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1999

Hietanen , S , Tuominen , L & Kuparinen , J 1999 , ' Benthic bacterial production in the northern Baltic Sea measured using a modified 14C-leucine uptake method ' , Aquatic Microbial Ecology , vol. 1999 , no. 20 , pp. 13-20 .

http://hdl.handle.net/10138/33873

publishedVersion

Vol. 20: 13–20, 1999 | AQUATIC MICROBIAL ECOLOGY
Aquat Microb Ecol | Published November 30

Benthic bacterial production in the northern Baltic Sea measured using a modified [^{14}C]leucine incorporation method

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ABSTRACT: Benthic bacterial production was measured with a modified [^{14}C]leucine incorporation method in the northern Baltic Sea in 1996 and 1997. Samples were incubated and TCA extractions were done in microcentrifuge tubes. Unincorporated label was removed by repeated centrifugation. Pellets were suspended in scintillation cocktail and counted as a gel using internal standardisation, a method that gave highly comparable counts with combustion of the sample. Bacterial production varied between 0.15 and 0.8 mg C l^{-1} h^{-1} (90 to 350 mg C m^{-2} d^{-1}). The highest production was found at Stn GF2 in the Gulf of Finland and the lowest in the northern Baltic Proper. Bacterial growth rates were 0.07 to 0.84 d^{-1}. Comparison of these results with published data from sedimentation trap measurements suggests that only a part of the benthic bacterial C demand can be explained by sedimentation.

KEY WORDS: Bacterial production · Sediment · Leucine · Baltic Sea

INTRODUCTION

Using leucine incorporation for measuring bacterial production has recently received increasing interest in sediment studies. This is partly explained by the controversy with the thymidine method, the previous method of choice (see review by Robarts & Zohary 1993). Leucine incorporation, which measures bacterial protein synthesis, gives a direct measurement of bacterial biomass change (or carbon production) because ca 60% of bacterial dry mass consists of proteins (Simon & Azam 1989). The ratio of cellular protein to carbon and the molar proportion of leucine compared to other amino acids has been found to be rather constant in natural bacterioplankton assemblages (Simon & Azam 1989). Therefore, conversion factors needed to calculate the results are quite constant, which makes the method attractive.

Various techniques have been used to study leucine incorporation in sediments (Table 1). Combustion is the only method to produce a homogenous and transparent sample for scintillation counting from a sample containing sediment particles. However, oxidizers are not very common, especially not onboard research vessels. Consequently, samples cannot be counted immediately after incubation, e.g. onboard a research vessel or at a field station, which makes on-site methodological adjustment (e.g. isotope concentration, incubation time) impossible. However, portable scintillation counters can be used on research vessels or at field stations that are normally not equipped with a scintillation counter.

The Baltic Sea is a brackish water area characterized by significant anthropogenic nutrient loading (Wulff et al. 1990). Although the (micro)biology of the Baltic Sea plankton has been widely studied (see e.g. Kuparinen & Kuosa 1993), the microbiological aspects of its sediment have remained less studied (but see Meyer-Reil 1986 and references therein, Piker & Reichardt 1991). In this study, we measured bacterial production in the sediments of the open areas of the northern Baltic Sea. Bacterial production was measured using a modified [^{14}C]leucine incorporation method in which centrifuga-

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Table 1. Methods used to study leucine incorporation in sediments

Method	Source
Macromolecules extracted from sediment with NaOH, EDTA and SDS followed by	
(a) precipitation by TCA and solubilization of protein by hot NaOH	Meyer-Reil & Charfreitag (1991), Marxsen (1996)
(b) dialysis	Tibbles et al. (1992)
(c) direct measurement	Epstein (1997)
Bacterial cells lysed with NaOH, after which TCA was added, samples heated to 95°C, cooled in ice, and filtered. The precipitated proteins solubilized in hot SDS	Moran & Hodson (1992)
Samples washed with ethanol followed by precipitation of DNA and protein by ice-cold TCA and solubilization of protein by hot NaOH	Moriarty et al. (1991), van Duyl & Kop (1994)
Samples washed with ethanol and the washed pellet combusted	Meyer-Reil (1986), Tuominen & Kairesalo (1992), Kairesalo et al. (1994), Tuominen (1995), Tuominen et al. (1996)

tion was used for macromolecule collection. This kind of method has previously been applied for water samples (Smith & Azam 1992) but not for sediment. The radioactivity of the sediment pellet was measured as a gel using internal standardisation in the scintillation counting, and was compared with the combustion method.

MATERIAL AND METHODS

Sediment samples were collected with a Gemini twin corer (inner diameter of the cores 80 mm) from the northern Baltic Sea during cruises onboard the RV 'Aranda' and RV 'Alkor' in 1996 and 1997 (Fig. 1). Only the top 1 cm was used in the incubations, except in the vertical distribution experiment (see below).

The routine procedure for measuring leucine incorporation was as follows: small amounts (normally 100 µl, see below) of sediment slurry were mixed in 1.5 ml microcentrifuge tubes with increasing concentrations of [^{14}C]leucine (11.1 to 11.3 GBq mmol^{-1}, Amersham, diluted with filtered seawater). The incubation volume was adjusted to 500 µl with filtered (0.2 µm pore-size, Minisart, Sartorius) near-bottom seawater. The [^{14}C]leucine additions to determine the saturation level varied in the beginning between 0.1 and 10.8 µM and later between ca 0.3 and 4 µM. No carrier was used in any incubations. Control samples (the results of which were subtracted before calculating the production rates) were prepared by adding the isotope into microcentrifuge tubes containing the sediment slurry plus 1 ml of ice-cold 10% TCA. Samples were incubated in the dark at *in situ* temperature. The optimal incubation time was determined on every cruise, except August 1996, by incubating a series of samples for 15 to 230 min (times varied from cruise to cruise). Incubation was terminated by adding 1 ml of ice-cold 10% TCA. Unincorporated isotope was removed from samples and macromolecules were collected by repeated centrifugation (10 min 10 000 × *g* at 4°C), once using 70 to 80% ethanol and twice using ice-cold 10% TCA. Washing with ethanol removed most of the pigments that lowered the scintillation counting efficiency.

After the washings, the caps of the tubes were removed (caps contained only traces of radioactivity) and the tubes were cut into 2 pieces and put into glass scintillation vials. Pellets were suspended in scintillation cocktail (Instagel or Instagel Plus, Packard) until no visible pellets were observed in the bottom of the microcentrifuge tubes. Samples were counted as gel

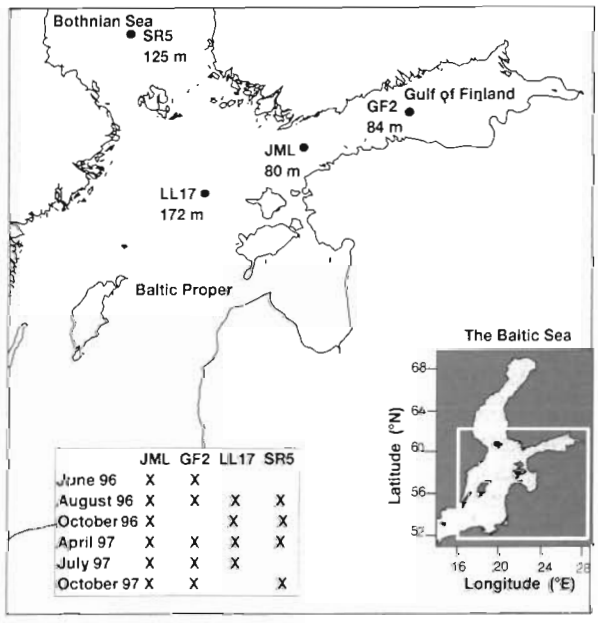


Fig. 1. Sampling stations and times

including the pieces of microcentrifuge tubes (1/3 H_2O, 2/3 Instagel according to manufacturer's recommendations) in a liquid scintillation counter. For sediment samples, the gel matrix is important since otherwise the sediment particles would settle to the bottom of the vial and distort the counting geometry. Since ^{14}C was used instead of ^{3}H, the possible effect of the small sediment particles suspended in the scintillation cocktail on the counting geometry was minor (cf. Amon & Benner 1998, Robarts 1998). The counts per minute (cpm) were converted to disintegrations per minute (dpm) using the counting efficiency measured by internal standardisation in which a standard capsule (ca 100 000 dpm, Wallac) was added to 3 control samples which were then re-counted. This gel method was tested against the combustion method (Tuominen 1995) in which the samples were dried at 60°C after similar washings using centrifugation and then combusted at 900°C (Junitek Oxidizer) prior to scintillation counting.

The results obtained were converted to bacterial carbon production (BCP, g) using the equation of Simon & Azam (1989):

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

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.

Time series incubations using different amounts of sediment (50 to 400 µl) were carried out 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 by incubating 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 Stns JML and GF2, bacterial production was measured from the 1 to 2 cm sediment layer in addition to the 0 to 1 cm top layer. At Stn SR5, the layers 0–1, 1–2 and 2–3 cm were analyzed due to a visually deeper oxidized layer.

Bacterial abundance in the sediment was counted from DAPI-stained samples using an epifluorescence microscope (excitation wave length 365 nm; Porter & Feig 1980). Samples were diluted 500 times, and stained at a DAPI concentration of 10 µg ml^{-1} for at least 20 min.

Statistical analysis. The gel method was compared to the combustion method by calculating regression equations with the 'new' gel method being the independent variable and the 'old' combustion method the dependent variable. If the methods gave 1:1 results, the constant of the regression equation should be zero and the coefficient one. In addition, linear correlations were calculated.

RESULTS AND DISCUSSION

Methodology

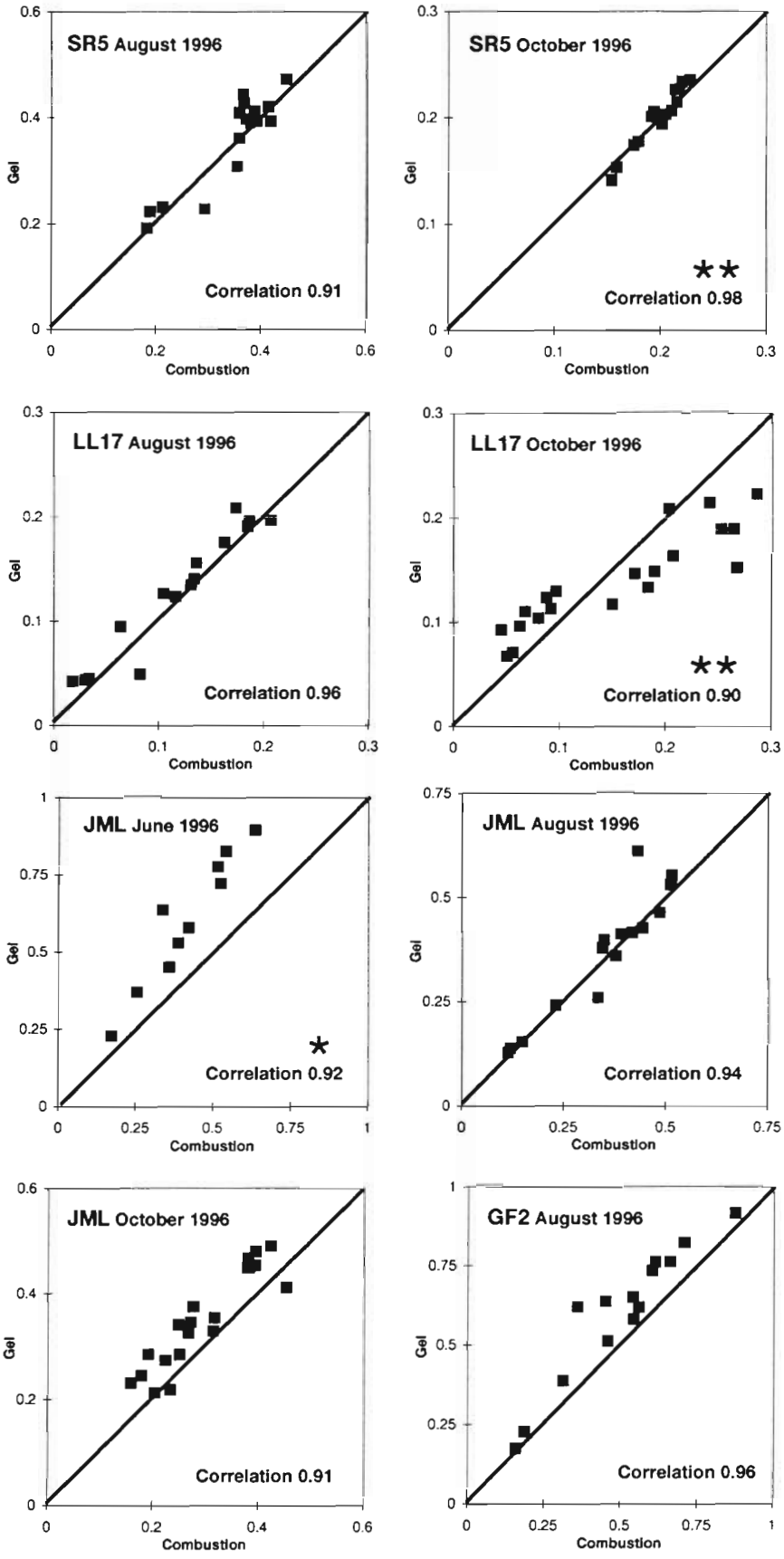
The linear correlation between leucine incorporation in samples measured as a gel and combusted samples was high. In 6 cases out of 8, the constant of the regression equation did not significantly differ from zero, and in 5 cases the value 1 was within the 95% confidence interval of the coefficient (Fig. 2). The gel method thus proved to be accurate for the northern Baltic mud sediments. It speeds up the analytical procedure and facilitates the counting of samples where an oxidizer is not available. The counting efficiency was almost constant within each station during each sampling occasion (coefficient of variation 0.2 to 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 between 2.5 and 3 µM of leucine (Fig. 3). This is much lower than 14 µM observed for 4 different lake sediments (Tuominen 1995) or 20 µM for a stream-bed sediment (Marxsen 1996). On the other hand, in a marine sediment a saturation level as low as 0.5 µM has been found (Moriarty et al. 1991). Obviously the adaptation by bacteria to the scarcity of easily degradable organic matter in deep (in this study 80 to 172 m) marine sediments as compared to lake and river sediments causes the lower saturation level. Time series incubations showed that the incorporation of [^{14}C]leucine was linear for at least 230 min (Fig. 4).

The amount of sediment used in the incubations did not affect the leucine incorporation rate during the 45 to 140 min used in routine incubations (Fig. 4). The decision to use 100 µl 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 and larger amounts increased the variance of the counting efficiency. Different amounts of near-bottom water to make a slurry had no effect on the leucine incorporation rate (data not shown).

Field observations

Bacterial production varied between 0.15 and 0.8 mg C l^{-1} h^{-1} at the 4 stations (Table 2). The highest pro-



duction was found at Stn GF2 in the Gulf of Finland, which is the most eutrophicated area in the present study (Wulff et al. 1990). This sediment area also shows the highest denitrification activity (Tuominen et al. 1998). The present bacterial production rates are comparable with values obtained from the North Sea (van Duyl & Kop 1994) if only stations deeper than 40 m (their Stns 3 to 11) are included. The shallower stations in the North Sea showed higher rates. Moriarty et al. (1991) measured bacterial production from the East Australian continental margin (depths 149 and 200 m) and found values which are very comparable with the rates from our deepest stations, SR5 and LL17. However, considerably higher rates (up to 5.7 mg C h^{-1} l^{-1}) have been measured from eutrophic freshwater sediments (depths 1 to 30 m; Moran & Hodson 1992, Tuominen 1995).

When comparing bacterial production rates from different studies, one has to be aware of the use of different conversion factors. In the present study (and in Tuominen 1995), the theoretical conversion factor of 1.5 kg C mol^{-1} leucine taken up (Simon & Azam 1989) was used. Since the calculations were carried out for results obtained at the saturating concentration of leucine, no isotope dilution factor was used. In some cases, however, saturation was not straightforward. Van Duyl & Kop (1994) used the same conversion factor in addition to their determined isotope dilution. In contrast to this, Moran & Hodson (1992) used a conversion factor of 8.6 kg C mol^{-1}, which they empirically determined for water samples. Bjørnsen & Kuparinen (1991) obtained an empirical conversion factor of 3.0 kg C mol^{-1} of leucine incorporated

Fig. 2. Correlation between the combustion and the gel method (bacterial production in mg C l^{-1} h^{-1}; for methods see text). Line represents 1:1 plot. ** = constant of the regression equation significantly different from zero ($p < 0.05$) and value 1 not within the 95% confidence interval of the coefficient (see 'Material and methods'). * = value 1 not within the 95% confidence interval of the coefficient

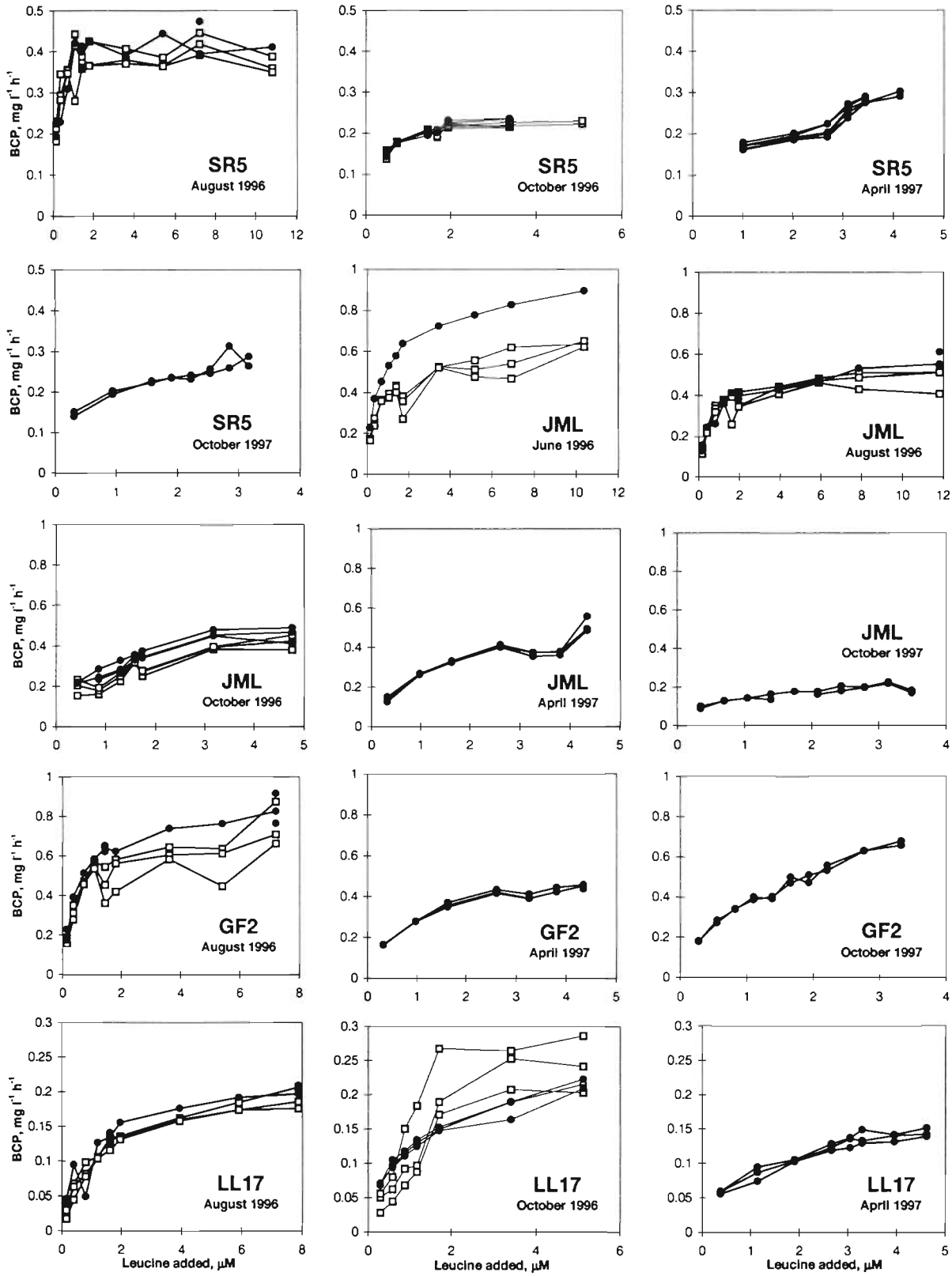


Fig. 3. Leucine incorporation versus added leucine concentration. Incorporation is given as bacterial carbon production (BCP) calculated using the conversion factor 1546 g C mol^{-1} leucine incorporated. □: combusted samples; ●: gel method. Unconnected data points refer to extra replicates. Note different scales for different stations

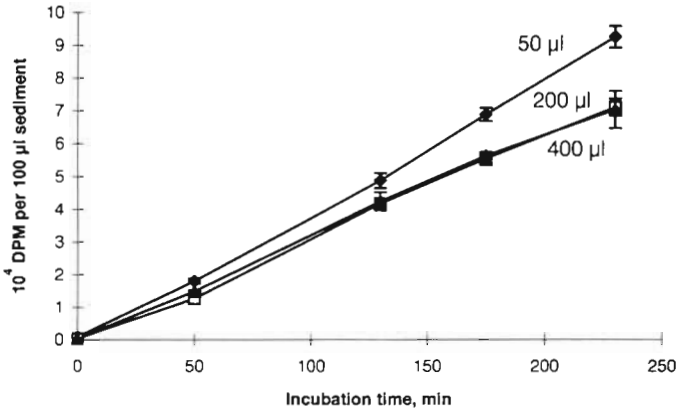


Fig. 4. Stn JML, July 1997. Time series incubations for different amounts of sediment used. Error bars show SD when larger than the symbol size. Results standardised to 100 µl of sediment

for water samples from the Southern Ocean. Our conversion factor may be underestimated since even if 'saturation' was achieved some isotope dilution is possible, and the true production may be as much as 2 times higher than estimated (Bjørnsen & Kuparinen 1991, Moran & Hodson 1992, van Duyl & Kop 1994).

At Stns GF2 and JML in the Gulf of Finland, 55 and 68%, respectively, of the measured bacterial production occurred in the top 1 cm of sediment (Fig. 5). In the Bothnian Sea (Stn SR5), where the oxygenated layer is deeper, the top 3 cm showed almost identical bacterial production (Fig. 5). However, it must be noted that bacterial production was not measured deeper than 2 or 3 cm. Moriarty et al. (1991) calculated from thymidine incorporation that 20 to 30% of bacterial production was in the top 1 cm, 60 to 70% in the top 2 cm and nearly 100% in the top 10 cm in the continental margin of eastern Australia.

Bacterial abundance varied between 0.32 and 2.23 × 10^9 cells ml^{-1} (Table 3). In the oligotrophic Bothnian Sea (Stn SR5), the abundance was slightly lower than in the Gulf of Finland. In the 1 to 2 cm layer the abundance was higher than in the 0 to 1 cm layer. This is obviously caused by the dilution effect of water in the topmost cm of sediment. Previous determinations of bacterial abundance in the Baltic Sea sediments are available from Stn SR5, where Mohammadi et al. (1993) counted 0.56 to 1.3 × 10^9 cells ml^{-1} using acridine orange staining. Bacterial growth rates varied between 0.07 and 0.84 d^{-1} (Table 3; leucine incorporation converted to cell production using the theoretical conversion factor of 0.082 × 10^{18} cells mol^{-1}; Simon & Azam 1989, Kirchman 1992). Previously published growth rates for sediment bacteria vary from 0.01 to 0.004 d^{-1} (Lake Vallentunasjön, depth 2.5 m, at +4°C; Bell & Ahlgren 1987) to 0.14–0.6 d^{-1} (East Australian continental margin, depth 140 and 200 m; Moriarty et al. 1991). The higher range of variation in the growth rates (12 times from the lowest to the highest) compared to bacterial abundance (7 times) in the present study suggests that bacterial abundance in the sediment varies less than the proportion of active bacteria within the bacterial population.

The overall variation of bacterial production in the sediment was much less than in the water column, where variation in orders of magnitude is typical (0 to 100 mg C m^{-3} d^{-1}; Kuparinen & Kuosa 1993). This is caused by the more stable conditions (temperature, availability of organic matter) compared to the water. Since the sediment was sampled only 3 times a year, it is possible that peaks have been missed. However, in laboratory experiments where settling algae have been added to sediment cores, we have found only moderate response in sediment bacterial production (maximum 5-fold; Tuominen et al. 1999 unpubl.).

If a growth yield of 40% is assumed as determined for sediment bacteria by Bell & Ahlgren (1987), sediment bacteria were calculated to require 0.4 to 0.8 g C

Table 2. Bacterial production in the sediments of the northern Baltic Sea

Stn	Bacterial production		
	mg C l^{-1} h^{-1} (in top 1 cm)	mg C m^{-2} d^{-1} (in top 1 cm)	mg C m^{-2} d^{-1} (in top 3 cm)[a]
SR5	0.2–0.4	48–96	160–320
JML	0.25–0.8	60–192	88–282
GF2	0.4–0.8	96–192	175–349
LL17	0.15–0.25	36–60	-[b]

[a]Calculated using the percentages from Fig. 5
[b]Depth distribution not analyzed because of the seemingly anaerobic sediment below 1 cm

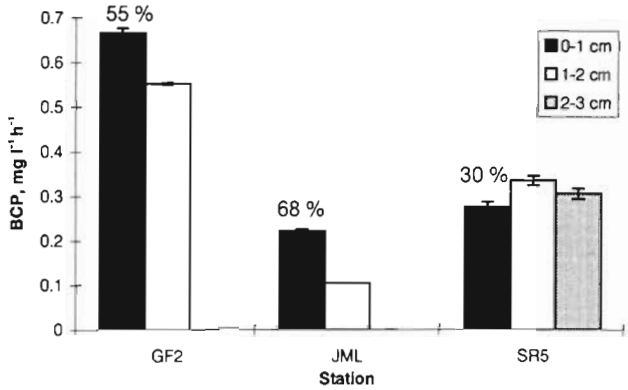


Fig. 5. October 1997. Depth distribution of bacterial carbon production in sediment. Error bars show range of 2 replicates. The proportion of BCP in the top 1 cm is shown by percentages

Table 3. Bacterial abundance (10^9 cells ml^{-1}) and growth rate (d^{-1}) in the surface sediments (0 to 1 cm unless otherwise stated) of the northern Baltic Sea

		Station							
		SR5		JML		GF2		LL17	
		Abundance	Growth rate	Abundance	Growth rate	Abundance	Growth rate	Abundance	Growth rate
Jun 1996				1.13	0.66	0.98	0.84		
Aug 1996		0.72	0.69	1.37	0.36	1.35	0.53		
Oct 1996		0.32	0.88	1.38	0.40	1.27	0.35		
Apr 1997		0.87	0.39	0.94	0.59	1.14	0.47	0.84	0.21
Oct 1997	0–1 cm	1.29	0.23	1.29	0.17	1.46	0.55		
	1–2 cm	1.56	0.27	2.01	0.07	2.23	0.32		
	2–3 cm	0.67	0.58						

m^{-2} d^{-1} in the Bothnian Sea (Stn SR5) and 0.4 to 0.9 g C m^{-2} d^{-1} in the Gulf of Finland (Stn GF2). On the contrary, measured sedimentation using sediment traps averaged only 0.077 g C m^{-2} d^{-1} at Stn SR5 (Lehtonen & Andersin 1998) and 0.1 to 0.2 g C m^{-2} d^{-1} at Stn GF2 (Kankaanpää et al. 1997, Leivuori & Vallius 1998). It appears, therefore, that sediment trap measurements underestimate the total available C for the benthic ecosystem, a conclusion drawn also by Lehtonen & Andersin (1998). There are 4 possible reasons for this: (1) Sediment trap measurements give too low an estimate of the amount of settling material. (2) Horizontal carbon flow near the sediment surface is important. (3) Bacteria utilize partly old, recycled carbon in the sediment. (4) Bacterial C demand was overestimated, which is, however, not probable since our conversion factor may be an underestimate and the growth yield an overestimate leading to even higher C demand. Growth yields as low as 10 to 25% have been determined for planktonic bacteria (del Giorgio et al. 1997). In conclusion, however, since the bacterial production results were of the same order of magnitude as the sedimentation data, it seems that the leucine incorporation technique to measure benthic bacterial production is an ecologically valid and meaningful assay.

Acknowledgements. We are indebted to the crews of the research vessels for help in the field, and to Prof. Imhoff and Dr Gocke for the opportunity to join the RV 'Alkor' cruise. We thank Antti Uusi-Rauva and Kaj-Roger Hurme (Isotope Section, University of Helsinki, Faculty of Agriculture and Forestry) for help with the Junitek Oxidizer. Markit Likolammi is acknowledged for counting the bacteria. This study was partly funded by the EU (BASYS project) through MAST programme (contract MAS3-CT96-0058).

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Editorial responsibility: David Karl, Honolulu, Hawaii, USA

Submitted: May 12, 1998; Accepted: June 2, 1999
Proofs received from author(s): October 22, 1999