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On the role of bacterial production and denitrification in some less-studied Baltic Sea habitats

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LIST OF ORIGINAL ARTICLES

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

I Hietanen, S., Kuparinen, J., Oja, R. J. & Tuominen, L. 2001: Different filtration treatments and centrifugation in measuring bacterial production in brackish waters. – Boreal Env. Res. 6: 221-229.

II Hietanen, S., Lehtimäki, J. M., Tuominen, L., Sivonen, K. & Kuparinen, J. 2002: *Nodularia* sp. (Cyanobacteria) incorporate leucine but not thymidine; importance for bacterial production measurements. – Aquat. Microb. Ecol. 28:99-104.

III Hietanen, S., Tuominen, L. & Kuparinen, J. 1999: Benthic bacterial production in the northern Baltic Sea measured using a modified [^{14}C]leucine incorporation method. – Aquat. Microb. Ecol. 20: 13-20.

IV Hietanen, S., Moisander, P. H., Kuparinen, J. & Tuominen, L. 2002: No sign of denitrification in a Baltic Sea cyanobacterial bloom. – Marine Ecology Progress Series 242: 73-82.

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THE AUTHOR'S CONTRIBUTION TO THE ARTICLES

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On the role of bacterial production and denitrification in some less-studied Baltic Sea habitats

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ABSTRACT

The role of planktonic bacteria in the cycling of carbon and nutrients within food webs has been intensively studied in the Baltic Sea during the 1980's and 90's. Less is known about the bacteria found in other Baltic Sea habitats, such as in the sediments or attached to algal aggregates. A general reason for this lack of information is the difficulty of the measurements. In this thesis, methods for measuring both bacterial production in various environments (pelagic, benthic and algal bloom-associated) and the denitrification of the attached bacteria are evaluated. The ecological significance of the results, achieved using these methods, is discussed.

The most commonly-used methods for measuring pelagic bacterial production are the ^{3}H-thymidine and ^{14}C-leucine incorporation methods, in which the bacterial cells are filtered on cellulose nitrate filters after incubation. The effects of using polycarbonate filters or centrifugation were tested against this method. The centrifugation method gave systematically lower results than the filtration method, and is not suitable for use as such in low-salinity brackish waters. The addition of salt could improve the method performance, but would need an additional step in the procedure (NaOH-boiling), making it less attractive for users. The use of polycarbonate filters also resulted in lower values in measuring ^{3}H-thymidine incorporation compared to the use of cellulose nitrate filters. No difference in filter performance was found when using ^{14}C-leucine. These results indicate that the choice of the post-incubation treatment procedure has a substantial effect on the final bacterial production estimate.

The leucine and thymidine incorporation ability of the most common bloom-forming cyanobacterial species in the Baltic Sea, *Aphanizomenon* sp. and *Nodularia* sp., was tested. All strains studied showed high leucine incorporation, whereas thymidine was either taken up at a very low rate or not at all. Therefore, heterotrophic bacterial production must be measured using the thymidine instead of the leucine incorporation method during cyanobacterial blooms.

A method of measuring benthic bacterial production was modified from a pelagic centrifugation method, tested and subsequently used for mud sediments of the Baltic Sea. Bacterial production varied between 36 and 350 mg C m^{-2} d^{-1}. On well-oxygenated sediments it equalled the integrated bacterial production of the whole water layer above. In deeper, oxygen-deficient areas, the benthic production only contributed less than 10% to the total bacterial production.

The production of algae-attached bacteria was measured in a cyanobacterial bloom in the northern Baltic Sea in July-August 2000. The algal filaments were heavily colonised by bacteria, and on a cellular basis the production of the attached bacteria did not differ from that of the free-living bacterioplankton. Thymidine uptake was saturated at around 100 nM, which is an unusually high concentration compared to most pelagic environments. A conversion factor based on increase in biovolume was 2.8×10^{16} μm^3 per mol. The daily production of the attached bacteria varied from 3.3 to 41.3 μg C m^{-3} d^{-1}. The production of the free-living pelagic bacteria in the same area has been measured to vary from 3.7 to 4.5 mg C m^{-3} d^{-1}, suggesting that the attached bacteria contribute only a very small fraction (1%) to the total bacterial production.

Bacterial denitrification (reduction of nitrate to molecular nitrogen) is an important nitrogen removal mechanism in the marine environment, and has been shown to be of considerable significance in the sediments of the Baltic Sea. In the late summers of 1999 and 2000, a sensitive ^{15}N-isotope pairing method was used to study denitrification by bacteria associated with the nitrogen-fixing cyanobacteria. Both the presence of bacterial species with genetic potential for denitrification, and the occurrence of anoxic patches were verified within aggregates, but no ecologically-significant nitrogen removal was detected in the cyanobacterial aggregates. The blooms of nitrogen-fixing cyanobacteria in the Baltic Sea must be seen solely as sources, not sinks, for nitrogen in the pelagic environment.

Key words: bacterial production, leucine, thymidine, denitrification, ^{15}N-isotope pairing, Baltic Sea, cyanobacterial aggregates

1 INTRODUCTION

The Baltic Sea is the largest brackish water basin in the world. It has several special features, including a surface salinity range of 2 to 20 psu (practical salinity units) due to extensive river discharge in the north and a connection to the North Sea in the south, severe salinity stratification with limited water exchange between the layers, and seasonal thermal stratification with surface temperatures ranging from ice cover in winter to more than 20 degrees in midsummer. The Baltic is a shallow sea, with a mean depth of 60 m. It has several distinct basins, separated from each other by shallow sills that prevent deep-water exchange between the basins. The main areas are the Gulf of Bothnia (including the Bothnian Sea and the Bothnian Bay), the Gulf of Finland, the Gulf of Riga and the Baltic Proper that includes the deepest basin near the island of Gotland. The permanent halocline is situated at a depth of around 60 to 70 m. Below that, no annual water exchange with the surface water occurs. For this reason, the deeper basins of the Baltic Sea regularly experience anoxia. The bottom water is replaced infrequently, when substantial volumes of North Sea water flow over the sills in the Danish Sounds and, entering the deep basins of the Baltic Sea, push less dense water ahead of them. Such events refresh the oxygen conditions of the sediments in the deep basins and force nutrient-enriched bottom water to the surface layer. Salinity stratification is less pronounced in the Gulf of Bothnia, and hence no stagnation occurs there. In the open Baltic Sea, large blooms of nitrogen-fixing cyanobacteria develop nearly every summer. The occurrence and intensity of such blooms has been suggested to have increased during the last few decades (Kahru & al. 1994).

Planktonic food webs have been extensively studied in the Baltic Sea, and the role of bacteria in the cycling of carbon and nutrients within food webs is well recognised (e.g. Lignell 1990, Lignell & al. 1992, 1993, Kivi & al. 1993, Heinänen & al. 1995, Heiskanen & al. 1996, Uitto & al. 1997). Consequently, bacterioplankton abundance, activity, and distribution (Autio 1990, Heinänen 1991, Heinänen & Kuparinen 1991, Höfle & Brettar 1995, Ameryk & al. 1999, Tuomi & al. 1999, Pinhassi & Hagström 2000) as well as factors controlling these parameters (Wikner & Hagström 1988, 1999, Kuuppo-Leinikki 1990, Kuuppo-Leinikki & Kuosa 1990, Autio 1992, 1998, Heinänen & Kuparinen 1992, Kuparinen & Heinänen 1993, Zweifel & al. 1993, 1995, Heinänen & al. 1995, Hoppe & al. 1998, Tuomi & Kuuppo 1999, Tuomi & al. 1999, Hagström & al. 2001) have been the focus of the microbial studies of the Baltic Sea during recent decades.

The benthic microbiology of the brackish Baltic Sea has, however, remained nearly unknown. Denitrification in northern Baltic Sea sediments has been measured using the ^{15}N-isotope pairing method (Nielsen 1992), and found to be of considerable importance (Tuominen & al. 1998, Gran & Pitkänen 1999). Less information is available on carbon cycling. Meyer-Reil (1986) and Piker & Reichardt (1991) have studied the microbial activity of the anoxic and nearly-anoxic sediments of Kiel Bight, and the benthic bacterial production of the southern Gulf of Riga, as well as that of a coastal station in Pojo Bay (south-west Finland), have been studied using the tritiated thymidine approach (Ekebom 1999, Tuomi & al. 1999). A general reason for the lack of information from larger areas of the Baltic Sea is the difficulty of the measurements. Sediment samples are not only logistically more demanding to obtain, but the production measurements are also more complicated than for pelagic samples.

Additionally the role of bacteria attached to algal aggregates (macroscopic "clumps" of algae clustered together) in the Baltic Sea has remained a mystery, although the importance of such a special growth environment in the Baltic Sea (Bursa 1968, Hoppe 1981) as well as in oceans, lakes, and rivers has been recognised (Alldredge & Silver 1988, Weiss & al. 1996, Zimmermann & Kausch 1996, Zimmermann 1997, Grossart & al. 1998, Azam & Long 2001, Zimmermann-Timm 2002). Various types of aggregates, known also as "marine snow", have been intensively studied in other aquatic environments. Most of the data relate to diatom aggregates, whose aggregation and disaggregation mechanisms, colonisation by both autotrophic and heterotrophic organisms and importance to the carbon flow have been extensively investigated. Cyanobacterial blooms appear less predictably than diatom blooms, especially due to their dependence on weather conditions, and consequently less is known about cyanobacterial aggregates.

Hoppe (1981) has described the aggregation and colonisation of a *Nodularia* bloom in the Baltic Sea. According to his observations, *Nodularia* aggregates develop as the cyanobacterial filaments age, change in appearance from straight to spiralled and start to produce mucus. Fungi and bacteria already colonise the filaments at this stage, but a massive increase in colonisation occurs once the filaments agglomerate. The high amount of bacteria within aggregates attracts small zooplankton to them, causing them to develop into "self-supporting systems" with a balanced input and output of organic matter. Hoppe (1981) points out that although the amount of cyanobacterial primary production-derived exudates decreases in the later stages of aggregate succession, the high standing stock of bacteria may be supported by the other organisms colonising the aggregate (zooplankton excretion, lysis of dead organisms and exudates from attached autotrophs, such as small diatoms). Aggregates of various types have been shown to be sites of elevated nutrient concentrations

and to provide anaerobic microenvironments within the pelagic environment (Paerl 1985, Alldredge & Cohen 1987, Paerl & Prufert 1987, Bianchi & al. 1992, Shanks & Reeder 1993, Ploug & al. 1997). Elevated nutrient, especially nitrogen, conditions also seem likely in *Nodularia* aggregates, as nitrogen-fixing cyanobacteria have been shown to release recently-fixed nitrogen into the surrounding water as dissolved organic nitrogen (Paerl 1984, Glibert & Bronk 1994, Ohlendieck & al. 2000). High heterotrophic activity is also likely to consume oxygen within the aggregate, and as the diffusion of oxygen into the aggregate may in addition be hindered physically by the mucus produced in the aggregate, anoxic conditions may be reached inside the aggregate, facilitating anaerobic processes like denitrification and sulphate reduction. No attempts to measure such processes in *in situ* conditions have been made so far, because of the lack of methods, neither has the heterotrophic production of the bacteria attached to *Nodularia* aggregates been quantified for the same reasons as in benthic bacterial production: the cyanobacteria-associated bacteria are difficult to sample, and there have been doubts about the suitability of the available methods in such procaryote-dominated surroundings.

In this thesis, methods to measure both the production of bacteria in various environments (pelagic, benthic and cyanobacteria-associated) and denitrification by attached bacteria are evaluated (Fig. 1). The ecological significance of the results, achieved using these methods, is discussed.

1.1 Bacterial production

The methods most commonly used to measure bacterial production nowadays are the ^{3}H-thymidine incorporation method by Fuhrman & Azam (1980, 1982), and the ^{3}H- or ^{14}C-leucine incorporation method by Kirchman & al. (1985). These methods have been criticised and subsequently modified, but the principles behind them have not dramatically changed. Azam & Fuhrman (1984) summarised the widely-accepted criteria for an exemplary method to estimate bacterial production. They stated that the method used should be specific for heterotrophic bacteria and that it should not rely on balanced growth. Furthermore, the growth rates should not be affected by the procedures involved in the measurements, and if any conversion factors were needed, these should be easy and reliable to produce.

The ^{3}H-thymidine method is based on measuring DNA production and, consequently, cell division. Thymidine has only one function in cells, namely participation in DNA synthesis, which makes it a good tool in bacterial production measurements. Several authors have demonstrated that thymidine in nanomolar concentrations is taken up exclusively by heterotrophic bacteria, but not by cyanobacteria, algae or zooplankton (Fuhrman & Azam 1982, Bern 1985, Robarts & Wicks 1989, Pedrós-Alió & al. 1993, Lehtimäki & al. 1997, **II**). However, it has also been shown that not all heterotrophic bacteria can incorporate thymidine (Pollard & Moriarty 1984, Davis 1989, Jeffrey & Paul 1990, Robarts &

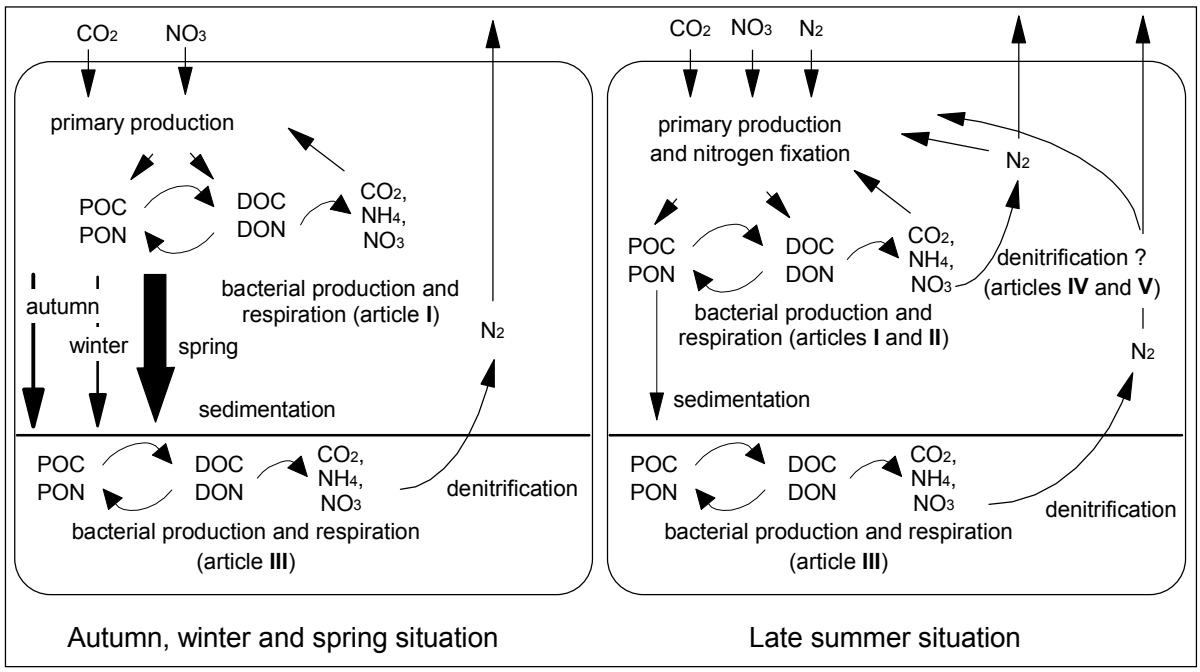

Fig. 1. A schematic summary presentation of carbon and nitrogen processes in the Baltic Sea and the contribution of this thesis to describing those processes.

Zohary 1993). In addition, the method relies on using conversion factors when translating the thymidine incorporation into cells created and further, from the cells to biomass (carbon and nitrogen) produced. These conversion factors have been shown to vary largely in different environments, neither is it clear which of the methods available is best for calculating such factors (Bell 1988, Moriarty 1988, reviewed by Ducklow & al. 1992). In addition to these major drawbacks, questions have also been raised about unspecific labelling after degradation of ^{3}H-thymidine in cells (Pollard & Moriarty 1984, Riemann 1984, Hollibaugh 1988, Kirschner & Velimirov 1999a), proper methods to extract and purify DNA from cells (reviewed by Robarts & Zohary 1993), intra- and extracellular dilution of the isotope (Moriarty 1986, Jeffrey & Paul 1988), non-saturable uptake kinetics (Hollibaugh 1988, Kaplan & al. 1992, Logan & Fleury 1993, Tuominen 1995) and the role of viral DNA synthesis in infected cells (Bratbak & al. 1992, Tuomi 1997).

Because of these uncertainties with the thymidine method, the leucine incorporation method has gained more adherents in recent years. The ^{3}H- or, nowadays more commonly, ^{14}C-leucine method is based on measuring protein synthesis in the cells. The main advantage of the leucine over the thymidine method is that no experimental conversion factors are needed, as the amount of leucine in bacterial proteins, the amount of protein per cell and the relation of carbon to protein are all relatively constant in marine bacterial assemblages (Simon & Azam 1989). Therefore, just by measuring the amount of leucine incorporated into the proteins, a theoretical minimum estimate of bacterial carbon production can be made. The isotope dilution can be measured relatively easily, and the "true" bacterial carbon production can then be calculated by multiplying the theoretical minimum value by the isotope dilution factor. Furthermore, the method is an order of magnitude more sensitive than the thymidine method, thus allowing measurements in low-activity environments (Simon & Azam 1989). Leucine in nanomolar concentrations was also considered to be taken up exclusively by heterotrophic bacteria (Kirchman & al. 1985, Riemann & Azam 1992), until Kamjunke & Jähnichen (2000) reported the significant leucine incorporation of an axenic culture (free of any other organisms, such as heterotrophic bacteria) of cyanobacterium *Microcystis aeruginosa* PCC 7806. They concluded that measurements with leucine in waters dominated by *M. aeruginosa* might yield overestimates of bacterial production. Until the experiments reported here, only this single cyanobacterium strain, belonging to a unicellular non-heterocystous genus, had been shown to incorporate leucine.

1.1.1 Pelagic bacteria

Routine measurements of bacterial production consist of incubating samples with a radioisotope, followed by filtration on either cellulose nitrate or polycarbonate filters, then extracting the cells and precipitating the macromolecules with ice-cold TCA before measuring in a scintillation counter the incorporated label trapped on the filters. In 1992, Smith and Azam suggested using centrifugation instead of filtration in sample processing, resulting in lower cost per sample as well as a reduced amount of radioactive waste. In this thesis, the results of several experiments between 1995 and 1999 in which this method was used parallel to the traditional filtration method are presented (**I**). Both ^{3}H-thymidine and ^{14}C-leucine were used to ascertain whether the method functions with both isotopes, as stated by Smith & Azam (1992). At the same time, two different filter types, cellulose nitrate and polycarbonate, were compared. Both filter types are commonly used in microbiological studies in the laboratories around the Baltic, but until this study, no comparison of the two had been published from this area.

The report of Kamjunke & Jähnichen (2000), of significant leucine incorporation by cyanobacterium *Microcystis aeruginosa*, called for further investigation of possible leucine incorporation by other cyanobacteria. In this thesis, results from laboratory experiments, in which axenic cultures of filamentous, heterocystous *Nodularia* and *Aphanizomenon* were used, are presented (**II**). The ability of these species, common in the Baltic Sea, to incorporate either leucine or thymidine was investigated to find a method suitable for studying heterotrophic bacterial production during cyanobacterial blooms, which are nearly an annual summer phenomenon in the Baltic Sea.

1.1.2 Benthic bacteria

Sediment microbiology has lately attracted increasing interest. The role of microbes as recyclers of nutrients and carbon in benthic ecosystems has been acknowledged, and studies have been published on benthic bacterial production in various environments including marine (e.g. van Duyl & Kop 1990, Meyer-Reil & Charfreitag 1991, Moriarty & al. 1991, Tibbles & al. 1992, Epstein 1997), lake (Moran & Hodson 1992, Kairesalo & al. 1995, Kirschner & Velimirov 1999a) and river (Marxsen 1996, Fischer & Pusch 1999) sediments. The small amount of data published from the brackish Baltic Sea has been collected mainly by using the thymidine method (Meyer-Reil 1986, Piker & Reichardt 1991, Ekebom 1999, Tuomi & al. 1999). The problems of using the thymidine method, however, are even more pronounced in sediment than in pelagic studies. Unspecific labelling, caused by the bacteria

metabolising the incorporated ^{3}H-thymidine to be used as a carbon or nitrogen source, instead of in DNA synthesis, has been shown to account for a large and variable fraction of the measured ^{3}H-label in samples (Kirschner & Velimirov 1999a). The amount of unspecific labelling can only be measured when DNA is separated from other macromolecules in complicated extraction processes, the efficiency of which must in addition be estimated. Furthermore, there is no tool for measuring the conversion factors between thymidine incorporation and cell production in sediments, and therefore theoretical conversion factors or those empirically estimated for water column bacteria have to be used. Thymidine also adsorbs to particles more effectively than leucine (Schuster & al. 1998), which implies that higher incubation concentrations of thymidine need to be used, leading to elevated costs of experimentation. On the other hand, it has been suggested that high thymidine concentrations enhance thymidine incorporation (Kirschner & Velimirov 1999a). This further complicates the isotope dilution measurements, in which increasing amounts of unlabelled thymidine are added to the samples. Leucine, even at micromolar concentrations, does not seem to have such an enhancing effect (Simon & Azam 1989, Marxsen 1996, Fischer & Pusch 1999, Kirschner & Velimirov 1999a). There is also less unspecific labelling when measuring ^{14}C-leucine incorporation (Kirchman & al. 1985, Simon & Azam 1989, Fischer & Pusch 1999, Kirschner & Velimirov 1999a), and no experimental conversion factors are needed (Simon & Azam 1989). In this thesis, an easy-to-use ^{14}C-leucine incorporation method for measuring benthic bacterial production is introduced, and results from field measurements from the northern Baltic Sea are presented (**III**).

1.1.3 Cyanobacteria-associated bacteria

Production of cyanobacteria-associated heterotrophic bacteria was measured using the thymidine incorporation method in July-August 2000 in the northern Baltic Sea. As no previous data from such an unusual growth environment existed, the saturation level, optimal incubation time and a conversion factor for the local microbial community were determined. The results of this experiment are discussed in this thesis and in article **IV**.

1.2 Denitrification

In general, primary production in oceans is nitrogen-limited. This is also the case in the brackish Baltic Sea (Granéli & al. 1990, Kivi & al. 1993), with the exceptions of the phosphorus-limited Bothnian Bay (Granéli & al. 1990, Andersson & al. 1996) and the easternmost Gulf of Finland (Pitkänen & Tamminen 1995). The Baltic Sea has a large and densely-populated catchment area that burdens the Baltic with considerable allochthonous nitrogen loading (592 kt N in 1995, Lääne & al. 2002). Additionally, nitrogen enters the system autochthonously via nitrogen fixation. A major part of nitrogen fixation occurs during periodic cyanobacterial bloom events, a common phenomenon in the Baltic Sea during warm summer months. The dominating bloom-forming cyanobacteria are *Aphanizomenon* sp. and *Nodularia* sp., which produce dense, satellite-detectable accumulations in the surface waters over hundreds of square kilometres (Kahru & al. 1994). Nitrogen fixation by the cyanobacteria in the Baltic has been studied intensively (Lindahl & al. 1980, Lindahl & Wallström 1985, Moisander & al. 1996, Ohlendieck & al. 2000, Wasmund & al. 2001). It has been calculated to contribute from ~10% (Melvasalo & al. 1983, Leppänen & al. 1988) to up to ~35% (Wasmund & al. 2001) of the annual nitrogen loading to the Baltic Sea. Meanwhile, few attempts have been made to follow and quantify the flux of the fixed nitrogen. Heiskanen & Kononen (1994) found no evidence of sedimentation of the gas-vacuolated cyanobacterial cells. They concluded that most of the cyanobacteria were decomposed, releasing nitrogen, within the surface layer. In the central Baltic Sea, 5-10% of the nitrogen newly-fixed during active growth of the filamentous cyanobacteria was incorporated into the picoplanktonic size fraction via exudates (Ohlendieck & al. 2000). The main nitrogen release mechanism was, however, lysis of the cyanobacterial cells as the bloom decayed (Ohlendieck & al. 2000). Grazing on the Baltic Sea cyanobacteria has been shown to be of minor importance (e.g. Sellner & al. 1994), and, therefore, direct transfer of the fixed nitrogen to higher trophic levels remains inefficient. In contrast, previous studies have indicated that cyanobacterial blooms support an active microbial community over the whole lifetime of the blooms (Bursa 1968, Hoppe 1981, Heinänen & al. 1995, Worm & Søndergaard 1998, Engström-Öst & al. 2002). These microbial interactions with cyanobacteria are not necessarily signs of decay, since the cyanobacteria provide heterotrophic bacteria with a source of labile substrates, while the heterotrophic bacteria provide cyanobacteria and aggregate-colonising autotrophs with a source of remineralised nutrients. In addition, the microenvironments of aggregates in various marine environments have been shown to harbour steep gas and chemical gradients (Paerl 1985, Alldredge & Cohen 1987, Paerl & Prufert 1987, Bianchi & al. 1992, Shanks & Reeder 1993, Ploug & al. 1997). Such microzones suggest the possibility for localised specialisation of the associated bacteria in anaerobic biogeochemical processes, such as sulphate reduction and denitrification.

Denitrification (reduction of nitrate *via* nitrite, nitric oxide and nitrous oxide to molecular nitrogen; $NO_3 \rightarrow NO_2 \rightarrow NO \rightarrow N_2O \rightarrow N_2$) is an important nitrogen removal mechanism in marine environments. It has also been shown to be of considerable significance in the sediments of the Baltic Sea (Stockenberg & Johnstone 1997, Tuominen & al. 1998, Gran & Pitkänen 1999). Denitrification has been calculated to remove about 23% and 31% of the annual nitrogen input to the Bothnian Bay and Bothnian Sea, respectively (Stockenberg & Johnstone 1997), and about 30% of the annual load in the Gulf of Finland (Tuominen & al. 1998). However, very little is known about denitrification in the water column of the Baltic Sea. Denitrification has been found to occur at the interface between anoxic, stagnant deep water and overlying oxic water in the central Baltic Proper (Rönner & Sörensson 1985, Brettar & Rheinheimer 1992). In those studies, denitrification was measured using the acetylene blockage method, now known to have some serious flaws (inhibition of nitrification: Seitzinger & al. 1993, reversal of blockage by sulphide: Sørensen & al. 1987, incomplete blockage by acetylene: Christensen & al. 1989). The conclusions as to the importance of the process are not clear (Rönner & Sörensson 1985, Brettar & Rheinheimer 1992).

The ^{15}N-isotope pairing method (Nielsen 1992) has proved to be an excellent tool for measuring denitrification in sediments (Lohse & al. 1996, Tuominen & al. 1998). It is based on adding ^{15}N-labelled nitrate to samples and, after incubation, measuring the relative increase in the partly ($^{14}N^{15}N$) and completely ($^{15}N^{15}N$) labelled N_2 molecules. From the changes in isotopic composition it is possible to calculate not only the denitrification rate based on the added nitrate but also the denitrification taking place in nature, that is, based on nitrate readily available or produced *in situ* by nitrification. A test was made as to whether the method could also be used to measure denitrification in a cyanobacterial bloom. In this thesis, results from two research cruises, in August 1999 and in July-August 2000 are presented (**V**, **IV**). In 1999, cyanobacterial aggregates were collected at the entrance to the Gulf of Finland. The denitrification in natural aggregates was measured without disturbing the *in situ* oxygen conditions. As no activity could be recorded, although bacteria with genetic potential for denitrification were present and respiration rates in aggregates were high enough to generate anoxia in the centre of the aggregate (calculated according to Ploug & al. 1997) in darkness, the experiments in 2000 were conducted under optimised conditions for denitrification. Artificial (net-concentrated) cyanobacterial scum was used and the oxygen concentration was allowed to decrease during incubation. Samples were withdrawn at different oxygen levels (oxic, hypoxic and anoxic), in order to monitor the onset of the denitrification process as a function of oxygen concentration. As denitrification sometimes does not proceed to the end (N_2) but ceases after the reduction of nitrite (NO_2) to nitrous oxide (N_2O) (Patureau & al. 1994, Frette & al. 1997, Gejlsbjerg & al. 1998), production of N_2O was also measured, although formation of N_2O during denitrification has been shown to account for less than 10% of the N_2 formation in various aquatic systems (Smith & DeLaune 1983, Seitzinger 1988, Capone 1991). In addition, bacterial numbers were calculated and heterotrophic bacterial production was measured (in 2000 only) to verify that the cyanobacterial bloom indeed supported high levels of heterotrophic activity.

2 MATERIALS AND METHODS

2.1 Pelagic bacterial production

2.1.1 Standard procedure

Samples were incubated with radiotracers, terminated either with TCA (trichloroacetic acid, final conc. 10% w/v) or formalin (final conc. 1% v/v), and stored refrigerated for a maximum of 12 hours before filtering under ice-cold conditions on cellulose nitrate filters (Ø 25 mm, pore size 0.2 µm, Sartorius) that had been briefly soaked in ice-cold 5% (w/v) TCA prior to use. Sample vials were rinsed with 2 ml of ice-cold 5% TCA and these solutions were added on filters. Filtration funnels were rinsed with 2 ml of ice-cold 5% TCA and removed. Cells on the filters were extracted five times with 1 ml of ice-cold 5% TCA, after which the filters were placed in scintillation vials. The filters were dissolved in scintillation liquid (Insta-Gel Plus, Packard) at room temperature overnight. The samples were shaken vigorously before assaying the radioactivity in a scintillation counter. The results were converted to bacterial carbon production (BCP, g) using the following equations:

$$BCP = leucine_{inc} \times (100/7.3) \times 131.2 \times 0.86$$

where $leucine_{inc}$ = moles of leucine incorporated, 7.3 = mol% of leucine in protein, 131.2 = formula weight of leucine, and 0.86 = conversion of a gram of protein produced to a gram of carbon (Simon and Azam 1989).

$$BCP = thymidine_{inc} \times CF1 \times CF2 \times CF3$$

where $thymidine_{inc}$ = moles of thymidine incorporated, CF1 = conversion factor between thymidine incorporation and cell production, CF2 = conversion factor between cell number and sample biovolume and CF3 = conversion factor between biovolume and

carbon content (all CFs either empirical or literature-derived) (Fuhrman & Azam 1980).

Isotope dilution was not determined in any of these studies; the results are therefore minimum estimates of carbon production.

2.1.2 The effect of using different filters or centrifugation

Water for the experiments was collected either from the pelagic surface waters of the Gulf of Finland (research cruises on board R/V Aranda and R/V Victor Bujnickij) or from coastal waters near Helsinki, Finland. All seasons were covered in sampling (Table 1). Water samples were either used immediately in experiments, or a nutrient-enriched, predator-free batch culture was prepared from them. A batch culture always consisted of 1800 ml of double glass-fibre filtered (GF/F, Whatman) seawater and a seawater inoculum of 200 ml that had been passed through a 0.8 µm pore size polycarbonate filter (Nuclepore, Poretics). The batch cultures were enriched to 5.7 µM N, 0.65 µM P and 16.7 µM C (calculated concentration after additions, natural levels not analysed). The absence of small flagellates in filtrates was checked by epifluorescence microscopy. Bacterial growth in batch cultures was monitored by Acridine Orange cell counts (Hobbie & al. 1977) and experiments were conducted when logarithmic growth was achieved.

All incubations were carried out in sterile vessels at *in situ* temperatures. High incubation concentrations of ^{14}C-leucine (130 to 270 nM; Amersham, 11.1-11.3 GBq mmol^{-1}) and ^{3}H-thymidine (20 to 70 nM; Amersham, 1.89 GBq µmol^{-1}) were used to ensure reliable counts. The incubation time varied from 1 to 3 hours depending on the incubation tem perature. Samples were run either in 3 replicates with one blank or 6 replicates with 3 blanks. Four different treatments were used: filtering on cellulose nitrate filters (CN), filtering on polycarbonate filters (PC), centrifuge method when incubating the samples in microcentrifuge tubes (C1), and centrifuge method when incubating a large sample volume and

Table 1. The differences detected in the final bacterial production estimate when polycarbonate filters or centrifugation were used instead of cellulose nitrate filtering. Experiments 1-12 were run using the ^{14}C-leucine and experiments 13-22 using the ^{3}H-thymidine incorporation method. PC = filtering the samples on polycarbonate filters, C1 = centrifuge method when incubating 1 ml samples in microcentrifuge tubes, CT = centrifuge method when incubating a large sample volume and transferring 1 ml subsamples after incubation to microcentrifuge tubes for processing. Significant (Dunnett's test, $p<0.05$) differences found when compared to the reference treatment (filtering on cellulose nitrate filter) after a one-way ANOVA for each experiment separately. Values in boldface when $p>0.05$ (no Dunnett's test run). * = significant difference, - = not significant difference, nd = not determined. Origin: P = pelagic, C = coastal. Sample: N = natural, B = batch.

Exp.	Date	Origin	Sample	*p*	Treatment		
					PC	C1	CT
1	May 1995	P	N	0.004	nd	*	nd
2	May 1995	P	B	0.048	-	-	*
3	June 1995	C	N	0.048	-	-	*
4	June 1995	C	B	**0.068**			
5	June 1995	C	N	**0.423**			
6	June 1995	C	B	0.002	*	-	-
7	June 1995	C	B	0.004	-	*	*
8	June 1995	C	B	0.000	-	*	*
9	June 1995	C	B	0.007	-	-	*
10	Dec. 1998	P	B	0.002	-	-	*
11	Dec. 1998	P	B	**0.174**			
12	May 1999	P	N	0.000	-	*	*
13	June 1995	C	B	0.000	-	*	*
14	June 1995	C	B	0.000	*	*	*
15	July 1995	P	N	0.002	-	*	*
16	July 1995	P	N	0.000	*	*	*
17	July 1995	P	N	0.000	*	*	*
18	Aug. 1995	C	B	0.000	*	*	*
19	Aug. 1995	C	B	0.000	-	*	*
20	Sep. 1995	C	B	0.004	-	*	*
21	Feb. 1996	P	B	0.000	*	*	*
22	Feb. 1996	P	B	0.000	*	*	*

transferring 1 ml subsamples after incubation to microcentrifuge tubes for processing (CT) (**I**: Fig. 1 CN, PC, C1 and CT). The results from the most commonly-used treatment, filtration on cellulose nitrate filters (Ø 25 mm, pore size 0.2 µm, Sartorius) (CN, see 2.1.1), were considered as reference results to which the results of the other three treatments were compared.

Different filters

The effect of using polycarbonate (Ø 25 mm, pore size 0.2 µm, Nuclepore or Poretics) filters instead of cellulose nitrate filters was tested. The cellulose nitrate filters are composed of a matrix of inert polymers and have a uniform microporous structure. The polycarbonate filters have a smooth flat surface and precisely controlled cylindrical pores. Both types of filters are hydrophilic and have a high non-specific binding capacity.

Subsamples of 5 to 10 ml were taken from large incubation vessels and filtered according to the standard procedure (see 2.1.1) using cellulose nitrate filters (treatment 1, reference results, **I**: Fig. 1 CN) and polycarbonate filters (treatment 2, **I**: Fig. 1 PC).

Centrifugation

The effect of using centrifugation instead of filtering to collect the macromolecules after incubation was tested. Two different approaches were tested. In the first approach, samples (1 ml) were incubated in microcentrifuge tubes (treatment 3, **I**: Fig. 1 C1). After termination of incubation, the samples were centrifuged in a cooled centrifuge for 10 minutes at +4 °C at 15 988 × *g* or 25 848 × *g* (Heraeus Contifuge 17RS). Supernatants were gently removed by suction, 1 ml of ice-cold 5% TCA was added, samples were vortexed well and centrifuged again. This was repeated altogether three times. After the last removal of supernatants, the microcentrifuge tubes were cut into three pieces and put into scintillation vials to which 10 ml of scintillation liquid (Insta-Gel Plus, Packard) was added. The samples were counted the next day in a scintillation counter. To see whether the incubation volume would have an effect on the results, in the second approach the subsamples (1 ml) were transferred to microcentrifuge tubes from the same large sample volume that was used for subsamples for the filtration treatments. These transferred 1 ml subsamples were then treated in the same way as the samples incubated in microcentrifuge tubes (treatment 4, **I**: Fig. 1 CT).

Data processing

Experiments were performed at all stages of annual plankton succession, and were spread over four years. Large variations in the environmental conditions and bacterial growth phases between the experiments resulted in orders of magnitude differences in average DPM (disintegrations per minute) counts. In order to see the differences between treatments, the DPM-data were scaled. Each DPM count was divided by the average DPM count of the reference method (CN, filtering on cellulose nitrate filter) of that experiment and the constant 1 was subtracted. This gave a result matrix in which all the data varied from -0.904 to 0.546. A value of -0.25 stands for a result 25% lower than achieved with the reference method and a value of 0.25 for a result 25% higher than achieved with it. Scaled results were analysed with SAS (version 6.12) both with a nested analysis of variance (differences resulting from different treatments nesting within different experiments) for the whole data set and also with one-way analysis of variance for each experiment separately. Different treatments were compared with the reference treatment by Dunnett's test when appropriate. ^{14}C-leucine and ^{3}H-thymidine incorporation results were analysed separately from each other.

2.1.3 Leucine and thymidine incorporation by cyanobacteria

The leucine and thymidine incorporation capacity of six axenic nodularin-producing and non-toxic *Nodularia* strains as well as one axenic *Aphanizomenon* strain were tested (Table 2). The *Microcystis aeruginosa* strain PCC 7806 was used as a positive control for leucine incorporation (Kamjunke & Jähnichen 2000), and coastal seawater was used as a positive control for both leucine and thymidine incorporation. The culturing methods of the *Nodularia* and *Microcystis* strains, as well as the way chlorophyll *a* concentrations were measured, are explained in detail in article **II**. The *Aphanizomenon* strain was cultured the same way as the *Nodularia* strains. Incorporation experiments were done using exponentially-growing cultures. Axenic *Nodularia* and *Microcystis* strains (grown in both nutrient-rich and nutrient-poor media) and the control seawater (ca. 2.4×10^6 heterotrophic bacterial cells ml^{-1}) were treated separately (no dual labelling) with three concentrations of the two substrates (69, 121 and 212 nM for leucine: Amersham, 10.9 GBq mmol^{-1}; 13, 30 and 121 nM for thymidine: Perkin-Elmer Life Sciences, 3.17 GBq µmol^{-1}). The axenic *Aphanizomenon* strain was treated with four concentrations of leucine (18, 76, 147 and 207 nM, Amersham, 11.1 GBq mmol^{-1}) and with three concentrations of thymidine (25, 42 and 90 nM, Amersham, 1.48 GBq µmol^{-1}), because the experiments using the *Aphanizomenon* strain were run a few months earlier than those using the other strains. Samples (5 ml; 3 replicates, 1 blank) were incubated with substrates in autoclaved scintillation vials in darkness at room temperature for one hour and then treated according to the standard procedure (see 2.1.1).

Table 2. Cyanobacterial strains tested for their capacity to take up leucine and thymidine, and the observed maximum uptake velocities per µg chlorophyll *a* (avg ± SD). Species identification for *Nodularia* according to Sivonen & al. (1989) and Laamanen & al. (2001) and for *Aphanizomenon* according to Lyra & al. (2001). Uptake velocities observed for Baltic Sea coastal seawater (2.4 × 10^6 heterotrophic bacterial cells ml^{-1}) were 650 and 175.1 pM h^{-1} for leucine and thymidine, respectively.

Species	Strain	Toxin	Geographical origin	Growth medium	Chl *a* µg l^{-1}	Max observed uptake velocity (µg chl *a*)$^{-1}$ leu pmol h^{-1}	thy pmol h^{-1}
N. baltica	BY1	+	Baltic Sea (plankton)	Z8XS	67	6.4 ± 0.93	0.16 ± 0.10
Nodularia sp.	PCC 7804	+	Dax, France	Z8XS	83	9.9 ± 0.98	0.02 ± 0.04
			(thermal spring)	Z8XS/ 4	71	2.6 ± 0.52	0.05 ± 0.01
N. harveyana	Hübel	-	Baltic Sea	Z8XS	47	2.1 ± 0.68	0.10 ± 0.06
	1983/300		(benthic microbial mat)	Z8XS/ 4	62	3.8 ± 0.15	0.04 ± 0.03
N. sphaerocarpa	UP16f	-	Baltic Sea (plankton)	Z8XS	105	1.4 ± 0.10	0.02 ± 0.04
N. sphaerocarpa	HKVV	-	Baltic Sea (water)	Z8XS	72	1.7 ± 0.11	0.04 ± 0.03
				Z8XS/ 4	66	9.4 ± 1.69	0.12 ± 0.04
N. sphaerocarpa	PCC	-	Spotted Lake, Canada	Z8XS	67	7.5 ± 2.25	0.02 ± 0.20
	73104/1		(alkaline soil)	Z8XS/ 4	58	12.1 ± 0.49	0.04 ± 0.03
Aphanizomenon sp.	TR183	-	Baltic Sea (plankton)	Z8X	192	11.2 ± 0.06	0.01 ± 0.00
M. aeruginosa	PCC 7806	+	Braakman Reservoir,	Z8	61	8.4 ± 0.54	0.28 ± 0.12
			the Netherlands (water)	Z8/ 4	61	4.0 ± 0.07	0.15 ± 0.02

2.2 Benthic bacterial production

Sediment samples were collected from the northern Baltic Sea on six cruises in 1996 and 1997 using a Gemini twin corer (inner diameter of the cores 80 mm) (**III**: Fig. 1). Only the topmost 1 cm was used in incubations, except in the vertical distribution experiment. The routine procedure for measuring leucine incorporation is explained in detail in **III**.

The gel method presented here was tested against the combustion method (Tuominen 1995) in which the samples were dried at 60 ºC after similar washings using centrifugation and then combusted at 900 ºC (Junitek Oxidizer) prior to scintillation counting. Regression equations were calculated with the "new" gel method as the independent variable and the "old" combustion method as the dependent variable. If the methods give identical results, the constant of the regression equation should be zero and the coefficient one. In addition, linear correlations were calculated.

Time series incubations using different amounts of sediment were carried out in July 1997 in the Gulf of Finland and in the northern Baltic Proper to test the effect of the volume of sediment used in the incubations. The possible effect of the filtered, near-bottom seawater (used to slurry the sediment and to adjust the incubation volume to 500 µl) was checked at the same time by incubating the samples with different amounts of seawater (0 to 400 µl).

In October 1997, an experiment was performed to study the vertical distribution of bacterial production. At two stations (GF2 and JML), the bacterial production in the 1-2 cm layer was measured, in addition to that in the 0-1 cm top layer. At a third station (SR5), the production of the 2-3 cm layer was also analysed, due to a visually deeper oxidised layer.

2.3 Production of cyanobacteria-associated bacteria

Study area and sampling

Cyanobacteria-associated bacteria were studied on a cruise of R/V Aranda in July-August 2000 in the northern Baltic Sea area (**IV**: Fig. 1, **IV**: Table 1). Wind kept the upper surface layer well mixed to a depth of approximately 20 m; no visible surface accumulations (cyanobacterial scums) were thus detected in the study area during the study period, despite high chlorophyll *a* values (up to 9 µg l^{-1}). To simulate a heavy surface bloom, a zooplankton net (100 µm mesh size) was used to collect cyanobacteria from the mixed layer. The resulting concentrated plankton suspension was diluted with surface water (1:1 v/v) to better reflect the natural cyanobacterial concentration in a surface scum. This "artificial

bloom sample" was then used in the measurements. The concentration factor was calculated using the diameter of the opening of the zooplankton net, the depth of each haul, the number of hauls per sample, the volume of the concentrate after combining the hauls and the diluting with surface water. The concentration factor varied from 729 to 1701 between different stations.

Samples (5 ml; 3 replicates, 1 formalin-killed blank) of the plankton concentrate collected by net-haul were incubated in acid-washed 6-ml glass vials using saturating thymidine concentration (~ 100 nM: Amersham, 1.70 GBq μmol^{-1}) at *in situ* temperatures in the dark for 20 to 60 min. After incubation the samples were treated according to the standard procedure (see 2.1.1).

Formalin-fixed heterotrophic bacteria in the concentrate were counted using DAPI (4´6-diamidino-2-phenylindole) staining and epifluorescence microscopy (Porter & Feig 1980) (**IV**).

The ratio between incorporated thymidine and cell production varies geographically and between growth conditions. A separate experiment was therefore carried out in which the conversion factor for the local microbial community was determined from the slope of regression between cumulative incorporated thymidine and bacterial biovolume in a batch culture (Bjørnsen & Kuparinen 1991, Tuomi 1997) (**IV**). Bacterial carbon production was calculated using the conversion factor determined (2.8×10^{16} μm^3 per mol) and the average carbon content, measured using X-ray microanalysis, of the bacterial cells in the study area (0.10 pg C μm^{-3}, Fagerbakke & al. 1996).

2.4 Denitrification studies

2.4.1 Denitrification and N_2O production

In August 1999, cyanobacterial aggregates were collected on a cruise of R/V Aranda at the entrance to the Gulf of Finland at a fixed sampling station (59º28′N, 22º53′E) on four days (4th, 5th, 8th and 10th of August). Sampling was done twice a day (morning and afternoon) using a zooplankton net (500 µm mesh) towed from the thermocline (20 m) up to the surface, following which the harvested aggregates were transferred gently with the use of a wide-bore pipette to various vials for incubations or analyses.

For the denitrification measurements, glass bottles (32 ml) equipped with diagonally-cut conical glass stoppers were used, enabling incubations in gas-tight, bubble-free conditions. Aggregates (5 or 10) were transferred to bottles (7-10 replicates per sampling occasion) filled with 10 µm filtered surface water. On every sampling occasion, a sample with 10 µm filtered surface water without aggregates was also prepared to check for ambient water activity. In addition, a sample without ^{15}N-addition was preserved for background subtraction. A $K^{15}NO_3$ solution (99 atom%, Europa Scientific Ltd, Cheshire, UK) was added to the bottles (100 µM final conc.) which were immediately closed and covered in aluminium foil. During incubation at the *in situ* temperature the samples were slowly rotated on a test tube mixer (Spiramix, Denley, Hampshire, UK) in order to keep the aggregates suspended (Ploug & Grossart 1999). The incubation time (17 to 27 hours) was decided on each occasion separately, based on the results from respiration measurements of parallel samples. The more "active" samples were incubated for a shorter time than the less active ones, in order to avoid lowering the ambient oxygen concentrations in the sample bottles. Samples were killed by adding 1 ml of $ZnCl_2$ solution (500 µg ml^{-1}) carefully under the surface, avoiding gas release from the samples. The samples were then transferred to 10 ml gas-tight Exetainers (Labco, High Wycombe, UK) containing 500 µl of $ZnCl_2$ solution. The mass ratios of N_2 ($^{28}N_2$, $^{29}N_2$ and $^{30}N_2$) formed in the aggregate samples and in the filtered (10 µm) water (for background subtraction) were later analysed using a mass spectrometer by the National Environmental Research Institute in Silkeborg, Denmark. Denitrification rates were calculated from the mass ratios according to Nielsen (1992).

N_2O production was measured from the aggregates harvested on the morning of August 10th. Single aggregates (n=24) were transferred to 60 ml ampoules that were filled beforehand with filtered (10 µm) surface water. The ampoules were closed air-free with butyl septums and aluminium caps (ScherfCroma, Germany). Blank samples (n=5) were treated immediately with 600 µl of formalin. The N_2O production of the ambient water (10 µm filtered surface water) was measured at the same time in a similar set-up using 3 replicates and 2 blanks. Samples were incubated in the dark at the *in situ* temperature for 9 hours. During incubation the ampoules were rolled slowly on a test tube mixer as mentioned above. After terminating the incubation by injecting 600 µl formalin though the septum equipped with an overflow needle the ampoules were stored upside-down at room temperature until analysis within a month. Prior to the analysis, 10 ml of sample water was replaced by 10 ml of ultra-pure helium, and samples were shaken vigorously and left to stabilise for an hour. 500 µl subsamples of the headspace were analysed using a Hewlett Packard 5890 series II gas chromatograph equipped with an HP 3396 A integrator, electron capture detector, and a Porapak Q column at 35 ºC, using an Argon (95%)-Methane (5%) mixture as the carrier gas. The amount of N_2O in the sample was calculated using the gas equation ($P \times V = n \times R \times T$, where P is pressure, V is volume, n is amount of gas moles, R is the ideal

gas constant, and T is temperature) and the Ostwald gas absorption coefficient (Weiss & Price 1980).

In July-August 2000, no aggregates were detected in the study area (**IV**: Fig. 1, **IV**: Table 1), and therefore the same concentrated plankton sample was used in the denitrification measurements as for measuring the production of cyanobacteria-associated bacteria (see 2.3). Glass vials (23 ml) equipped with diagonally-cut conical glass stoppers were filled with the collected plankton concentrate, using 2 replicates for each of the 3 points in time (oxic, hypoxic and anoxic conditions, see below). $K^{15}NO_3$ solution was added to the vials (100 µM final conc.) and the samples were incubated as in 1999. The time instants at which the incubations were terminated were decided upon based on the respiration measurements made with the parallel samples (see 2.4.3). The first two replicate vials were removed from incubation after 2-3 hours when the oxygen concentration was still high (~ 100 µM O_2). The next two replicate vials were removed when the conditions in the vials were hypoxic (~ 40 µM O_2) and the last two were incubated until anoxic conditions had prevailed for several hours. This plan allowed us to follow the onset of denitrification activity as a function of O_2 availability. The activity in the samples and in one blank at the beginning of each incubation was terminated using $ZnCl_2$ solution. The samples were transferred to Exetainers and the mass ratios of N_2 formed during incubations and those of the original plankton concentrate (for background subtraction) were analysed by the National Environmental Research Institute. The significance of the changes in mass ratios was evaluated using a paired t-test. Denitrification was only considered to occur in samples in which both ^{15}N-labelled fractions ($^{29}N_2$ and $^{30}N_2$) increased significantly ($p<0.05$). Denitrification (as nmol N_2 formed) was calculated from the differences between the isotopic compositions at the end and at the beginning of the incubation. The denitrification rate per hour (nmol N_2 l^{-1} h^{-1}), however, was calculated using only the incubation time when anoxic conditions prevailed, as the denitrifying bacteria are facultative anaerobes that switch to denitrification as a form of anaerobic metabolism when the oxygen concentration decreases (Tiedje 1988).

N_2O production was measured in each plankton concentrate sample parallel to denitrification. Samples (23 ml glass vials with septums, no replicates) were incubated following the same incubation plan as in the denitrification measurements, sampling at times when oxic, hypoxic and anoxic conditions prevailed. In order to measure both natural N_2O production and N_2O production potential, two sample sets were prepared. Altogether 8 ampoules were filled with phytoplankton suspension. Half of the ampoules were enriched with the KNO_3 solution to a final concentration of 100 µM while the other 4 remained at natural nutrient concentrations. Background subtraction blanks (one with and one without KNO_3 addition) were prepared by adding 250 µl formalin before sealing the ampoules. During incubation at the *in situ* temperature, the samples were slowly rotated on a test tube mixer. Incubations were terminated at the same time as in the denitrification measurements by injecting 250 µl formalin through the septum with an overflow needle inserted. Samples were later analysed in the same way as in 1999.

2.4.2 Attached bacteria

On August 10th, 1999, aggregates were collected in the morning (n=9) and in the evening (n=8) to enumerate the attached heterotrophic bacteria. In 2000, the number of bacteria attached to cyanobacterial filaments was counted from 10 ml samples of the plankton concentrate. The procedure used is described in detail in articles **IV** and **V**. The methods used to investigate whether species capable of denitrification colonised the aggregates are presented in article **V**.

2.4.3 Oxygen production and respiration

In 1999, the oxygen production (photosynthesis) and respiration of the natural aggregates was measured in the morning and the afternoon of the sampling days using a micro-Winkler (titration) method. Aggregates (1-3) were transferred to numbered, volume-calibrated (circa 22 ml) glass-stoppered glass vials filled with 10 µm filtered surface water. Three similar series of five replicate samples were used for 1) initial oxygen concentration, 2) oxygen production and 3) respiration measurements. Samples were incubated either in the dark (respiration) or in the light (oxygen production) at the *in situ* temperature for 3 to 6 hours. During incubation the vials were rolled slowly on a test tube mixer. Oxygen production and respiration were calculated from the changes in oxygen concentrations in the vials. No ambient water subtractions were made, as respiration in seawater is negligible over such short incubation times (Kuparinen 1987). The measured respiration rates were compared to the rates needed to create anoxia at the centre of a spherical aggregate of a similar volume according to Ploug & al. (1997) using the oxygen diffusion coefficient, the aggregate diameter and the temperature, salinity and oxygen concentration at the sample station. The aggregates were assumed to have a full diffusion boundary layer, as cyanobacterial filaments are positively buoyant due to intracellular gas vacuoles (no sinking-induced erosion of the diffusion boundary layer) and often rich in mucus, further slowing the diffusion of gases in and out of the aggregates.

In 2000, four replicate samples of the plankton concentrate in volume-calibrated, approximately 11 ml vials were used for the initial oxygen concentration measurement and four replicates were incubated at the *in situ* temperature in the dark for 90 to 150 min to measure the community respiration. The linearity of oxygen decrease was tested twice during the cruise and the respiration was found to be linear for at least 6 hours, down to 80 µM O_2 (data not shown). Results from the respiration measurements were used to adjust the incubation times for the denitrification and N_2O production samples.

3 RESULTS

3.1 Pelagic bacterial production

3.1.1 The effect of using different filters or centrifugation

Polycarbonate filters

Using polycarbonate instead of cellulose nitrate filters did not have a systematic effect on incorporation rates when measuring ^{14}C-leucine incorporation (Fig. 2, PC, shaded bars). A nested ANOVA revealed no significant differences between the filters, and in the one-way ANOVA run for each experiment separately, only once out of 11 experiments did the results from the polycarbonate filters differ significantly from those from the cellulose nitrate filters (Table 1, PC, experiments 1-12).

In contrast, filtering the samples on polycarbonate instead of cellulose nitrate filters when using ^{3}H-thymidine made a surprisingly large difference in the results (Fig. 2, PC, white bars). In all ten cases the results were lower than those achieved using the reference treatment (CN), and in 6 cases out of 10 the difference was significant (Table 1, PC, experiments 13-22). No correlations between significant differences and season, sample type or the ratio of blank/sample were found.

Centrifugation treatments

When measuring ^{14}C-leucine incorporation, the results from the centrifugation treatments were lower than those from the reference treatment 19 times out of 23 (Fig. 2, C1 and CT), and a nested ANOVA showed significant differences between treatments. When analysed with a one-way ANOVA separately for each experiment, significant differences in the cases when samples were incubated in microcentrifuge tubes were only found 4 times out of 12 (Table 1, C1). The difference was more pronounced when subsamples from a larger incubation volume were transferred to microcentrifuge tubes; in 7 cases out of 11 the difference was found to be significant (Table 1, CT). Significant differences seemed to be related neither to the season of the sampling, nor to whether the sample was natural or from a batch culture. There was no correlation between the ratio of blank DPM/sample DPM and the significant differences found.

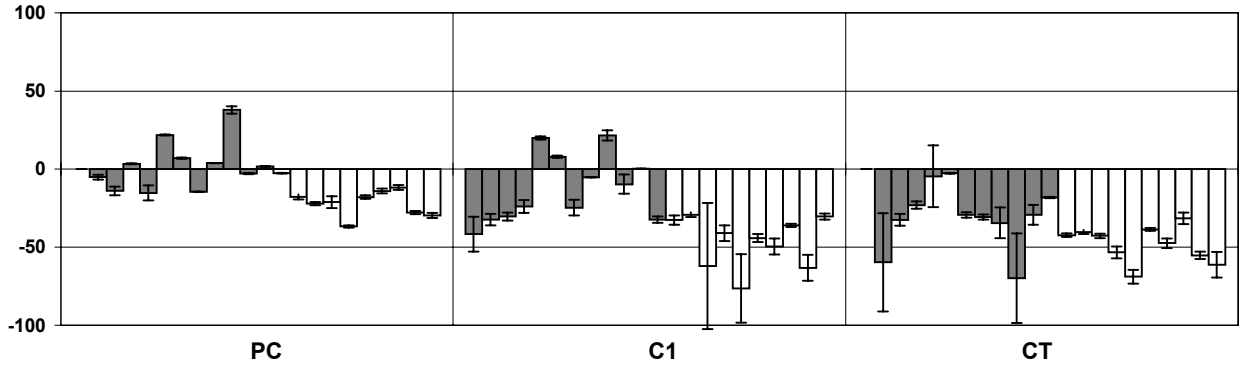

Fig. 2. Percentual difference in results achieved by using other treatments compared to those achieved using the reference treatment (cellulose nitrate filtering) (avg ± SD). The negative bars show values lower than those achieved with the reference method whereas the positive bars show values higher than those achieved with it. Shaded bars: experiments using leucine; white bars: experiments using thymidine. PC = filtering the samples on polycarbonate filters, C1 = centrifuge method when incubating 1 ml samples in microcentrifuge tubes, CT = centrifuge method when incubating a large sample volume and transferring 1 ml subsamples after incubation to microcentrifuge tubes for processing.

To find a reason for the systematically lower results of the centrifugation treatments compared to the filtration, in one experiment (Exp. 12, May 1999, using ^{14}C-leucine) both the supernatants and the corresponding filtrates were collected and their radioactivity measured in a scintillation counter. Adding the supernatant or filtrate counts to those of the corresponding samples should give a 100% yield of the radioactivity added to the samples. However, while for all the filtration treatments this proved to be true (yield 97% to 102%), the yield from both of the centrifugation treatments varied from 76% to 81%. No explanation was found for this loss of label in the centrifugation treatments.

When ^{3}H-thymidine incorporation was measured, the centrifugation method failed to produce results comparable to the filtration methods. All 10 experiments with the centrifugation treatment showed significantly lower results (range 30-75%) compared to the reference treatment (CN), both when incubating samples in microcentrifuge tubes (C1), and when transferring subsamples to the tubes (CT) (Fig. 2, C1 and CT, Table 1, C1 and CT).

3.1.2 Leucine and thymidine incorporation by cyanobacteria

All strains studied incorporated leucine (Fig. 3). The incorporation rate varied between the strains and was significantly (t-test, p<0.01) higher for the strain HKVV (Fig. 3e) starved of nutrients than for that grown in abundant nutrient conditions, whereas it was significantly lower for the strain PCC 7804 (Fig. 3c) starved of nutrients than for that grown in abundant nutrient conditions. For *M. aeruginosa*, the incorporation rate was significantly higher in the culture grown in abundant nutrient conditions (Fig. 3a).

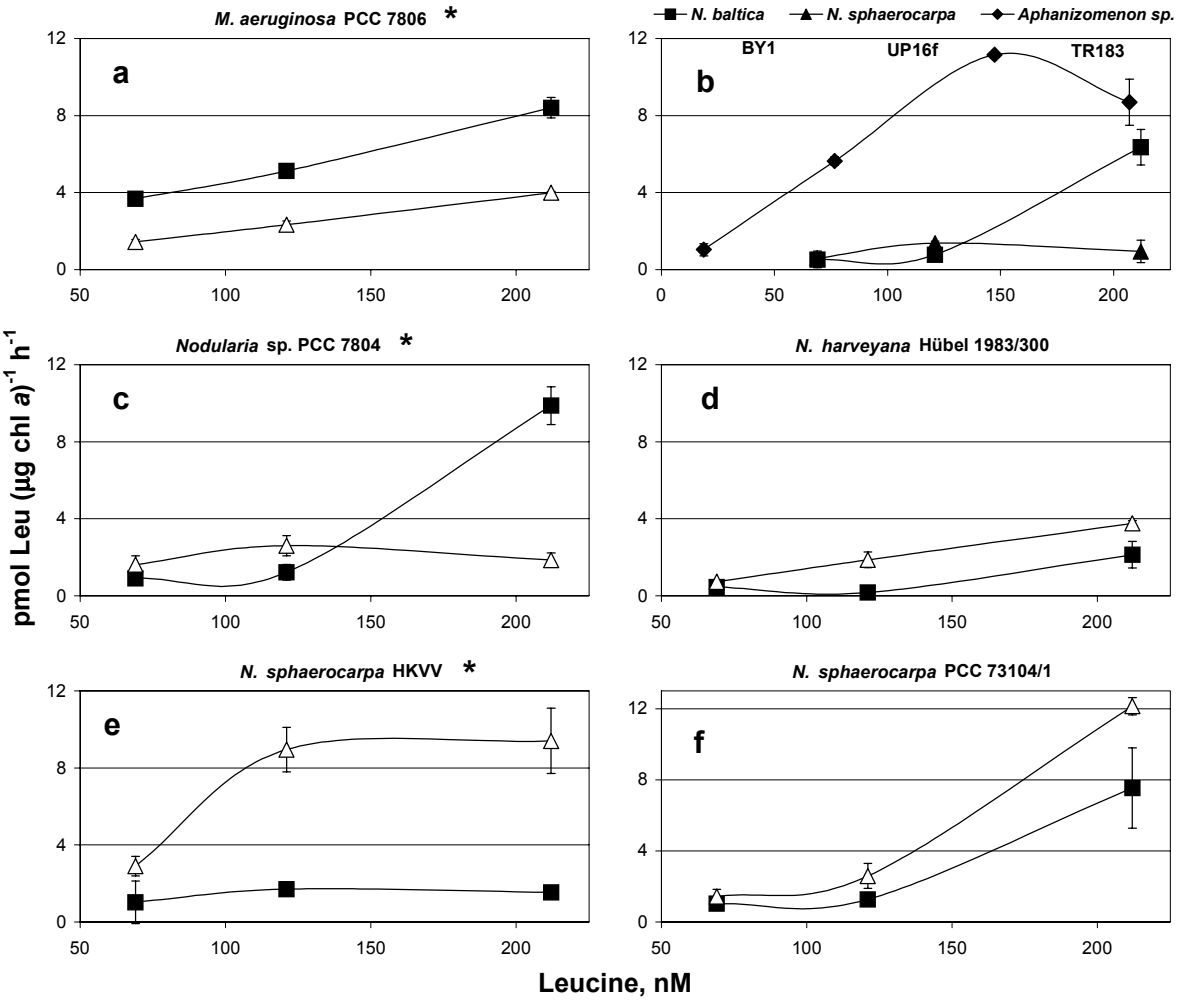

Fig. 3. Cyanobacterial leucine uptake velocity pmol leucine (µg chl *a*)$^{-1}$ h^{-1} at increasing leucine concentrations (avg ± SD). Cyanobacteria were grown in nutrient-rich (filled symbols) and nutrient-poor (open symbols) growth media. * marks significant (p < 0.01) differences in uptake between the cultures grown in nutrient-rich and nutrient-poor media.

No significant difference in uptake was detected in the other two strains grown in nutrient-rich and nutrient-poor conditions (Fig. 3d, f). Evidently, most of the strains were not saturated even at the highest leucine concentration used. The blank counts of cyanobacteria samples were on average 45% of the true samples (DPM-difference between blanks and samples ranging from 52 to 2685 DPM, average 699 DPM) whereas the blank counts were 15% of the sample counts in seawater (DPM-difference between blanks and samples 1792 - 2115 DPM, average 1965 DPM). In contrast to leucine, thymidine was either incorporated at a very low rate, or not incorporated at all (Fig. 4), blanks giving higher values than the samples. Some of the strains seemed not to be saturated even at the extremely high thymidine concentration used (Fig. 4a, b, d, e), although the incorporation rate was very low. The blank counts were on average 89% of the cyanobacterial sample counts, while in seawater they were 9% of the sample. The DPM values after blank subtraction averaged 338 at the lowest and 3334 at the highest thymidine concentration when negative values were included, and 555 and 4187, respectively, when negative values were marked as zero. The corresponding average values in the seawater sample (ca. 2.4×10^6 heterotrophic bacterial cells ml^{-1}) were 35979 at the lowest and 119385 at the highest incubation concentration.

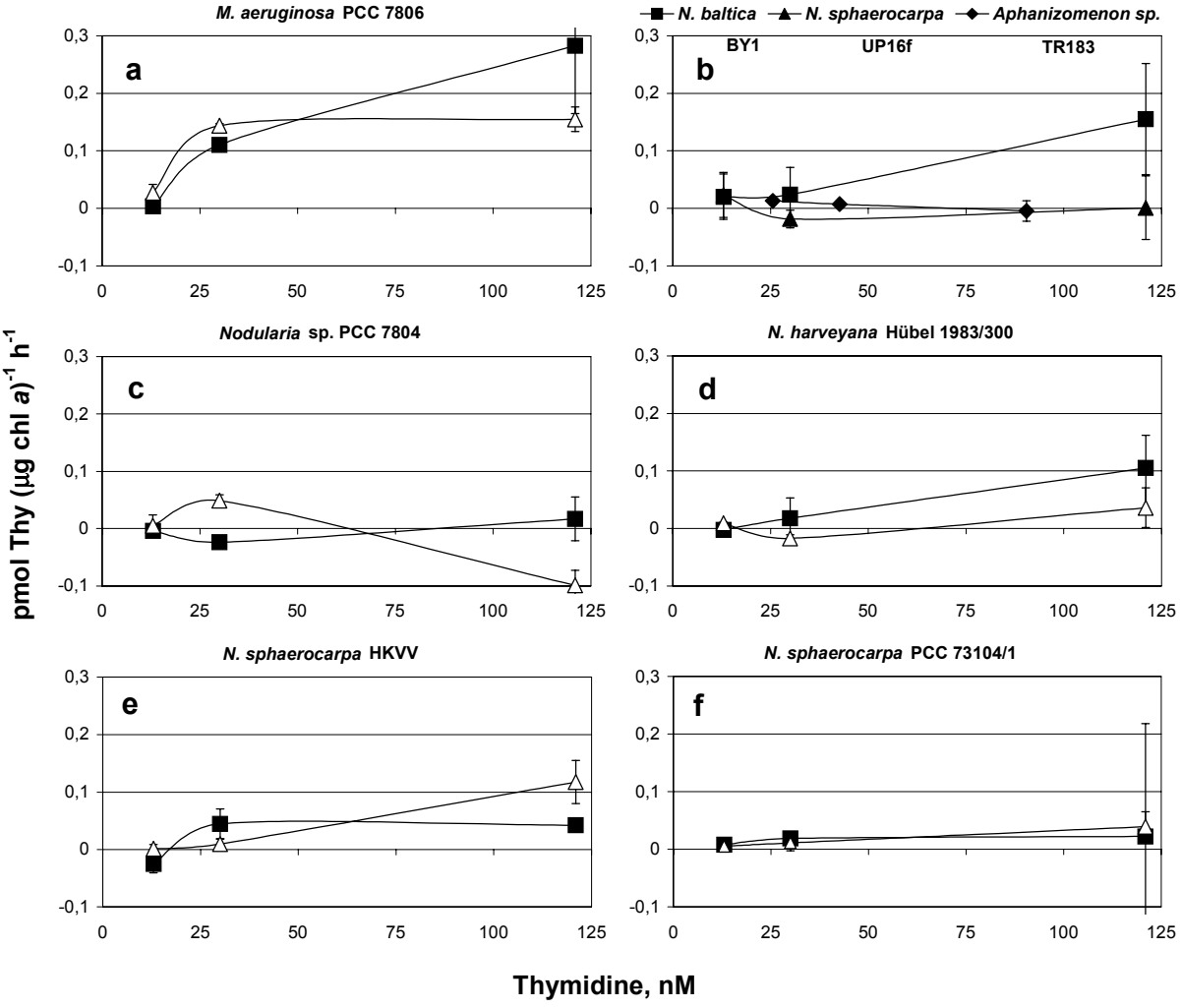

Fig. 4. Cyanobacterial thymidine uptake velocity pmol thymidine (µg chl *a*)$^{-1}$ h^{-1} at increasing thymidine concentrations (avg ± SD). Cyanobacteria were grown in nutrient-rich (filled symbols) and nutrient-poor (open symbols) growth media.

3.2 Benthic bacterial production

The linear correlation of leucine incorporation between the samples measured as a gel and as combusted samples was high. In 6 cases out of 8, the constant of the regression equation did not differ significantly from zero, and in 5 cases the value of 1 for the coefficient was within the 95% confidence interval (**III**: Fig. 2). The counting efficiency was almost constant on each sampling occasion (CV% 0.2-6.4%), so the use of an internal standard for each sampling site provided a reliable DPM conversion. The counting efficiency varied, however, between the sampling stations and seasonally within stations (from 46 to 87%).

The saturation level at the different stations and sampling times varied from 2.5 to 3 µM of leucine (**III**: Fig. 3). Time series incubations showed that ^{14}C-leucine incorporation was linear for at least 230 min (**III**: Fig. 4). The amount of sediment used in the incubations did not affect the leucine incorporation rate during the 45 to 140 min routine incubations (**III**: Fig. 4). The decision to use 100 µl of sediment in routine incubations was based on practical sample handling. This amount can be pipetted reliably using an adjustable dispenser (Finnpipette Stepper, Labsystems). Smaller amounts of sediment were difficult to measure accurately, while larger amounts increased the variance of the counting efficiency. Differences in the amounts of near-bottom water (0-400 µl) to make a slurry had no effect on the leucine incorporation rate (data not shown).

Bacterial production in the top 0-1 cm layer varied from 36 to 192 mg C m^{-2} d^{-1} at the 4 stations (Fig. 5). At stations GF2 and JML in the Gulf of Finland, where the production was also measured in the 1-2 cm layer, 55 and 68%, respectively, of the measured 0-2 cm layer bacterial production occurred in the top 1 cm of the sediment (**III**: Fig. 5). In the Bothnian Sea (Stn SR5), where the oxygenated layer is deeper, hardly any differences between the three topmost 1 cm layers studied could be detected (**III**: Fig. 5). According to these estimates, the benthic bacterial production of the 2 or 3 cm deep sediment layers at these stations ranged from 90 to 350 mg C m^{-2} d^{-1}. At the deep station in the northern Baltic Proper (LL17, 172 m), no vertical distribution of production was studied, due to seemingly anoxic sediments below 1 cm.

3.3 Production of cyanobacteria-associated bacteria

The thymidine uptake of the cyanobacteria-associated bacteria in the plankton concentrate was saturated at approximately 100 nM thymidine (Fig. 6) and the incorporation was linear for at least 60 min. The thymidine incorporation rate in the samples varied from 61 to 597 pM h^{-1}. Bacterial carbon production calculated using the conversion factor determined from cell numbers (3.5×10^{17} cells per mol) (Fig. 7), an average cell volume (0.077 µm^3, Tuomi 1997) and carbon content (0.1 pg µm^{-3}, Fagerbakke & al. 1996) resulted in a bacterial carbon production rate of 0.17 – 1.61 µg C l^{-1} h^{-1}. When calculated using the conversion factor determined from biovolume increase (2.8×10^{16} µm^3 per mol) (Fig. 7) and the average carbon content, the value for bacterial production in the plankton concentrate was essentially the same, 0.17 - 1.67 µg C l^{-1} h^{-1}. Cell numbers varied from 0.54 to 3.6×10^8 cells ml^{-1}.

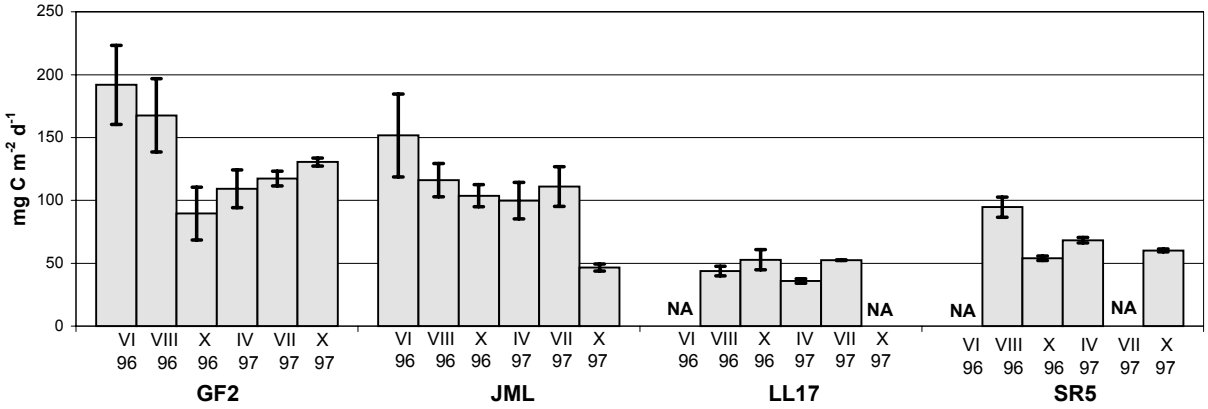

Fig. 5. Seasonal variation of the benthic bacterial production mg C m^{-2} d^{-1} (avg ± SD) at the stations studied. NA = not analysed.

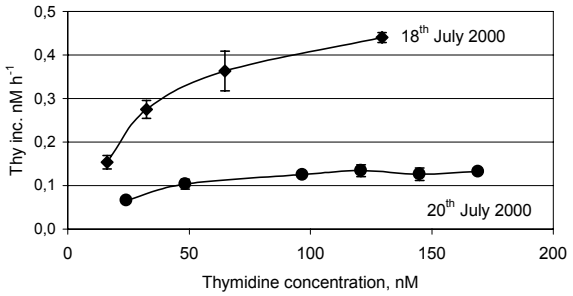

Fig. 6. Thymidine incorporation saturation curves of the cyanobacteria-associated bacteria in the plankton concentrate, July 2000.

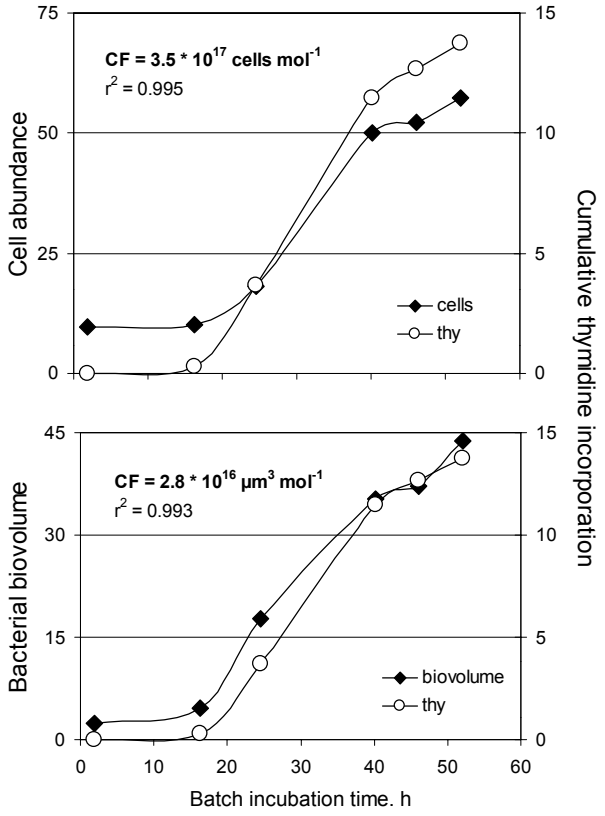

Fig. 7. Results from a batch culture experiment to determine conversion factor for thymidine incorporation of the cyanobacteria-associated bacteria in August 2000. Bacterial cells (10^8 cells l^{-1}), cumulative thymidine incorporation (nmol l^{-1}) and bacterial biovolume (μm^3 l^{-1}) as a function of incubation time of the batch culture.

3.4 Denitrification

Aggregates collected from the Gulf of Finland in August 1999 were densely colonised by bacteria. On the day of enumeration (August 10th), single aggregates had an average of 3.3×10^8 cells attached to them, compared to the average of 1.3×10^6 cells ml^{-1} in the surrounding water. When calculated against the average volume of a single aggregate on the sampling day, 0.034 ml, the enrichment factor over ambient water was more than 7000. The aggregates were shown to harbour bacteria with genetic potential for denitrification (**V**). Unfortunately, no information on the abundance of the aggregates at the sampling site is available. The net oxygen production (photosynthesis minus respiration) of the aggregates varied between 0 and 240 nmol O_2 per aggregate h^{-1}, and respiration from 10 to 100 nmol O_2 per aggregate h^{-1}. Daily averages run from 3.9 to 128.3 nmol O_2 produced per aggregate h^{-1}, and from 27.3 to 70.2 nmol O_2 respired per aggregate h^{-1} (**V**: Table 1). The respiration rate was high enough to result in anoxia in the centre of the aggregate when incubated in darkness in all cases except on the morning of 4th August and on the afternoon of 10th August. In the light, no anoxia would be expected, as the gross oxygen production within the aggregates always exceeded the measured dark respiration, making the aggregates net autotrophic (more oxygen produced than respired) in the light.

In the plankton concentrate samples of 2000, bacterial cell numbers varied from 0.54 to 3.6×10^8 ml^{-1} and bacterial production varied from 0.17 to 1.67 µg C l^{-1} h^{-1} (calculated using the biovolume-based conversion factor). Community respiration in the plankton concentrate varied between 12.3 and 60.0 µM h^{-1}.

The isotope pairing method is based on adding ^{15}N-labelled nitrate to samples and, after incubation, measuring the relative increase of the partly ($^{14}N^{15}N$) and completely ($^{15}N^{15}N$) labelled N_2 molecules. From the changes in isotopic composition it is possible to calculate not only the denitrification rate based on the added nitrate but also the denitrification taking place in nature, that is, based on nitrate readily available or produced *in situ* by nitrification.

In the natural aggregate samples collected at the entrance to the Gulf of Finland in August 1999, both a relative increase and a decrease of the heavier isotope was detected. In 29 of the 32 samples analysed, either one or both of the fractions was lower after incubation than before it, thus making calculations senseless. The activity in the remaining 3 samples varied from 0.16 to 1.50 pmol N_2 per aggregate h^{-1} based on added nitrate and from 0.04 to 0.23 pmol N_2 per aggregate h^{-1} based on *in situ* available nitrate. A small amount of N_2O (a side product of nitrification and an intermediate product of denitrification) was produced in 15 out of 19 aggregate samples, whereas no N_2O production was detected in the 3 ambient water samples. In some aggregate samples, as well as in all ambient water samples, the amount of N_2O after incubation was lower than at the beginning of incubation. The range of changes compared to the zero time measurement was from -9 to 71 pmol per aggregate h^{-1}.

The following year, no aggregates were available and net-collected samples were used instead in the

experiments, in which optimal conditions for denitrification were created to see whether the activity could be enhanced to a detectable level. In the 23 plankton concentrate samples from August 2000, both an increase and a decrease of the heavier isotopes were again detected. In some samples the increase in one fraction and decrease in the other were measured, showing the limits of the method in such a low-activity environment. Only in three cases could a significant increase (t-test, $p<0.05$) be detected in both isotopic fractions at the same time (**IV**: Fig. 2a) and the results of these three samples were calculated further. In the 3 occasions out of 23 when calculating denitrification was meaningful, the rate based on the added $^{15}NO_3$ varied between 1.3 and 3.9 nmol N_2 l^{-1} h^{-1}, whereas the rate based on naturally-occurring $^{14}NO_3$ varied between 0.8 and 1.8 nmol N_2 l^{-1} h^{-1} (**IV**: Fig. 3). The amount of N_2O slightly decreased during incubation in most cases, but clear increases were noticed in six cases out of 23 measurements when the samples were enriched with NO_3 (**IV**: Fig. 2b). The N_2O production rates varied between –0.55 and 0.39 nmol l^{-1} h^{-1} in the natural samples and between –0.41 and 12.48 nmol l^{-1} h^{-1} in the enriched samples. In the three above-mentioned samples, the natural N_2O production rate varied from –0.55 to –0.05 nmol l^{-1} h^{-1} and in the enriched samples from 0.13 to 4.40 nmol l^{-1} h^{-1}.

4 DISCUSSION

4.1 Pelagic bacterial production

4.1.1 The effect of using different filters or centrifugation

Polycarbonate filters

The filters tested here were both of pore size 0.2 µm. Blanks were always lower when using polycarbonate filters. No significant difference could be found between the filters when ^{14}C-leucine incorporation was measured, except in 1 out of 11 experiments. However, when measuring ^{3}H-thymidine incorporation, the results were always higher when cellulose nitrate filters were used, compared to those of the polycarbonate filters. Hollibaugh & Wong (1992) as well as Kirchman (1992) and Kiene & Linn (1999) came up with similar results. Higher counts from thicker, matrix-type cellulose nitrate filters could be an artefact of unincorporated isotope binding within the filter. However, after performing re-filtering experiments, Hollibaugh & Wong (1992) concluded that some of the originally "particular" material became "dissolved" when passing through the polycarbonate filter. Lower counts would therefore result from inefficient particle trapping on polycarbonate filters, not from "over-efficient" (abiotic) trapping of unincorporated isotope on mixed cellulose ester filters. Kiene & Linn (1999), likewise, suggested that desiccation of cells at the end of filtering, when the filters run dry, could cause rupture of the cells and therefore loss of label.

That differences were found when using thymidine, but not when using leucine, might be related to the different sensitivity of the corresponding labelled macromolecules to the conditions during precipitation. ^{3}H-thymidine labels DNA, whereas ^{14}C-leucine is incorporated into proteins. TCA is used to precipitate macromolecules (such as DNA, RNA, proteins and lipids), and to wash away, without breaking the cells, the excess label not incorporated – although possibly uptaken - in the samples (Riemann 1984). When precipitating DNA, extra care must be taken to keep the conditions ice-cold, as a rise of even a few degrees may hydrolyse parts of the DNA and lead to losses of the label (Riemann 1984). The protein fraction is more resistant to temperature: originally a hot-TCA extraction was used to separate the proteins from other macromolecules (Kirchman & al. 1985). The experiments of Chin-Leo & Kirchman (1988) and Wicks & Robarts (1988), however, proved this to be unnecessary, and nowadays the TCA extractions in the leucine method are commonly done at room temperature. The cellulose nitrate filters are sponge-like and have a fast flow rate. Therefore, they not only make the filtering procedure quicker, but may also keep the temperature of the cells on the filter more stable than do the very thin polycarbonate filters. Even when the cellulose nitrate filters “run dry” between the washing rounds, they still retain some of the TCA, whereas the polycarbonate filters do not, exposing the cells more to the room temperature. The fragility of the DNA fraction might also explain why differences were only found when using polycarbonate filters. If the cells indeed break due to desiccation at the end of the filtering, as suggested by Kiene & Linn (1999), then the macromolecules are possibly more exposed to the TCA treatment, and some of the labelled DNA is lost. Breakage of the bacterial cells does not, however, seem likely, as the method commonly used to calculate bacteria in water samples relies on filtering the cells on polycarbonate filters similar to those used in these studies (Hobbie & al. 1977). The filtered bacteria in such samples seem unharmed by the filtering procedure, judging from the clearly-defined cell shapes.

Centrifugation

On 31 times out of 43 the results of the centrifugation treatment were significantly different (in all these cases: lower) compared to the reference treatment (filtering on cellulose nitrate filters). Smith & Azam (1992) added scintillation liquid (0.5 ml)

straight into the microcentrifuge tubes, and placed these into scintillation vials to be counted, whereas here the microcentrifuge tubes were cut in scintillation vials, resuspending the precipitate in a larger amount of scintillation liquid. This was likely to improve counting efficiency by making samples more homogenous and diminishing the effect of plastic tubes on the counting (radioactivity dissolved in 10 ml of scintillation liquid, instead of in 0.5 ml inside a microcentrifuge vial, standing inside yet another vial). In our samples, there was no difference in the counting efficiency between the filter and centrifugation samples, either when using ^{14}C-leucine, or when using ^{3}H-thymidine. The other major difference in treating the sample was the number of "washing rounds" (mixing the sample with 5% TCA, centrifuging and removing the supernatant). Smith & Azam (1992) washed their samples once only (either with 5% TCA or with 80% ethanol) after the first precipitation with TCA. In the preliminary tests for the experiments reported here it was noticed that three washes with 5% TCA were needed to remove unincorporated isotope from samples. On the other hand, the filters were also washed more extensively than in the experiments of Smith & Azam (1992) (2 ml + 5 × 1 ml compared to 2 × 1 ml). In the experiments reported here, the effect of extracting the samples with 80% ethanol was not tested, but this step would have been expected to affect the samples in the same way in all treatments.

One reason for the systematically lower results achieved with the centrifugation treatments might be that not only the unincorporated label but also the sample was washed away in the repeated washing rounds. Collecting the supernatants and filtering them on cellulose nitrate filters proved that this was indeed the case. Up to 30% of the incorporated isotope was in some samples found in the combined supernatants. Decreasing the number of washing rounds, however, resulted in higher blank values, and thus after blank subtractions the final results were the same. It seems that the 10 min centrifuging at 15 988 × *g* (used by Smith & Azam 1992 and Kirschner & Velimirov 1999b) or 25 848 × *g* is not enough to make the pellet stick to the tube while the supernatant is sucked out. Smith & Azam (1992) tested the effect of adding either bovine serum albumin or sodium deoxycholate as co-precipitants in the samples. They found no significant difference in the results between the samples with and without a co-precipitant. Kirschner & Velimirov (1999b) used the centrifugation method for freshwater samples and tested the effects of various co-precipitants (humic extract, tris-buffered saturated ammonium sulphate solution (SAS), tris-buffered sodium chloride (NaCl) and non-buffered SAS and NaCl). They found out that for freshwater samples, an addition of co-precipitant was needed in order to get reliable results, and that the highest counts were always achieved using unbuffered NaCl. The sample water used in our experiments was brackish with low salinity (5 to 7 psu), and therefore adding a co-precipitant could indeed have improved the method performance. Results from the southern Baltic Sea show that the correlation between the centrifugation and filtration methods (using PC filters) is only adequate during winter (Hanna Giesenhagen, Institut für Meereskunde an der Universität Kiel, personal communication), when salty water regularly flows into the Baltic Sea from the North Sea. This also suggests that salt plays an important role as a co-precipitate. Kirschner & Velimirov (1999b) only got reliable results when using 3.5% NaCl (final conc.), which is comparable to the salinity of the oceans. They pointed out that using a co-precipitant results in a visible pellet, making withdrawal of the sample by accident less likely, but that the precipitated proteins within the pellets need to be redissolved by boiling in NaOH for 20 minutes prior to adding the scintillation liquid. Co-precipitation with NaCl, therefore, adds an extra step to the centrifugation treatment procedure.

In addition to the above-mentioned problems, there was also the unexplained label loss when using the centrifugation method. Not only was some incorporated isotope lost in the washing rounds of the samples, subsequently to be found in the supernatant, but some of the added isotope (around 20%) vanished altogether from the samples during the processing. This was especially surprising as there were less steps at the sample treatment procedure (transferring subsamples etc.), known to cause some loss of the label in each step, than in the traditional filtration method. So far, no explanation for this phenomenon has been found.

In the light of all these uncertainties, the centrifugation method cannot be recommended – at least without an addition of salt to the samples, consequently resulting in the need to redissolve the precipitate by boiling in NaOH – as the method of choice for bacterial production measurements in the brackish Baltic Sea.

4.1.2 Leucine and thymidine incorporation by cyanobacteria

The high blank values noticed in the experiments using axenic cyanobacterial cultures probably reflect extensive isotope adsorption to the cyanobacterial surfaces, which are often covered with mucus. Adsorption to mucus has been shown to be even more extensive for thymidine than for leucine (Schuster & al. 1998). In any case, adsorption can be expected to be similar in both blanks and samples, and hence blank subtraction effected a reliable elimination of adsorbed (not incorporated) isotope from the calculations.

The leucine incorporation per µg chlorophyll *a* varied from 0.95 to 12.4 pmol h^{-1} at the highest incubation concentration (> 200 nM). Some strains were clearly not saturated, a fact that might be related to leucine adsorption to the mucus. Only after all the adsorption sites are occupied will there be free leucine left for uptake (Schuster & al. 1998). Under non-saturated conditions, internal and external isotope dilution can be expected to occur, leading the measured values to be underestimates of the real uptake capacity. A conservative estimate of the effect of cyanobacteria on the heterotrophic bacterial production measurements using the leucine method can be calculated. In late summer 1990 (at chlorophyll *a* of 1.8 µg l^{-1}), heterotrophic bacteria in the Gulf of Finland incorporated 90 pM leucine h^{-1} (Heinänen & Kuparinen 1992). In the Gulf of Finland, late summer cyanobacterial blooms have been shown to reach chlorophyll *a* concentrations of up to 18 µg l^{-1} (Heinänen & al. 1995). At such a chlorophyll *a* concentration, the leucine incorporation of the cyanobacteria (17-218 pM h^{-1}) would be equal to or even higher than that of the heterotrophic bacteria. Kamjunke & Jähnichen (2000) calculated the overestimate caused by a *Microcystis* bloom in a eutrophic lake to be in the same range (20-140%). They pointed out that during maximum biomass, cyanobacteria are often already senescent, and therefore less likely to actively incorporate leucine, but that they nevertheless may cause significant overestimation of heterotrophic production. Although the late summer chlorophyll *a* (mainly cyanobacteria) in the northern Baltic Sea occasionally reaches peaks as high as those mentioned above, a more typical value is between 3 and 4 µg l^{-1} (Lignell 1990, Heinänen 1991, Kivi & al. 1993, Heiskanen & Kononen 1994, Ohlendieck & al. 2000). In addition, if the weather is calm, the cyanobacteria accumulate on the surface, with a very small biomass in the rest of the water column. Thus, the results from the earlier studies using the leucine method during cyanobacterial blooms are probably not seriously biased, with maybe the exception of overestimates in the samples collected from the uppermost, surface scum layer. In contrast, when the thymidine method has been used without determining the thymidine saturation level in the bloom samples, non-saturating conditions may have prevailed, as suggested by the high level of isotope adsorption to the cyanobacterial filaments. Heinänen (1993) found no saturation of thymidine incorporation even at the highest concentration tested (28 nM) in late summer in the Baltic Sea (Table 3), and in the experiments reported in article **IV**, a 100 nM thymidine concentration was needed to saturate incorporation (Table 3). As mentioned above, a non-saturating thymidine concentration causes isotope dilution in the samples and results in underestimates of the bacterial production.

In these experiments the *Microcystis aeruginosa* strain incorporated leucine at a rate that was only 10% of that measured by Kamjunke & Jähnichen (2000). Obviously, the growth conditions (nutrients, light, growth phase) have a large effect on incorporation. Here, the cyanobacterial leucine and thymidine incorporation was measured in the dark, as this is the standard method for measuring heterotrophic bacterial production. Kamjunke & Jähnichen (2000) reported enhanced leucine incorporation by *M. aeruginosa* in light, and this effect must be kept in mind if light incubations for bacterial production measurements are started on a routine basis, as suggested by Morán & al. (2001).

Kamjunke & Jähnichen (2000) suggested that the leucine uptake by *M. aeruginosa* PCC 7806 could be related to the toxin production, as leucine is one constituent of microcystin-LR. Several strains of *Nodularia* produce a toxin, nodularin, which does not contain leucine, and all the *Nodularia* strains tested here incorporated leucine independently of their ability to produce nodularin, as did also the tested, non-toxic *Aphanizomenon* sp. TR183 strain. Leucine also did not seem to be taken up as a source of nitrogen or carbon, as both significantly higher (*N. sphaerocarpa* HKVV) and significantly lower (*Nodularia* sp. PCC 7804, *M. aeruginosa* PCC 7806) uptake was detected in cultures starved of nutrients, compared to those grown in abundant nutrient conditions. This suggests that the cyanobacteria incorporate this small amino acid to use it as a building block for other cell products, such as proteins, as the heterotrophic bacteria do. So far, the only cyanobacteria shown to incorporate leucine are *Nodularia* (**II**), *Aphanizomenon* (this study) and *Microcystis* (Kamjunke & Jähnichen 2000, **II**). Experiments with *Synechococcus* showed no incorporation at a leucine concentration of 24 nM (Torreton & Dufour 1996), neither was incorporation detected in experiments with coccoid cyanobacteria at a 0.5 nM concentration (Kirchman & al. 1985). However, more information is needed on the potential leucine incorporation of other common cyanobacterial species, such as *Anabaena*. More experiments using axenic strains should be performed using leucine concentrations relevant to eutrophic aquatic environments, as it is possible that leucine is also taken up by other bloom-forming cyanobacteria than *Microcystis*, *Aphanizomenon* and *Nodularia*. Obviously, the leucine incorporation method should not be used during cyanobacterial blooms.

Table 3. Summary of the bacterial parameters from the various growth environments in the northern Baltic Sea.

	Free-living	**Cyanobacteria-associated**	**Sediment***
Leucine saturation level, µM	0.04-0.1 [1]		2.5-3 [III]
BCP mg l^{-1} d^{-1}, leucine method	0.242 [1]		3.6-19.2 [III]
Thymidine saturation level, nM	5-21, >28 [2, 3]	100 [IV]	50-400 [3]
Conversion factor 10^{18} cells mol^{-1}	0.32-6.89 [3, 4, 5, 6]	0.35 [IV]	2.5 [3 **]
Conversion factor 10^{17} µm^3 mol^{-1}	1.1-1.37 [7]	0.28 [IV]	
BCP µg C l^{-1} d^{-1}, thymidine method	2.8-14.9 [1, 8, 9, 10]	0.0003-0.04 [IV]	1000-150000 [3, 11]
Cell abundance 10^6 cells ml^{-1}	2.8-10 [1, 4, 5, 10]	0.03-0.21 [IV]	320-1380 [III]
Denitrification, nM N_2 h^{-1}	0.9-2.1 [12, 13]	1.3 [IV]	300-1350 [14]
Remarks	Denitrification limited to oxic-anoxic interface	Leucine method not possible due cyano-bacterial leucine uptake	

* calculated for top 1 cm
** calculated for the water column bacteria
[1] Heinänen & al. 1995
[2] Heinänen 1993
[3] Tuomi & al. 1999
[4] Heinänen & Kuparinen 1991
[5] Autio 1992
[6] Heinänen & Kuparinen 1992
[7] Tuomi 1997
[8] Autio 1990
[9] Lignell 1990
[10] Heinänen 1991
[11] Ekebom 1999
[12] Rönner & Sörensson 1985
[13] Brettar & Rheinheimer 1992
[14] Tuominen & al. 1998

4.2 Benthic bacterial production

4.2.1 Methodology

The protein extraction efficiency of the gel method was not tested, nor was it compared to the often-used alkaline-extraction method. The gel method, however, gave results which were exactly the same as the combustion method that has been successfully used for mud sediments in lakes (Tuominen & Kairesalo 1992, Tuominen 1995). The combustion method, in which unincorporated leucine is removed by repeated washings with ethanol, has been criticised for measuring the total leucine uptake instead of its incorporation into proteins (Fischer & Pusch 1999). In the gel method, 5% TCA is used, but no difference in washing efficiency could be detected between washing the samples with TCA, ethanol or both. The washing round with ethanol was included in the routine as it removed most of the pigments, thus increasing the counting efficiency. The blank counts were generally well below 10% of the sample counts, and the standard deviation between replicate samples was lower than with the combustion method. Taking all this into account, the gel method is a reliable, relatively cheap (no filters, small amounts of reagents, less radioactive waste created), fast and easy-to-use way to measure benthic bacterial production in mud sediments. In addition, it allows samples to be already counted on board, which facilitates on-site methodological adjustments (isotope concentration, incubation time). In these experiments no unlabelled carrier was used, and the DPM counts (after blank subtraction) ranged from about 3 000 to about 25 000 DPM. The sensitivity of the assay would not, consequently, be compromised by the use of unlabelled carrier to lower the costs. In mud sediments of the Baltic Sea, the saturation level varied between the stations and seasons, but uptake was usually saturated at 2.5 to 3 µM of leucine (Table 3), and was linear for at least 230 min. The counting efficiency varied more, and needs to be measured using internal standardisation for each station and season.

When comparing bacterial production rates from different studies, one has to keep in mind the use of different conversion factors. In studies where carbon production is measured from the leucine uptake, the theoretical conversion factor of 1.5 kg C per mol leucine taken up (Simon & Azam 1989) is most often used. When the thymidine incorporation method is used, additional conversion factors for incorporation to cell production, cell volume and carbon per cell must be determined, making comparisons between studies difficult. Such conversion factors cannot be derived experimentally from sediment samples, and therefore values obtained from the water column must be used, adding to the uncertainty of

the final results (Table 3). Conversion factors (for the water column, only) for the leucine method have also been determined. Bjørnsen & Kuparinen (1991) calculated a conversion factor of 3.0 kg C per mol leucine for the Southern Ocean. Simon & Azam (1989), using the theoretical value and their experimentally-determined isotope dilution factor of two, came up with essentially the same value (3.1 kg per mol) for the marine environment. Heinänen & Kuparinen (1992) reported a value of 6.8×10^{15} μm^3 per mol leucine incorporated for the northern Baltic Proper, but did not calculate the final carbon production estimate. Using the carbon content measured by Fagerbakke & al. (1996), 0.1 pg μm^{-3}, a conversion factor of 0.68 kg C per mol leucine taken up could be calculated for the brackish water bacteria. Moran & Hodson (1992) came up with a factor of 8.6 kg C per mol leucine for freshwater marsh samples. The high variation between the factors derived from different environments suggests that the isotope dilution varies markedly between the environments. Other imaginable explanations include methodological inaccuracies in sizing the bacteria and measuring the carbon content, and the possibility that the bacterial protein and carbon content and their relation to each other vary more than theoretically expected. Bacteria are known to regulate their macromolecular synthesis according to changes in environmental factors (eg. Marouga & Kjelleberg 1996, Fegatella & Cavicchioli 2000), but the bacterial protein:dry weight and carbon:dry weight ratios have been found to be constant (63±1% and 54±1%) for the size range of 0.026-0.4 μm^3 of pelagic marine bacteria (Simon & Azam 1989). As the proteins and carbon comprise such major fractions of the cell dry weight, changes in protein and carbon content are not likely to cause variations in the value of the conversion factor on an orders-of-magnitude scale.

4.2.2 Field observations

Very little data exists on benthic bacterial production of the Baltic Sea. We found bacterial production in the top 0-1 cm layer to vary from 36 to 192 mg C m^{-2} d^{-1} in the northern Baltic Sea (Table 3). Meyer-Reil (1986) measured the bacterial production of a muddy sand sediment (water depth 18 m) in the Kiel Bight (southern Baltic Sea) to vary between 0.37 and 0.67 mg C m^{-2} d^{-1} in the top layer (leucine method, data calculated from figures). In the Gulf of Riga (at a water depth < 45 m), production was found to fluctuate between 300 and 1500 mg C m^{-2} d^{-1}, with the highest values occurring in the spring (Tuomi & al. 1999, thymidine method). At the coastal station of Pojo Bay, south-west Finland (water depth 30-35 m), bacterial productivity ranged from 10 mg C m^{-2} d^{-1} in April to 463 mg C m^{-2} d^{-1} in August (Ekebom 1999, thymidine method). In our experiments (water depths 80-172 m), the top layer productivity varied from 36 to 192 mg C m^{-2} d^{-1} (Fig. 5). Using the leucine incorporation method, values similar to ours have also been measured in silty littoral freshwater sediment (16-598 mg C m^{-2} d^{-1}, Kirschner & Velimirov 1999a) and the mud sediments of eutrophic lakes (36-137 mg C m^{-2} d^{-1}, Tuominen 1995), whereas higher productivities have been reported from sandy river sediments (550 mg C m^{-2} d^{-1}, Marxsen 1996; 1200 mg C m^{-2} d^{-1}, Fischer & Pusch 1999). In our experiments, the calculations were carried out for counts obtained at a saturating concentration of leucine, and hence no isotope dilution factor was used. In some cases, however, saturation was not straightforward. The results may still be underestimates, since even if "saturation" was achieved in our samples, some isotope dilution is possible, and the true production would then be higher than estimated (Tibbles & al. 1992, Fischer & Pusch 1999, Kirschner & Velimirov 1999a).

The importance of including benthic production measurements in the overall study of the carbon cycle in the Baltic Sea is emphasized when comparing the benthic bacterial production to the integrated bacterial production in the water layers above. In areas where oxygen concentrations can be expected to be adequate, the benthic bacterial production reaches the same values per square metre as does the bacterial production in the whole water column above (Table 4). In deeper areas, however, the oxygen conditions are usually poor, less sedimenting material reaches the sea floor, and the benthic bacterial production is quite low (Table 4).

The overall variation of bacterial production in sediment is much smaller than in the water column, where variations of orders of magnitude are typical (Kuparinen & Kuosa 1993). The conditions experienced by sediment bacteria are more stable than those in the water column in terms of temperature and available organic matter. Temperature has been suggested as controlling the productivity in shallow water sediments (up to about 50 m) in temperate areas (Goedkoop & Johnson 1996, Ekebom 1999, Kirschner & Velimirov 1999a, Tuomi & al. 1999). On deeper bottoms, such as those studied here (around 100 m or more), where temperature conditions are more stable, the availability of organic carbon most probably plays a more important role. At the two stations for which all seasons were covered in the sampling (GF2 and JML), productivity was highest in the early summer, suggesting that the sedimentation of the spring bloom affects the benthic bacterial productivity. As the sediment was only sampled 3 times a year, it is possible that peaks were missed. In laboratory experiments where settling

Table 4. Average bacterial production in the top layers of the sediment and in the water layer above in different areas of the northern Baltic Sea. The depth column shows the depth at the sediment sampling stations and the average depth of the area, used for calculating the integrated water column production.

Area	Depth, m	Production mg C m^{-2} d^{-1} Water column	Benthic	Ref.
Bothnian Sea	155	310		1
Stn SR5	125		160-320	**III**
Northern Baltic Proper	236	870		1
Stn LL17	172		36-60	**III**
Entrance to the Gulf of Finland	90	232-259		2
Stn JML	80		88-282	**III**
Gulf of Finland	90	190		1
Stn GF2	84		175-349	**III**

1 Heinänen 1992
2 Heinänen & al. 1995

algae have been added to lake sediment cores, an extremely quick (effects seen two hours after diatom addition), strong (13 times the production compared to the controls), but short-lived (only for 1-2 days) effect on bacterial production has been demonstrated (Goedkoop & al. 1997). However, using Baltic Sea sediment and spring bloom algae in similar experiments, only a moderate response in bacterial production was found (Tuominen & al. 1999).

In the continental margin of eastern Australia, 20 to 30% of the benthic bacterial production, calculated using thymidine incorporation, was in the top 1 cm, 60 to 70% in the top 2 cm and nearly 100% in the top 10 cm of the sediment (Moriarty & al. 1991). A similar distribution of activity has also been recorded in a salt marsh lagoon (Tibbles & al. 1992) and sandy river sediments (Marxsen 1996). In the Gulf of Finland, more than half of the activity of the 2 cm studied was in the top 1 cm, while the activity was equally divided between all three 1 cm sediment layers studied in the Bothnian Sea. Unfortunately, no deeper layers were studied, as they appeared anoxic and slurrying the layers would have introduced oxygen into the sediment, disturbing the natural state and possibly changing the production rate. Oxygen penetration into sediments and the vertical stratification of bacterial production related to the oxygen conditions call for more research.

Growth efficiencies of sediment bacteria have been measured to vary from 10 to 40% (Bell & Ahlgren 1987, Törnblom 1996, Goedkoop & al. 1997). Using a conservative growth yield of 40%, sediment bacteria were calculated to require 0.4 to 0.8 g C m^{-2} d^{-1} in the Bothnian Sea and 0.4 to 0.9 g C m^{-2} d^{-1} in the Gulf of Finland. Sedimentation measured using sediment traps has been shown to average only 0.077 g C m^{-2} d^{-1} in the Bothnian Sea (Lehtonen & Andersin 1998) and 0.1-0.2 g C m^{-2} d^{-1} in the Gulf of Finland (Kankaanpää & al. 1997, Leivuori & Vallius 1998). The actual carbon demand may even exceed the calculated value, as the measured production may be underestimated due to isotope dilution, and the growth yield used may be an overestimate. It therefore seems that the bacteria partly utilise recycled carbon within the sediment, and that possibly a horizontal flow of carbon near the sediment surface also provides benthic bacteria with the substrates needed. In addition, sediment trap measurements may underestimate the actual settling rates of organic matter (Lehtonen & Andersin 1998).

4.3 Production of cyanobacteria-associated bacteria

The net-haul collecting of samples in the northern Baltic Sea in July-August 2000, using a 100 µm mesh, was not likely to enrich the free-living bacteria in the samples, and it is logical to assume that the high number of bacteria in the samples is a result of them being attached to the collected cyanobacteria. The measured bacterial activity, consequently, relates mainly to the attached community, instead of the free-living bacteria.

Thymidine uptake was saturated at around 100 nM, which is an unusually high concentration compared to most pelagic environments (Table 3). The ambient thymidine saturation concentrations reported from the Gulf of Finland and northern Baltic Proper area are around 5 nM, with temporary peaks as high as 21 nM (Heinänen & Kuparinen 1992, Heinänen 1993, Heinänen & al. 1995, Tuomi 1997) (Table 3). A possible reason for such a high satura-

tion level is the large amount of algal products in the sample. Decaying blooms especially are known to produce large amounts of colloidal dissolved organic matter (Kepkay & al. 1993) that provides adsorption sites for thymidine (Schuster & al. 1998). As the colloids efficiently adsorb thymidine, there will only be free thymidine left for bacterial uptake after all the adsorption sites are occupied (Schuster & al. 1998). Lehtimäki & al. (1997) also noticed that in non-axenic cyanobacterial cultures up to 50 nM (*Nodularia*-cultures) and even 200 nM (*Aphanizomenon*-cultures) thymidine concentrations were needed to saturate the bacterial thymidine incorporation. In our samples the thymidine incorporation was positively correlated with the chlorophyll *a* concentration, which might be a sign of the cyanobacteria dominating the incorporation. However, no thymidine incorporation was detected in axenic cultures of *Nodularia* and *Aphanizomenon* (Lehtimäki & al. 1997, **II**), the dominant species in our samples. The correlation more probably indicated a close coupling between autotrophic and heterotrophic production in our samples.

According to several studies, attached bacteria are more productive per cell than the free-living bacteria (Alldredge & Gotschalk 1990, Simon & al. 1990, Worm & Søndergaard 1998). However, other results suggest that the bacteria attached to marine snow do not grow any faster than the free-living bacteria (Alldredge & al. 1986, Worm & al. 2001). In 2000, both the thymidine incorporation and the cell numbers were 10-20 times higher in the concentrated samples than those measured for the free-living bacterioplankton in the northern Baltic Sea (Heinänen 1991, Heinänen & Kuparinen 1991, Tuomi 1997). Hence, although the filaments were heavily colonised by bacteria, on a cellular basis the thymidine uptake of the attached bacteria did not differ from that of the free-living bacterioplankton.

The conversion factor between thymidine incorporation and cell production was only 3.5×10^{17} cells per mol thymidine incorporated. This value is up to 20 times lower than those measured earlier in the same area (5×10^{17} cells mol^{-1}, Heinänen & Kuparinen 1992; $1.96\text{-}6.89 \times 10^{18}$ cells mol^{-1}, Autio 1992) (Table 3). The conversion factor based on increase in biovolume was 2.8×10^{16} μm^3 per mol, which is about four times lower than that reported for free-living bacteria in the northern Baltic Sea in August ($1.1\text{-}1.37 \times 10^{17}$ μm^3 mol^{-1}, Tuomi 1997) (Table 3). The biovolume-based approach is especially attractive for estimating the production of attached bacteria, as the size measurements are done in the batch culture, in which the bacteria can be counted using Acridine Orange. When counting bacteria attached to surfaces (sediment, macrophytes, algae etc.), DAPI staining must be used to effectively distinguish the bacterial cells from the matrix. DAPI, unlike Acridine Orange, does not stain the outer membrane of the cells, and therefore the measured sizes could be underestimates of actual cell sizes (Suzuki & al. 1993). The low conversion factors measured in the present study may also reflect the fact that conditions in a filtered batch culture differ dramatically from those experienced by the attached bacteria in nature. It is also possible that the bacteria growing in such a batch culture do not originate from the attached community at all, but from the free-living fraction of the sample. However, there is no other known way to estimate the conversion factor between thymidine incorporation and cell or biovolume production.

When the biovolume-related conversion factor was used, the attached bacteria were calculated to produce 4.1-40.1 mg C m^{-3} d^{-1} in the concentrated samples. Using the relevant concentration factor for each sampling occasion, the daily production of the attached bacteria, when the cyanobacterial filaments are suspended in the water mass, could be calculated to vary from 3.3 to 41.3 µg C m^{-3} d^{-1}. Daily bacterial production in the northern Baltic proper has been measured to vary from 3.7 to 4.5 mg C m^{-3} d^{-1} (Autio 1990, Heinänen & al. 1995), suggesting that the attached bacteria contribute only a very small fraction (1%) to the total bacterial production (Table 3). This situation may, however, change drastically if the weather conditions allow the cyanobacterial filaments to coil and form aggregates. Cyanobacterial aggregates are characterised by elevated nutrient and organic matter concentrations as well as high heterotrophic production (Hoppe 1981, Worm & Søndergaard 1998), and cyanobacteria-associated bacteria have been shown to contribute up to 50% of the total bacterial production, measured using the thymidine incorporation method, in an eutrophic lake (Worm & Søndergaard 1998). In August 1999, the cyanobacterial aggregates in the Gulf of Finland were heavily colonised by bacteria. Unfortunately, the abundance of the aggregates was not determined. The abundance of various types of aggregates has been measured to vary from 0.1 to more than 200 per litre in oceans, lakes and rivers (Alldredge & al. 1986, Alldredge & Gotschalk 1990, Weiss & al. 1996, Zimmermann 1997, Grossart & al. 1998). Had the aggregate abundance been just 5 aggregates per litre, the number of aggregate-attached bacteria would already have exceeded the abundance of the free-living heterotrophic bacteria in the Gulf of Finland in late summer 1999. One must keep in mind, however, that cyanobacterial blooms occur randomly, and the occurrence, species composition and duration of the blooms depend on several environmental factors, such as the weather conditions. Summers without visible blooms of cyanobacteria are rare (e.g. 1998), but so also are years with massive blooms (e.g. 1997 and 2002). Accordingly, the significance of bacteria attached to cyanobacterial filaments and aggregates may vary widely. Further

experiments using naturally-occurring aggregates and unconcentrated cyanobacterial samples are needed to evaluate the role of the attached bacteria in various bloom situations.

4.4 Denitrification

In the Gulf of Finland in August 1999, the first aggregates were collected for experiments when *Nodularia spumigena* was growing healthily and filaments were strongly coiled into spherical aggregates (August 4th and 5th). The aggregates were small and consisted mainly of cyanobacteria. Within days the *Nodularia spumigena* filaments became lighter in colour and aggregates became yellowish and more buoyant (samples on August 8th). The change of aggregate appearance was mainly due to decaying *Nodularia spumigena* filaments, but also due to diatoms (*Nitzchia* sp.), microheterotrophs and fecal pellets that were collecting in the bundles. At this stage aggregates were also densely colonised by bacteria, but still packed into tight, round bundles. Later (samples on August 10th), the bundles became bigger but also looser, and thus less dense, with the *Nodularia spumigena* bloom showing signs of decay, with a large proportion of empty filaments in the aggregates. A high number of diatoms (*Nitzchia* sp.) was recorded within the *Nodularia spumigena* aggregates both as free cells and within gel-like bags. Aggregate appearance varied from tight spheres of about 3 mm diameter on the first sampling day via even tinier, 2 mm diameter packages to about 4 mm diameter loose roundish bundles on the last sampling day. While this means an 8-fold difference in biovolume, the dry weight of the aggregates barely doubled, reflecting the loose packing of the decaying algae and colonising organisms. The carbon and nitrogen content (POC and PON) varied greatly from day to day, but the C:N-ratio lowered only a little during the whole sampling period (**V**: Table 1). Judging by the high colonisation, oxygen production, and respiration rates in the aggregates, these were indeed hot spots of activity in the pelagic environment.

In July-August 2000, it was necessary to use net-concentrated cyanobacterial biomass instead of natural aggregates, as the sampling period was windy and no surface scums and aggregates formed, despite the high cyanobacterial biomass. The chlorophyll *a* content of the concentrate ranged from 143 to 2300 µg l^{-1} (**IV**: Table 1), which is several orders of magnitude higher than in naturally-occurring concentrations during cyanobacterial blooms in the Baltic (3-4 µg l^{-1}, Heiskanen & Kononen 1994, Heinänen & al. 1995, Ohlendieck & al. 2000). Usually the reported values reflect the integrated chlorophyll *a* values in the whole mixed surface layer. In the experiments reported here, conditions in the uppermost surface layer in calm conditions, a cyanobacterial surface "scum" - more specifically, the conditions experienced by attached bacteria inside cyanobacterial aggregates - were simulated. The zooplankton community in the concentrated sample was probably different from a natural community because the 100-µm mesh selectively collected large zooplankton, allowing smaller grazers to pass through. Herbivore-enhanced nutrient and carbon release together with reduced grazing pressure on heterotrophic bacteria most probably favoured the growth of the bacteria in the concentrated samples. However, for the purposes of measuring the activities of the attached bacteria, the concentrate gave a reasonable simulation of a cyanobacterial aggregate.

The presence of species capable of denitrification was verified in 1999, as nitrite reductase (the key enzyme of the denitrification pathway) gene fragments could be amplified from the aggregate-derived DNA (**V**). Interestingly, no evidence of the presence of nitrifiers was found in the aggregates. This was especially surprising, as nitrifiers were detected in the cyanobacterial filaments that had not yet aggregated, at the same sampling time and place (Jaana Tuomainen, University of Kuopio, unpublished results), suggesting that aggregation alters the conditions experienced by attached bacteria drastically enough to shape the microbial community structure. Such a process has also been shown to happen in marine phytodetrital aggregates, in which specific bacterial populations, different from those that predominate in free-living bacterioplankton, develop (DeLong & al. 1993).

In 1999, anoxic microzones were calculated to develop in aggregates in the dark, especially as they were very compact and, in the later stage, also rich in gel-like substances, produced by the decaying algae, which further restrain oxygen diffusion in the aggregate. In aggregate-like environments with fluctuating oxygen conditions, such as intertidal microbial mats and waste-water treatment plants, denitrification is quickly initiated as anoxia establishes (Joye & Paerl 1993, Frette & al. 1997). Nitrification, likewise, adapts rapidly to lowered oxygen availability and is most efficient in suboxic conditions (Kester & al. 1997). In the present study, the aggregate samples were incubated in darkness (a prerequisite for the formation of anoxia in the aggregates) for 17-27 hours, which is much longer than they would experience in nature. However, no denitrification (with the exception of the very low rates found in three samples) and only a negligible production of N_2O could be observed. N_2O is used by denitrifiers as an intermediate in the denitrification process, and the lack of increase, or even a decrease in the concentration of N_2O could also have been interpreted as being the result of active uptake and reduction of the formed N_2O to N_2. The failure of the very sensitive ^{15}N-isotope pairing method to demonstrate any sign of denitrification in the sam-

ples, however, confirms the conclusion that denitrification, if happening at all, was not intense enough in the samples to be measured.

In the samples from 2000, dissolved O_2 decreased linearly in the phytoplankton concentrate to at least 80 µM over a period of time ranging, within different experiments, from 5 to 14 h. This allowed monitoring the onset of anaerobic processes in a system analogous to the natural formation of anoxic microniches. However, even though the samples were incubated on average for ten hours after reaching anaerobic conditions, no sign of denitrification could be detected, with the exception of extremely low rates in 3 samples out of the 23 studied. In the three cases where denitrification could be calculated, the rate was approximately 1.3 nmol N_2 l^{-1} h^{-1} in the concentrate (Table 3). Denitrification at the oxic-anoxic interface of the Baltic Sea deep waters has been estimated to be within the same range (2.1 nmol N_2 l^{-1} h^{-1}; Rönner & Sörensson 1985, 0.9 nmol N_2 l^{-1} h^{-1}, Brettar & Rheinheimer 1992) whereas the activity in sediments in the northern Baltic Proper varies from 3 to 13.5 µmol N_2 m^{-2} h^{-1} (Tuominen & al. 1998) (Table 3). No increases in N_2O concentrations, possibly indicating incomplete denitrification reactions, were observed in our samples (except in six enriched samples). Comparison of the measured denitrification rates with the simultaneously measured N_2 fixation rates, 0.03-1.84 µmol N_2 l^{-1} h^{-1} in the concentrate (**IV**), demonstrated that even with the unnaturally long anoxic conditions prevailing for the denitrification, the cyanobacterial bloom acted as a source, not a sink, for nitrogen in the aquatic ecosystem.

Denitrification is controlled by several factors, the most important of which are the availability of nitrate and organic carbon, and the absence of oxygen. Many studies have confirmed that the substrate (nitrate and carbon) availability plays a more important role than the oxygen status. It has also been suggested that denitrification in natural marine environments, such as sediments and the oxic-anoxic interfaces of deep waters, may be limited by carbon availability (Tiedje & al. 1982, Rönner & Sörensson 1985, Brettar & Rheinheimer 1992, Joye & Paerl 1993). In our samples, nitrate limitation can be ruled out, because NO_3 was added at a level of 100 µM (natural NO_3 concentrations were around 0.1 µM). The possible denitrifiers were also not likely to be limited by carbon availability, because the fresh and decaying phytoplankton provided a rich source of dissolved organic compounds.

Lack of nitrifiers in aggregates might lead to a lack of NO_3, inhibiting the induction of denitrification enzyme synthesis. However, nitrifiers were present in the surrounding cyanobacterial bloom (Jaana Tuomainen, University of Kuopio, unpublished results), and enriching the samples with NO_3 to an incubation concentration of 100 µM did not enhance the denitrification to a measurable level. Presumably the energy-demanding synthesis of denitrification enzymes is not induced in aggregate-attached bacteria in the Baltic Sea because the frequency and magnitude of favourable conditions (anoxia only during the night, in limited microniches) is low compared to the occurrence of oxic conditions.

The experiments presented here confirmed that the cyanobacterial filaments were colonised by heterotrophic bacteria and that these bacteria were actively growing. The aggregate-attached bacteria were shown to harbour genetic potential for denitrification, and the slow emergence of denitrification activity in the samples after extended anoxia shows that the bacteria were capable of switching to denitrification as a form of anaerobic metabolism. However, neither in natural aggregates, nor in conditions analogous to the formation of anoxic microniches within cyanobacterial aggregates, could a clear switch to denitrification as a form of anaerobic metabolism be detected, except when anoxic conditions prevailed for several hours. It is, therefore, unlikely that denitrification plays any significant role in the Baltic Sea cyanobacterial blooms, in which anoxic microzones may be present only fleetingly.

5 CONCLUSIONS

5.1 Methodological aspects

- The centrifugation method is not suitable for use in low-salinity brackish waters without the addition of salt, which in turn adds extra steps to the procedure, making it less attractive for users.
- The use of polycarbonate filters, when measuring ^{3}H-thymidine incorporation, leads to results lower than those achieved using cellulose nitrate filters.
- During cyanobacterial blooms, the ^{3}H-thymidine instead of the ^{14}C-leucine incorporation method should be used, as cyanobacteria incorporate leucine, causing an overestimate of bacterial production.
- The bacterial production in muddy sediments can easily and reliably be measured using the gel method presented.
- When measuring the production of cyanobacteria-associated bacteria, a high incubation concentration of ^{3}H-thymidine is needed. The conversion factor for carbon production needs to be determined experimentally, as it differs substantially from those published for the free-living bacteria.

5.2 Ecological aspects

- Benthic bacterial production in the northern Baltic Sea was high and equalled the integrated bacterial production of the whole water column above on the well-oxygenated sediments, whereas in low-oxygen areas it was much lower and only contributed less than 10% of total bacterial production.
- Cyanobacteria-associated bacteria contributed only a small fraction to the total bacterial production during a cyanobacterial bloom in the northern Baltic Sea, but aggregation of the cyanobacterial filaments may enhance production.
- The carbon demand of benthic bacteria in the northern Baltic Sea exceeds the measured sedimentation rates. The benthic bacteria seem to rely on recycled carbon within the sediment and on a horizontal flow of carbon near the sediment surface.
- No denitrification could be detected in natural aggregates despite the observed genetic potential and favourable environmental conditions, neither could denitrification be induced in experiments in which incubation conditions were modified to enhance denitrification. According to these results, no ecologically-significant nitrogen removal happens in the Baltic Sea cyanobacterial blooms. Thus, they must be seen solely as sources, not sinks of nitrogen in the pelagic environment.

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