

Succession of sea-ice bacterial communities in the Baltic Sea fast ice

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Abstract Coastal fast ice and underlying water of the northern Baltic Sea were sampled throughout the entire ice winter from January to late March in 2002 to study the succession of bacterial biomass, secondary production and community structure. Temperature gradient gel electrophoresis (TGGE) and sequencing of TGGE fragments were applied in the community structure analysis. Chlorophyll-*a* and composition of autotrophic and heterotrophic assemblages were also examined. Overall succession of ice organism assemblages consisted of a low-productive stage, the main algal bloom, and a heterotrophic post-bloom situation, as typical for the study area. The most important groups of organisms in ice in terms of biomass were dinoflagellates, plasticidic flagellates, rotifers and ciliates. Ice bacteria showed a specific succession not directly dependent on the overall succession events of ice organisms. Sequenced 16S rDNA fragments were mainly affiliated to α -, β -, and γ -proteobacterial phyla

and *Cytophaga–Flavobacterium–Bacteroides*-group, and related to sequences from cold environments, also from the Baltic Sea. Temporal clustering of the TGGE fingerprints was stronger than spatial, although lower ice and underlying water communities always clustered together, pointing to the importance of ice maturity and ice–water interactions in shaping the bacterial communities.

Keywords Sea ice · Bacteria · Algae · Baltic Sea · 16S rDNA · TGGE · Phylogeny

Introduction

In addition to the Polar seas, seasonal sea-ice cover is formed in some temperate sea areas, such as the White Sea, Ohkotsk Sea and the Baltic Sea (Dieckmann and Hellmer 2003). In the Baltic Sea, seasonal sea-ice is an important feature, with ice covering a mean of 40% of the sea area during winter (Granskog et al. 2006). Ice conditions vary considerably in different parts of the Baltic Sea, ice winter lasting over half a year in the northernmost part of the Baltic Sea, the Bothnian Bay, whereas in the southern Baltic Sea ice appears only during severe winters. Despite the brackish nature of the parent water, Baltic sea-ice is structurally similar and comparable to polar sea-ice (Kawamura et al. 2001; Granskog et al. 2006).

Heterotrophic bacteria are the most abundant group of prokaryotic organisms in polar and Baltic sea-ice and also important with regard to biomass (Lizotte 2003; Kaartokallio 2004; Mock and Thomas 2005). Together with unicellular algae they represent the two major organism groups within the sea-ice, and have also been most intensively studied to date (Mock and Thomas 2005; Granskog et al. 2006). Bacteria have multiple roles in sea-ice food webs in decomposing

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particulate organic matter, producing DOM and recycling autochthonous and allochthonous DOM via the microbial loop. Bacterial secondary production supplies particulate carbon, which subsequently serves as an important food source for several groups of bacterivores in the ice food webs.

Abundance and biomass of sea-ice heterotrophic bacteria varies widely, the highest biomass usually being found in association with high algal biomasses. A rich diversity of bacteria has been found in both Antarctic and Arctic sea-ice (Brinkmeyer et al. 2003), mainly species from α - and γ - subclasses of phylum Proteobacteria and *Cytophaga–Flavobacteria–Bacteroides* (CFB) group (phylum Bacteroidetes) being represented. In addition, phylotypes from β -subclass of phylum Proteobacteria, characteristic for freshwater environments, dominate Arctic ice melt ponds in summer. In general, sea-ice bacterial phylotypes are very similar in both polar areas, which implies the occurrence of similar selection mechanisms in these two geographically separated environments (Brinkmeyer et al. 2003; Mock and Thomas 2005). Two so far published studies containing information on the Baltic Sea ice bacterial diversity give different views. Petri and Imhoff (2001) did not find bacteria related to polar sea-ice bacteria but, interestingly, reported the occurrence of 16S rDNA sequences related to fermenting bacteria and anoxygenic phototrophic purple sulphur bacteria, which implies the occurrence of anoxic microzones in the sea-ice environment. Kaartokallio et al. (2005) found sequences closely related to polar sea-ice bacteria from Baltic Sea ice, belonging to α - and γ -Proteobacteria as well as CFB group. In addition, phylotypes from β -Proteobacteria and Actinobacteria were found. In general, the same bacterial groups seem to be present in Baltic Sea water during summer (Pinhassi and Hagström 2000; Sipura et al. 2005; Tuomainen et al. 2006) and wintertime sea-ice. Evidently, some of the species present in summertime seawater also inhabit sea ice. In a study by Kaartokallio et al. (2005), the sequences related to α -Proteobacteria and Actinobacteria were identical with those found in summertime Baltic Sea water (Sipura et al. 2005) whereas the sequences related to γ -Proteobacteria and Flavobacteria were close to or identical with sequences found from Arctic and Antarctic sea ice and other cold habitats.

Biomass accumulation and succession in sea-ice habitats typically follow the seasonal increase in solar radiation during winter (Cota et al. 1991). The sea-ice organism assemblages in the SW coast of Finland seem to have a succession sequence reminiscent to polar sea ice. The succession begins with the incorporation of organisms into ice upon ice formation, followed by a low-productive winter stage in January–February, and ice algal production maximum in March. In late winter, after the algal production maximum, the importance of heterotrophic processes increases (Kaartokallio 2004; Granskog et al. 2006 and

references therein). Based on the bacterial activity and biomass measurements, it is apparent that sea-ice bacterial communities also undergo a succession during the various phases of ice growth (Grossmann and Dieckmann 1994; Mock et al. 1997; Brierley and Thomas 2002; Kaartokallio 2004). The main selective factors affecting the succession of sea-ice bacterial communities are suggested to be extreme physical conditions, high concentrations of nutrients and available organic substrates, and the presence of copious attachment sites in ice (Brierley and Thomas 2002; Junge et al. 2002). In Antarctic sea-ice, the enrichment of psychrophiles may be initiated early in ice formation along with the establishment of ice algal community, and the number of psychrophilic bacterial taxa is significantly higher in ice containing a diatom assemblage (Bowman et al. 1997). The increased biodiversity of bacteria appears to be linked to a higher algal biomass in sea-ice, as well as to an increasing proportion of CFB clones (Bowman et al. 1997). The ice bacterial communities, even in clear ice, seem to be metabolically more active than communities in the underlying water, and different diatom species dominance in communities may also lead to dissimilar succession of bacterial production (Brown and Bowman 2001). Association to particles is common among the ice bacteria, as over 50% of bacteria are attached to particles or surfaces, such as sediment grains, detritus, ice crystal boundaries and other organisms (Sullivan and Palmisano 1984; Junge et al. 2004). This may be linked to the reported high culturability of sea-ice bacteria (Junge et al. 2002) and forms an important mechanism for survival and growth in subzero temperatures.

Studies revealing actual changes in bacterial community composition at a single sampling location along the ice winter progress are up to now lacking. Here, as first, we present the results of a study on structure and succession of Baltic Sea fast ice bacterial communities in relation to the bacterial activity, biomass and overall development of ice assemblages. As both the sea ice properties and the organism communities in the study area typically change during winter (e.g. Kaartokallio 2004), we wanted to find out the response of sea-ice bacterial communities. For that purpose, separate ice layers were studied at several chosen periods, and also other components of ice biota were included to this work.

Materials and methods

Study site and sampling

The time series dataset were collected during the mild winter of 2002 in semi-enclosed, shallow Santala Bay at a coastal location in SW Finland (Fig. 1). Sampling, including

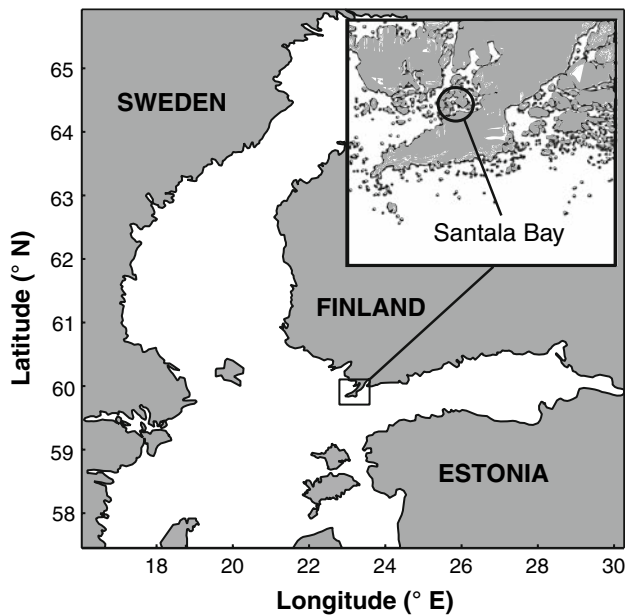


Fig. 1 Northern Baltic Sea with the insert showing the detailed map of study site

ice and under-ice water samples, was performed at 1- to 2-week intervals throughout the entire ice season from January to late March. Sampled sea ice was land-fast level ice with a maximum thickness of 27 cm. Snow cover was generally absent during the study period. Water depth at the sampling site was 6 m. A Kovacs CRREL-type power core auger was used to drill two replicate ice cores at each sampling occasion. Ice temperature was measured from the ice core at 5-cm intervals immediately after drilling, using a Testo 110 electrical thermometer. Cores were cut into 5-cm sections with a handsaw and placed in carefully washed plastic containers. To avoid contamination, ice cores were handled with gloves. Water samples were obtained from immediately under ice using a 1-L Ruttner-type water sampler employed through a borehole. All samples were kept in dark and cold until analyses. One of the ice cores was immediately sub-sampled in the laboratory for bacterial secondary production measurements and then thawed along with the replicate core in a cold room at +5°C for 24–38 h. All the ice samples were melted without the addition of filtered seawater. Due to generally low salinity difference (<7 psu) between Baltic Sea brines and melted ice sample, direct melting has been shown to be suitable method in biological Baltic Sea ice studies (Kaartokallio 2004).

Salinity and chlorophyll-*a*

Salinity was measured from all samples (thawed ice core sections and water) using an YSI 63 temperature-conductivity-pH meter, calibrated with YSI standard solutions. For determination of Chl-*a*, 50 mL aliquots of sample water

(thawed ice or water) were filtered through 25-mm Whatman GF/F filters. The filters were placed in 10 mL 96% v/v ethanol and Chl-*a* was extracted at room temperature in dark for 24 h. The extract was filtered through a GF/F filter and fluorescence was measured with a Shimadzu RFPC 5001 fluorometer, calibrated with pure Chl-*a*. The Chl-*a* concentrations were calculated according to HELCOM (1988).

Abundance of ice organisms

For bacterial and flagellate abundance measurement, subsamples of 20 mL were taken from the thawed ice and water samples and fixed with 25% electron microscopy grade glutaraldehyde (Sigma, final concentration 1%). Prior to counting, 5–10 mL of each sample was filtered onto a black 0.2- μm polycarbonate filter (Osmonics) and stained with 0.015% acridine orange solution for bacteria and 0.030% proflavine solution for flagellates. Total bacterial numbers (TBN) and flagellate abundance were counted using a Leitz Aristoplan epifluorescence microscope equipped with I3 and M3 filters and PL Fluotar 100 \times 12.5/20 oil immersion objective. The TBN was calculated from at least 200 cells recorded in a minimum of 20 fields in a New Porton E11 counting grid. The abundance of ice algae, ciliates and metazoans (rotifers, copepods) present in ice were counted from acid Lugol-fixed 50 mL samples by inverted light microscopy using a Leica inverted microscope and the Utermöhl counting technique (Utermöhl 1958). For counting, 50 mL of sample water was settled >12 h and counted with phase contrast illumination using 125–500 \times magnifications.

Bacterial secondary production

For bacterial production measurement, samples containing known amount of ice crush and concentrated seawater (Kaartokallio 2004) were prepared as follows. Each intact 5–10 cm ice core section was crushed using a spike and electrical ice cube crusher. Approximately 10 mL of crushed ice was weighed in a scintillation vial. To better simulate brine pocket salinity and ensure even distribution of labelled substrate, 2–4 mL of 2 \times concentrated (by evaporation), filtered (through 0.2 μm) seawater from sampling area was added to the scintillation vials. All the work was done in a cold room at +5°C.

Bacterial production was measured immediately after sample collection using the ^{14}C -leucine (Kirchman et al. 1985) and ^3H -thymidine (Fuhrman and Azam 1980, 1982) incorporation methods and dual labelling. Two aliquots and a formaldehyde-killed absorption blank were amended with L-[U- ^{14}C] leucine (Amersham, UK, sp. act. 307 mCi mmol^{-1}) diluted with carrier leucine (1:5) and [methyl- ^3H] thymidine (NEN, USA, specific activity 84.3 Ci mmol^{-1}).

The used concentrations, 14 nM for thymidine and 1,100 nM (ice samples) and 400 nM (water samples) for leucine, were tested to be above the saturating concentration. Samples were incubated in the dark at -0.2°C in a cooled incubator (LMS 205, LMS, UK) for 5–14 h, the incubation was stopped with addition of formaldehyde and samples were processed using the standard cold-TCA extraction procedure. A Wallac WinSpectral 1414 counter and InstaGel (Perkin-Elmer) cocktail were used in scintillation counting. Average TTI-based community turnover times for bacteria were calculated using conversion factor of 1.1×10^9 cells nmol^{-1} (Riemann and Bjørnsen 1987).

Molecular analysis of the microbial community structure

For community structure analysis, ice associated microbes were collected from the original melted ice samples on 23 January, 26 February, and 18 March, by filtering the maximum possible amount of sample water (220–700 mL) onto a 47 mm diameter Supor 200 polyethersulfone filter (Pall Corp.; East Hills, NY, USA). The filters were stored in a lysis buffer consisting of 40 mM EDTA, 400 mM NaCl, 750 mM sucrose and 50 mM Tris HCl (pH 8.3) at -80°C .

Total DNA was extracted with a modified method by González et al. (1996). The frozen membrane was cut to small pieces on a cold (-20°C), sterile surface, and the pieces were transferred to 2 mL of STE-buffer (10 mM Tris, 1 mM EDTA, 100 mM NaCl, pH 8.0). Lysozyme was added to a final concentration of 1 mg mL^{-1} , and the sample was incubated 30 min at $+37^{\circ}\text{C}$. SDS and proteinase K were added to final concentrations of 0.5% and $150 \mu\text{g mL}^{-1}$, respectively. The sample was boiled for 1 min, cooled to room temperature, and extracted sequentially with phenol (pH 8.0), phenol–chloroform–isoamyl alcohol (25:24:1, v/v/v) and chloroform–isoamyl alcohol (24:1, v/v). DNA was precipitated from the supernatant with isopropanol ($>2 \text{ h}$ at -20°C), washed with 70% ethanol, and dissolved in TE-buffer (10 mM Tris, 1 mM EDTA, pH 8.0).

For the TGGE analysis, 50 ng of total DNA was amplified with primers GM5F (see Muyzer et al. 1993 for the primer and Santegoeds et al. 1998 for GC-clamp sequence) and Cy5TM-labelled primer 518R (Muyzer et al. 1993; Sigma-Genosys, Sigma-Aldrich, Suffolk, UK). Amplification was performed in a 50 μL reaction mix containing $1 \times$ DyNAzyme buffer (Finnzymes, Espoo, Finland), 20 pmol of each primer, 0.2 mM of each dNTP, 3 mM MgCl_2 , 100 ng μL^{-1} BSA (bovine serum albumin), and 1.2 U DyNAzyme II polymerase (Finnzymes). A touchdown program (one cycle of 5 min at $+95^{\circ}\text{C}$, 1 min at $+65^{\circ}\text{C}$, 3 min at $+72^{\circ}\text{C}$; 19 cycles of 1 min at $+95^{\circ}\text{C}$, 1 min at $+64^{\circ}\text{C}$, 3 min at $+72^{\circ}\text{C}$, with 0.5°C -reduction of annealing temperature in each step; 10 cycles of 1 min at $+95^{\circ}\text{C}$, 1 min at $+55^{\circ}\text{C}$, 3 min at $+72^{\circ}\text{C}$, with 10 min final extension) in

PTC-200 Thermal Cycler (MJ Research, Waltham, MA, USA) was used. PCR products were separated in a TGGE gel (8% acrylamide, 8 M urea, 20% formamide and 0.1% glycerol) using a TGGE System apparatus (Biometra, Goettingen, Germany) with $1 \times$ TAE running buffer, and $+37^{\circ}\text{C}$ to $+56^{\circ}\text{C}$ thermal gradient. TGGE gels were scanned with a STORM[®] imager (Molecular Dynamics, Sunnyvale, CA, USA).

TGGE-data analysis

The TGGE community fingerprint patterns were analyzed with BioNumerics program (Version 4.0, Applied Maths BVBA, St. Martens-Latem, Belgium). Gel was normalised and clustering of the sample communities was calculated with Ward method, using Pearson coefficient, from the curve data deduced from the genetic population fingerprints. The reliability of the dendrogram was tested by calculating the cophenetic correlations of the nodes, with the tools available in the BioNumerics program package. Discriminative analysis of TGGE profiles with pre-defined groups (sampling date or ice layer) was performed with BioNumerics.

Sequencing and phylogenetic analysis

Fragments were cut from the TGGE gel with a sterile scalpel, put in 100 μL of sterile water and incubated for 10 min in room temperature, heated for 12 min at $+98^{\circ}\text{C}$, and centrifuged with maximum speed for 2 min in room temperature. Sodium acetate (final concentration 300 mM), glycogen carrier (final concentration $100 \mu\text{g mL}^{-1}$), $4.5 \times$ volume ethanol was added to the supernatant, and DNA was precipitated for 30 min at -80°C . After centrifugation (10 min, 12,000g at $+4^{\circ}\text{C}$), the DNA was dissolved in 10 μL of sterile PCR-grade water. The extracted fragments (4 μL) were amplified with primers otherwise identical to the ones used in the PCR for TGGE analysis, but lacking the GC-clamp and the Cy-5 label. The amplicons were cloned for sequencing with TOPO TA cloning kit (Invitrogen corp, Carlsbad, CA), grown in TOP10 *E. coli* (Invitrogen) and purified with QIAprep Spin Miniprep Kit (QIAGEN, Hilden, Germany). Cloned fragments were sequenced with ALF DNA sequencer (Amersham Pharmacia Biotech, Uppsala, Sweden) with Thermo Sequenase fluorescent labelled primer cycle sequencing kit with 7-deaza-dGTP (Amersham Pharmacia Biotech, Piscataway, NJ; Murray 1989; Sanger et al. 1977; Tabor and Richardson 1995), using T3 and T7 sequencing primers. The vector and primer areas were cleaved from the Baltic Sea ice 16s rDNA fragments with the tools of the EMBOSS package (Rice et al. 2000), and the sequences were deposited to the EMBL database under accession

numbers AM062742–AM062760. Sequences were compared with the EMBL database sequence data with BLAST (<http://www.ncbi.nlm.nih.gov/BLAST/>). Phylogenetic analyses were done with PHYLIP software package (version 3.63; Felsenstein 1993) using Fitch–Margoliash method, and the bootstrap values were determined using 500 replicates. The phylogenetic tree was drawn with the Treeview program (version 1.6.6; Page 1996).

Results

Ice physical environment

Winter 2002 at our study site was mild, characterized by extended periods of warm weather with air temperatures exceeding 0°C. Due to high temperatures, ice reached a maximum thickness of only 27 cm. Ice formation was quick in early January, and ice devoid of snow cover was already 22 cm thick at the first sampling date. Ice bulk salinity varied from 0.1 to 0.6 psu and underlying water salinity from 1.3–3.2 psu. Salinity of underlying water was lower than normal salinity of seawater in the study area (5–6 psu), most likely reflecting the effect of warm weather-induced freshwater input from ice melting. Snow cover thickness and air temperature during sampling occasions varied between 0 and 5 cm and –4.8 and +5.0°C, respectively.

Chlorophyll-*a*, autotrophic and heterotrophic organisms

Simultaneously with rapid growth of ice, a well-developed algal community arose throughout the whole ice column with chlorophyll values up to 30 $\mu\text{g L}^{-1}$ in the lower layer (Fig. 2). The bulk of algal biomass (about 80%) was composed by an autotrophic dinoflagellate, *Scrippsiella hangoei*, reaching abundances up to 60,000 cells L^{-1} . In early February, the ice got snow cover, which, together with the short midwinter daylength, inhibited algal growth and led to lowered chlorophyll-*a* concentrations in both upper and lower ice (Fig. 2). However, the dinoflagellate *S. hangoei* remained a major element with abundances up to 10,000 cells L^{-1} , with a considerable share of small, unidentified plasticid flagellates (1,000–6,000 cells mL^{-1}). In late February, simultaneously with increasing solar radiation, the snow melted, and another algal growth burst took place in the lower ice layer with chlorophyll-*a* values up to over 30 $\mu\text{g L}^{-1}$, again by *S. hangoei* (30,000 cells L^{-1}). The algal community changed in March into one dominated by a green alga, *Dictyosphaerium* sp. (up to 300,000 cells L^{-1}), and small plasticid flagellates (about 1,500 cells mL^{-1}). Chlorophyll-*a* concentrations in melted ice samples generally exceeded the

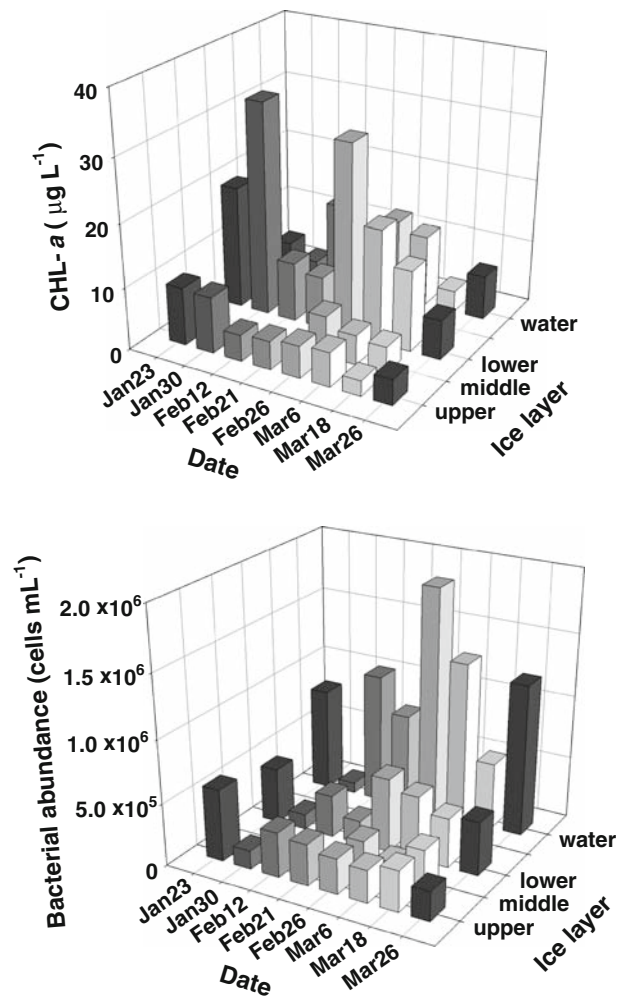


Fig. 2 Chlorophyll-*a* concentration and abundance of heterotrophic bacteria in sea ice and underlying water

concentrations in under-ice water, but interestingly, *S. hangoei* showed reverse succession in water with the highest abundance during the low chlorophyll period in the ice in February.

Heterotrophic flagellates were always numerous in the ice with numbers ranging from 500 to 4,000 mL^{-1} , without clear succession. The composition of other ice heterotrophic organism assemblages was rather similar with high abundances only at the lowermost layer with the exception of the first sampling. Rotifers, mainly of genus *Synchaeta*, dominated the heterotrophic component (abundances up to 160 individuals L^{-1}) together with small (10–30 μm) ciliates (3,000–8,000 cells L^{-1}). *Synchaeta* sp. was abundant also in January in the upper layer of the ice. *Synchaeta* sp. was present at water under the ice during the whole winter. Ciliate abundances in water were low in January, but reached numbers comparable to those of lower ice layers (4,000–7,000 cells L^{-1}) in February and March.

Bacterial productivity and abundance

Total leucine incorporation (TLI), reflecting bacterial protein synthesis, was initially low just after ice formation, increasing until the mid-February and reaching its highest values following the ice algal growth burst in late March (Fig. 3). Total thymidine incorporation (TTI), a proxy for bacterial cell production, did not increase from initial values by February but showed elevated values in March, especially in the lower ice layer after the algal growth maximum. In under-ice water both TLI and TTI were highly variable, typically exceeding TLI and TTI in ice. Except the low TTI values in January and February, TTI and TLI did generally follow the same temporal patterns in ice and under-ice water, although values were higher in water. TTI/TLI ratio (Fig. 3) was elevated after ice formation, then lower in January and February and increased again during and after the ice algal bloom in late March and early February. In contrast to the TLI and TTI values, TLI/TTI ratios were similar in different ice layers and underlying water. Average community bacterial turnover times calculated from TTI-based cell production values and TBN were 5.8 days on 23 January and 2.0, 4.1 and 2.8 days in 30 January, 12 February and 21 February, respectively. Bacterial abundance (Fig. 2) remained rather stable over the season, with only slightly elevated abundance associated with the initial incorporation into ice in January and ice algal bloom in lower ice in March. Bacterial abundance in under-ice water always exceeded the abundance in melted ice. Compared to vertical variations of Chlorophyll-*a* in the ice column, both bacterial abundance and production parameters were more evenly distributed in the ice column.

Analysis of the microbial community structure

The TGGE-fingerprints included disappearing and appearing, as well as “constant” bands (Fig. 4). The fingerprint pattern varied in each sampling occasion, reflecting differences in the microbial community structures. In cluster analysis, the January communities clustered separately, as did the March sample communities. The February communities were separated between January and March clusters, the middle and uppermost ice layer communities resembling more the January communities, whereas the February lowest ice layer and water communities clustered with the March communities (Fig. 4). The temporal and spatial grouping of the communities was further tested by discriminative analyses, where the differentiation by sampling time was stronger than by position in the ice (Fig. 5a, b, respectively).

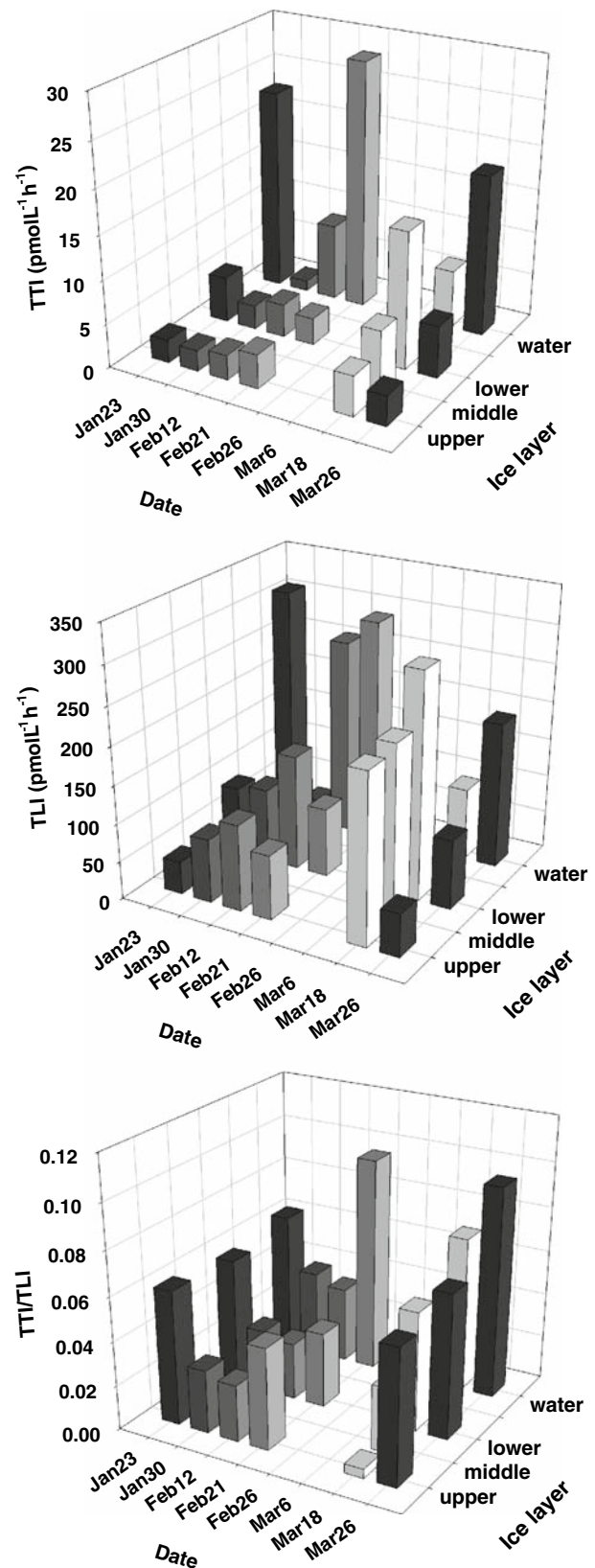


Fig. 3 Total thymidine incorporation (TTI), total leucine incorporation (TLI) and TTI/TLI ratio in sea ice and underlying water

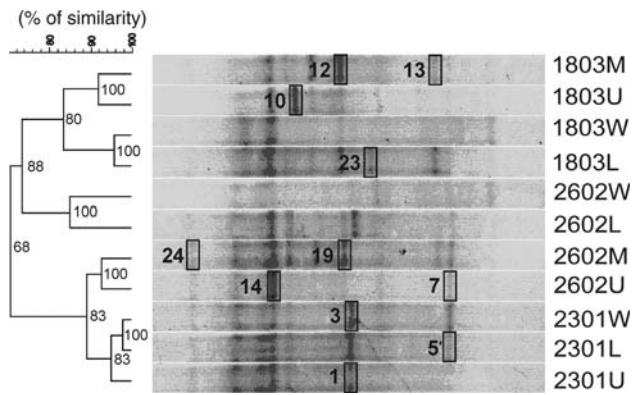


Fig. 4 Cluster analysis of the Baltic Sea ice TGGE-profiles calculated with Ward method, using the Pearson coefficient. Cophenetic correlation values are given in the nodes. The scale-bar represents the similarity percentage between community profiles. Samples are coded by sampling date, and the position in the ice (*U* upper, *M* middle, *L* lowest section) or in water below the ice (*W*)

Sequence analysis

The extracted TGGE fragments were 168–194 bp long. Comparison with EMBL database revealed the closest relative sequences to be mainly from Arctic or otherwise cold environments, also from the Baltic Sea (Table 1; Fig. 6). Some Baltic Sea ice 16S rDNA fragments had low similarity to EMBL sequences, but perfect matches were found as well (Table 1). The Baltic Sea ice sequences were affiliated to α -, β -, and γ -proteobacterial phyla as well as to the Cyanobacteria and *Cytophaga–Flavobacterium–Bacteroides* (CFB)-group, β -proteobacterial and CFB associations being most abundant (Fig. 6). The similarity values within the Baltic Sea ice sequence group varied between 61 and 98%. α -Proteobacterial associations were found among January and February ice-derived sequences, whereas CFB-group-related sequences were derived from February to March ice. β -Proteobacterial associations were found among sequences from all the three sampling months. The only sequences related with γ -proteobacteria and Cyanobacteria-associated cluster originated from the March ice samples, and January ice samples produced the only α -proteobacterial SAR11 cluster-associated sequence (Fig. 6). There was a division of taxonomic affiliations also in relation to source ice layer, as CFB-group-related sequences were located in the upper and middle ice layers, whereas no α -proteobacteria-associated sequences could be found from the uppermost ice layer. β -Proteobacteria-related sequences appeared in all the ice layers as well as in underlying water (Fig. 6).

Discussion

The overall succession of ice organism assemblages largely followed a sequence described previously for a similar winter

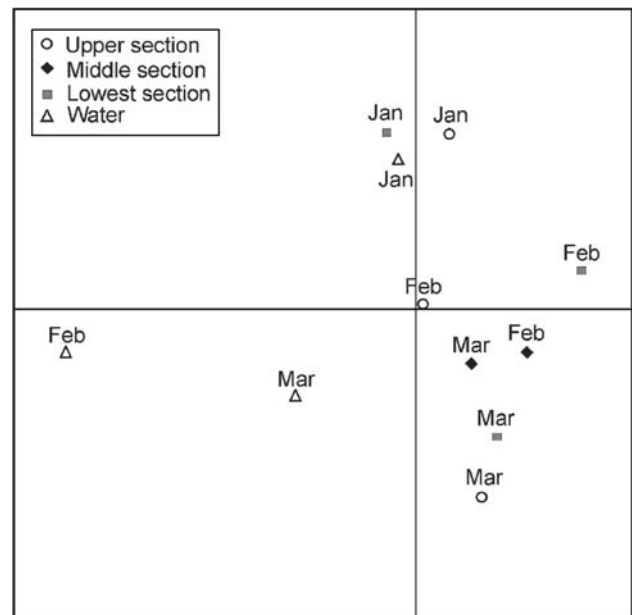
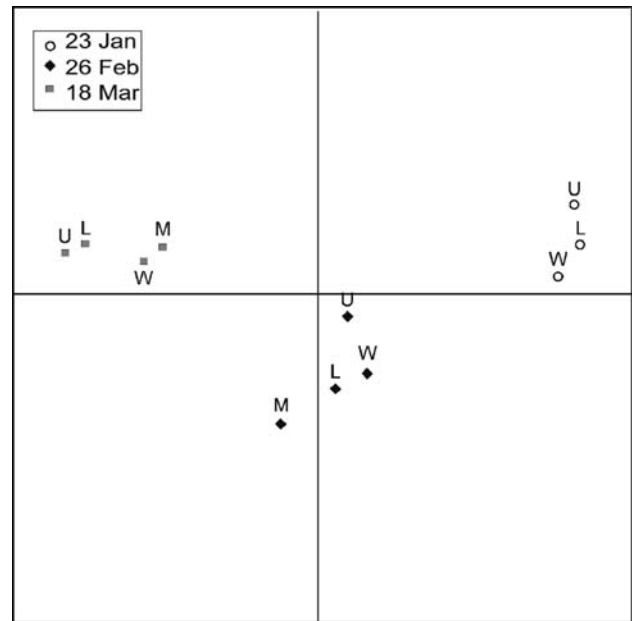


Fig. 5 Discriminative analysis of TGGE-fingerprints of the Baltic Sea ice and water associated microbial communities using predefined grouping. **a** Grouping according to sampling time (January, February, March); **b** Grouping according to sample position in the ice (*U* upper, *M* middle, *L* lowest section), or in water below the ice (*W*)

in the study area (Kaartokallio 2004), with a small ice algal bloom—this time consisting mainly of dinoflagellates, and not diatoms—in January, followed by a low-productive stage, the main ice algal bloom in late February–early March, and a more heterotrophic post-bloom situation in late March. The amount of ice algal biomass was highest in the lower ice layers, while heterotrophic bacteria and their activity were more equally distributed in the ice column. Dinoflagellates are able to swim from under-ice water to the lower layers of

Table 1 Characterization of the sequences from the Baltic Sea ice TGGE community fingerprint analysis

Fragment ID	Closest relative sequences			Similarity (%)
	EMBL #	Source	Taxonomic affiliation	
2301U1 = 2301W3	AY989054	Uncultured soil bacterium clone L1A.7E01, Alaska, USA	β -Proteobacteria	97
2301W3 ^a	AF494503	Uncultured <i>Sphingomonadaceae</i> bacterium clone GOBB3-B04-14-6, Baltic Sea estuary	α -Proteobacteria	97
2301L5 = 2602U7, 2602M24, 1803M13 ^b	AJ319679	Uncultured bacterium clone FBR-E, Polyphenol degrading bioreactor, Kärkölä, Finland	–	96
	EF103212	<i>Comamonadaceae</i> bacterium MSCB-15, mountain snow, Western China	β -Proteobacteria	96
2602U7 ^a	AY771760	Arctic <i>Flavobacterium degerlachei</i> clone SE47	CFB group	100
2602U14	AM168011	<i>Flavobacterium</i> sp. VAS37, Baltic Sea marine sediment	CFB group	100
2602M19 = 1803M12	DQ512791	<i>Flavobacterium</i> sp. PDD-14b-7, tropospheric cloud water (1465m asl)	CFB group	97
1803M12 ^a	AF468425	Arctic sea ice bacterium ARK10164, Arctic sea ice-melt pond	CFB group	96
2602M19 ^a	AY771761	<i>Loktanella salsilacus</i> clone SE49, Arctic environment	α -Proteobacteria	95
2602M24	EF215729	Uncultured <i>Rhodobacterales</i> bacterium clone GL2-04, Qingdao coast, China	α -Proteobacteria	97
1803U10 ^b	AB242910	<i>Escherichia coli</i> strain JCM1649	γ -Proteobacteria	96
	DQ007435	<i>Flavobacterium</i> sp. BSi20510, Arctic sea ice	CFB group	98
	DQ187866	Uncultured γ -proteobacterium clone 45, 100 m deep seawater, Taiwan	γ -Proteobacteria	95
1803M12 ^a	AY429700	<i>Flexibacteraceae</i> bacterium P3, TCE and cis-DCE contaminated groundwater	CFB group	98
1803M13 ^b	AY168755	<i>Hydrogenophaga</i> sp. YED6-4, arsenite-oxidizing biofilm	β -Proteobacteria	96
1803L23	AM040908	Uncultured bacterium bandDNA14-2, Lake Loclat, Neuchatel, Switzerland	Eubacteria (Cyanobacteria)	90

The most closely related sequences from the EMBL database with the sequence similarities are given. The fragment ID refers to the numbering used in Fig. 1, with the sample code in front of the fragment number. Similarities given cover the whole fragment area

^a Co-migrating fragment

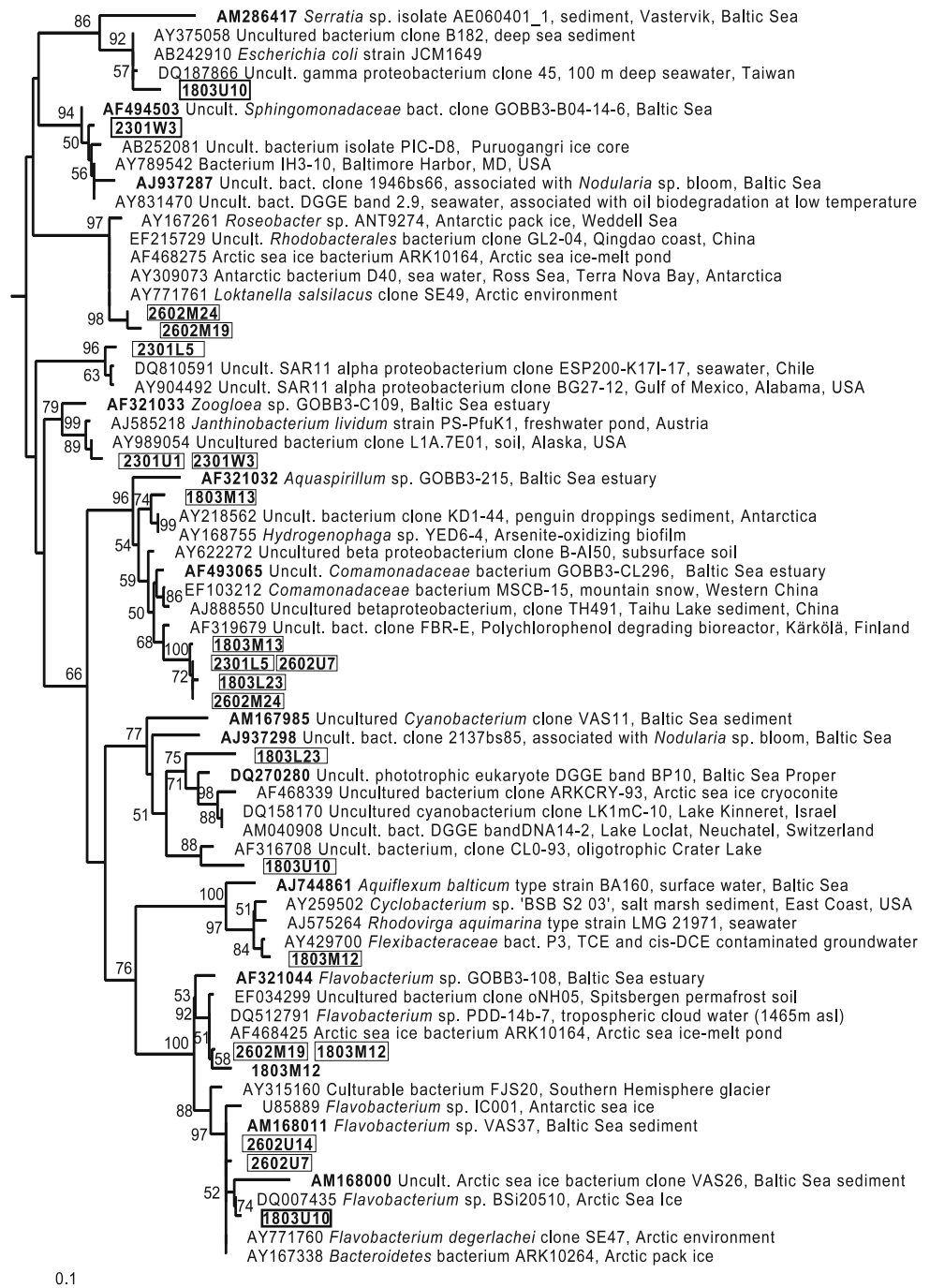
^b All sequences associated with the fragment were different

the ice, and back, which may explain the rapid concentration and disappearance of ice biomass. The dominating algal groups during the last sampling period were coccoid green algae (Order Chlorococcales, attributed to the genus *Dictyosphaerium*) and small flagellates. Above mentioned green algae are not capable to move, which may have led to effects by increasing irradiation. The three sampling occasions for bacterial community structure study were thus located in different phases of the overall development of ice organism assemblages; 23 January representing the initial situation shortly after ice formation, 26 February the ice algal bloom and 18 March the heterotrophic post-bloom situation.

Ice bacteria were initially abundant, but inactive in comparison to under-ice water as seen in TLI and TTI. The high TTI/TLI ratio in the first sampling occasion on 23 January

points to actively dividing cells, indicating that bacterial assemblage incorporated recently from seawater was changing and adapting to life in ice. High bacterial abundance and low TLI values soon after ice formation have been reported also earlier by Kaartokallio (2004). TLI increased in late January, followed by an increase in abundance in early February. Relatively short community turnover times, stable bacterial abundance, as well as discrepancies between bacterial abundance and TLI, point to efficient grazing on ice bacteria (see also Kaartokallio 2004). Meltwater flushing of the ice matrix, initiated by warm weather, may partly explain the similarity of communities in the lower ice layer and the underlying water, as bacteria can move along with brine/melt-water movements at the ice water interface.

Fig. 6 A Fitch-Margoliash tree of Baltic Sea ice-derived 16S rDNA fragments (primers omitted), showing the relationships with the closest reference sequences, and the most related Baltic Sea-associated 16S rDNA sequences from the EMBL database. The coding of the Baltic Sea ice-derived sequences (in boxes) refers to the fragment ID used in Table 1. All Baltic Sea sequences are in *boldface*. The bootstrap values (500 replicas) over 50% are given in the nodes. *Methanosarcina baltica* 16S rDNA sequence (EMBL accession no. AJ238648) was used as outgroup



The sequenced 16S rDNA fragments here were unfortunately too short to enable species level identification of the Baltic Sea ice associated bacteria, but group level affiliations can, nevertheless, be drawn. Majority of the most related EMBL entries were of Arctic origin or from other cold, extreme environments, such as Alaskan soil, mountain snow or stratospheric clouds. This suggests that despite generally higher temperatures and lower salinities of the Baltic Sea-ice internal brine channel environment compared to Polar sea ice, extremophilic adaptations are needed

also in Baltic Sea ice. Sequences related to all four groups typically encountered in sea-ice environment (α -, β -, γ -Proteobacteria and CFB-group) were found, and the majority of sequenced fragments clustered with β -proteobacterial and CFB-group reference sequences (Fig. 6). β -Proteobacterial assemblage has been shown to be important in terms of abundance as well as biomass production in a marine estuary with freshwater influence (Cottrell and Kirchman 2003). The CFB bacteria have been suggested to possess specific capabilities enabling their prosperity in cold (Junge

et al. 2002) and their fraction of total Arctic sea ice bacteria has been shown to grow with decreasing temperature and increasing salinity (Junge et al. 2004). Most of the CFB-related 16S rDNA sequences in this study had highest similarities (97–100%) with *Flavobacterium* sp. (Table 1, Fig. 6), indicating the abundance and possible ecological importance of these bacteria in Baltic Sea ice.

Our results differ from the Baltic Sea ice associated bacterial assemblage from Kiel Bight, described by Petri and Imhoff (2001), but are in line with the findings of Kaartokallio et al. (2005) conducted in the same study area as this study. The disagreement with the results by Petri and Imhoff (2001) may be due to geographical differences between SW coast of Finland and Kiel Bight. Especially the sporadic sea-ice occurrence in the Southern Baltic Sea (Kiel Bight) could be one explanation, potentially affecting the presence of ice-related psychrophiles in the parent water bacterial populations. In the study by Kaartokallio et al. (2005), the majority of Baltic Sea ice-derived 16S rDNA sequences were related to γ - and α -proteobacteria and CFB-group, but β -proteobacterial sequences were found as well. The sequences gained within this study could unfortunately not be compared with these Baltic Sea ice sequences (Kaartokallio et al. 2005), as the target 16S rDNA areas did not overlap. The value suggested to be the threshold for two 16S rDNA sequences to originate from the same species is 97% (Stackebrandt and Goebel 1994). Thus the low similarities of the Baltic Sea ice derived sequences to 16S rRNA reference gene sequences (Table 1) suggests the presence of possibly novel bacterial species. However, as many of the 16S rRNA entries in EMBL are only partial, these “unknown” Baltic Sea ice 16S rDNA sequences may also originate from the area outside of these partial EMBL entries.

The Baltic Sea ice bacterial communities evolved throughout the sampling period, as indicated by the changes in the community fingerprint patterns. We interpret the tight clustering in January (Fig. 4) to reflect the recent incorporation of bacterial communities originating from parent water into the ice. In all the three sampling occasions, the lower ice and under-ice water communities clustered together (Fig. 4), pointing to an active exchange between the two environments and to the importance of brine transport processes at ice-water interface, in addition to the exchange of matter (Granskog et al. 2006), as community shaping factors. According to the discriminative analyses, temporal differentiation of Baltic Sea ice associated microbial communities was stronger than spatial differentiation, which underlines the importance of ice maturity (including algal succession) as a community shaping factor.

The optimization of either PCR (e.g., using different primer combination) or TGGE conditions did not improve

the separation efficiency of the TGGE analysis, or increase the number of fragments in this study. Co-migration of the TGGE-fragments reduces the number of bands and thus has an effect on the community fingerprint patterns. However, despite the potential biases of this methodology (e.g., Crosby and Criddle 2003; Kisand and Wikner 2003; Sekiguchi et al. 2001), also recognized here, molecular fingerprinting is a useful tool for studying spatial or temporal differences in microbial population structures. The primer 518R used, is not purely specific to prokaryotic DNA (Lopez et al. 2003) and one of the sequenced TGGE fragments here (1803L23) had resemblance to a sequence of eukaryotic origin (DQ270280, Fig. 6). The presence of eukaryotic DNA in the samples may employ a fraction of the primers otherwise available for annealing to prokaryotic targets, and thus limit the detection range of bacteria (Lopez et al. 2003). This should be kept in mind when interpreting the results of the community analysis, but as all samples were treated similarly, an equal “masking” effect was assumed for all samples.

In conclusion, specific, stable and well-adapted bacterial community developed in the studied Baltic Sea fast ice environment. The results show that the Baltic Sea fast ice bacterial community undergoes a succession sequence during the ice winter that is not directly dependent on overall succession of ice organism assemblages but has its own specific characteristics. Based on our limited dataset, we suggest the parent water bacterial community structure, the exchange processes at ice–water interface, the maturity of ice as well as the association to ice algal communities (providing substrate) to be the main factors affecting the development and diversity of Baltic Sea ice bacterial communities, which is also consistent with earlier studies in polar sea-ice. To verify these findings and to study the physical and both bottom–up and top–down food web controls of ice bacterial community structure and succession in more detail, further experimental and explorative studies will be needed.

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References

- Bowman JP, McCammon SA, Brown MV, Nichols DS, McMeekin TA (1997) Diversity and association of psychrophilic bacteria in Antarctic sea ice. *Appl Environ Microbiol* 63:3068–3078
- Brierley AS, Thomas DN (2002) Ecology of southern ocean pack ice. *Adv Mar Biol* 43:171–276

- Brinkmeyer R, Knittel K, Jürgens J, Weyland H, Amann R, Helmke E (2003) Diversity and structure of bacterial communities in Arctic versus Antarctic pack ice. *Appl Environ Microbiol* 69:6610–6619
- Brown MV, Bowman JP (2001) A molecular phylogenetic survey of sea-ice microbial communities (SIMCO). *FEMS Microbiol Ecol* 35:267–275
- Cota GF, Legendre L, Gosselin M, Ingram RG (1991) Ecology of bottom ice algae: I. Environmental controls and variability. *J Mar Syst* 2:257–277
- Cottrell MT, Kirchman DL (2003) Contribution of major bacterial groups to bacterial biomass production (thymidine and leucine incorporation) in the Delaware estuary. *Limnol Oceanogr* 48:168–178
- Crosby LD, Criddle CS (2003) Understanding bias in microbial community analysis techniques due to *rrn* operon copy number heterogeneity. *Biotechniques* 34:790–802
- Dieckmann GS, Hellmer HH (2003) The importance of sea ice: an overview. In: Thomas DN, Dieckmann GS (eds) *Sea ice: an introduction to its physics, chemistry, biology and geology*. Blackwell Science, Oxford, pp 1–21
- Felsenstein J (1993) PHYLIP (phylogeny inference package). Version 3.5c. Department of Genetics, University of Washington, Seattle
- Fuhrman JA, Azam F (1980) Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. *Appl Environ Microbiol* 39:1085–1095
- Fuhrman JA, Azam F (1982) Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. *Mar Biol* 66:109–120
- Gonzalez JM, Whitman WB, Hodson RE, Moran MA (1996) Identifying numerically abundant culturable bacteria from complex communities: an example from a lignin enrichment culture. *Appl Environ Microbiol* 62:4433–4440
- Granskog M, Kaartokallio H, Kuosa H, Thomas DN, Vainio J (2006) Sea ice in the Baltic Sea—a review. *Estuar Coast Shelf Sci* 70:145–160
- Grossmann S, Dieckmann G (1994) Bacterial standing stock, activity and carbon production during formation and growth of sea ice in the Weddell Sea, Antarctica. *Appl Environ Microbiol* 60:2746–2753
- HELCOM (1988) Guidelines for the Baltic monitoring programme for the third stage; Part D. Biological Determinands, Report no. 27D
- Junge K, Imhoff F, Staley K, Deming W (2002) Phylogenetic diversity of numerically important Arctic sea-ice bacteria cultured at sub-zero temperature. *Microb Ecol* 43:315–328
- Junge K, Eicken H, Deming JW (2004) Bacterial activity at -2 to -20°C in Arctic wintertime sea ice. *Appl Environ Microbiol* 70:550–557
- Kaartokallio H (2004) Food web components, and physical and chemical properties of Baltic Sea ice. *Mar Ecol Prog Ser* 273:49–63
- Kaartokallio H, Laamanen M, Sivonen K (2005) Responses of Baltic sea ice and open-water natural bacterial communities to salinity change. *Appl Environ Microbiol* 71:4364–4371
- Kawamura T, Shirasawa K, Ishikawa N, Lindfors A, Rasmus K, Granskog MA, Ehn J, Leppäranta M, Martma T, Vaikmäe R (2001) Time-series observations of the structure and properties of brackish ice in the Gulf of Finland. *Ann Glaciol* 33:1–4
- Kirchman D, K'nees E, Hodson R (1985) Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. *Appl Environ Microbiol* 49:599–607
- Kisand V, Wikner J (2003) Limited resolution of 16S rDNA DGGE caused by melting properties and closely related DNA sequences. *J Microbiol Methods* 54:183–191
- Lizotte MP (2003) The microbiology of sea ice. In: Thomas DN, Dieckmann GS (eds) *Sea ice: an introduction to its physics, chemistry, biology and geology*. Blackwell Science, Oxford, pp 184–210
- Lopez I, Ruiz-Larrea F, Cocolin L, Orr E, Phister T, Marshall M, VanderGheynst J, Mills DA (2003) Design and evaluation of PCR primers for analysis of bacterial populations in wine by denaturing gradient gel electrophoresis. *Appl Environ Microbiol* 69:6801–6807
- Mock T, Thomas DN (2005) Recent advances in sea-ice microbiology. *Environ Microbiol* 7:605–619
- Mock T, Meiners KM, Giesenhausen HC (1997) Bacteria in sea ice and underlying brackish water at $54^{\circ}26'50''\text{N}$ (Baltic Sea, Kiel Bight). *Mar Ecol Prog Ser* 158:23–40
- Murray V (1989) Improved double-stranded sequencing using the linear polymerase chain reaction. *Nucleic Acids Res* 17:8889
- Muyzer G, de Vaal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl Environ Microbiol* 59:695–700
- Page RDM (1996) TREEVIEW: An application to display phylogenetic trees on personal computers. *Comput Appl Biosci* 12:357–358
- Petri R, Imhoff JF (2001) Genetic analysis of sea-ice bacterial communities of the Western Baltic Sea using an improved double gradient method. *Polar Biol* 24:252–257
- Pinhassi J, Hagström Å (2000) Seasonal succession in marine bacterioplankton. *Aquat Microb Ecol* 21:245–256
- Rice P, Longden I, Bleasby A (2000) EMBOSS: the European molecular biology open software suite. *Trends Genet* 16:276–277
- Riemann B, Bjørnsen PK (1987) Advances in estimating bacterial biomass and growth in aquatic systems. *Arch Hydrobiol (Beih Ergebn Limnol)* 118:385–402
- Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain terminating inhibitors. *Proc Natl Acad Sci USA* 74:5463–5467
- Santegoeds CM, Ferdelman TG, Muyzer G, de Beer D (1998) Structural and functional analysis of sulfate-reducing populations in bacterial biofilms. *Appl Environ Microbiol* 64:3731–3739
- Sekiguchi H, Tomioka N, Nakahara T, Uchiyama H (2001) A single band does not always represent single bacterial strains in denaturing gradient gel electrophoresis analysis. *Biotechnol Lett* 23:1205–1208
- Sipura J, Haukka K, Helminen H, Lagus A, Suomela J, Sivonen K (2005) Effect of nutrient enrichment on bacterioplankton biomass and community composition in mesocosms in the Archipelago Sea, northern Baltic. *J Plankton Res* 27:1261–1272
- Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int J Syst Bacteriol* 44:846–849
- Sullivan CW, Palmisano AC (1984) Sea ice microbial communities: distribution, abundance, and diversity of ice bacteria in McMurdo Sound, Antarctica, in 1980. *Appl Environ Microbiol* 47:788–795
- Tabor S, Richardson CC (1995) A single residue in DNA polymerases of the *Escherichia coli* DNA polymerase I family is critical for distinguishing between deoxy- and dideoxynucleotides. *Proc Natl Acad Sci USA* 92:6339–6343
- Tuomainen J, Hietanen S, Kuparinen J, Martikainen PJ, Servomaa K (2006) Community structure of the bacteria associated with *Nodularia* sp. (Cyanobacteria) aggregates in the Baltic Sea. *Microb Ecol* 52:513–522
- Utermöhl H (1958) Zur Vervollkommnung der quantitativen Phytoplankton-Metodik. *Mitt Int Ver Theor Angew Limnol* 9:1–38