her of own brand of special skills combined with great enthusiasm that formed a unique harmony working together like a smoothly oiled engine with expert help from more experienced taxonomists such as Seija and Guy Hällfors, who have shared their passion for species identification, teaching me the very basic skills, such as isolation of cells, and sharing all of their knowledge that has taken a lifetime of dedicated devotion to obtain.

I owe my greatest gratitude to my supervisors: Adjunct professor Riitta Autio and Prof. Harri Kuosa for letting me quite freely try out and test my own ideas, and for their patience. Yet, most importantly, for always believing in me, even when I myself could not and I think no one else did either. Towards the end these hopeless feelings became more frequent. Thank you both, I could not have coped without your support.

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