

Benthic bacterial secondary production measured via simultaneous ^3H -thymidine and ^{14}C -leucine incorporation, and its implication for the carbon cycle of a shallow macrophyte-dominated backwater system

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Abstract

The role of the benthic bacterial community in the carbon cycle of a temperate, macrophyte-dominated oxbow was investigated in a 2-yr study from 1994 to 1996. Simultaneous incorporation of ^3H -thymidine into DNA and ^{14}C -leucine into proteins was monitored as a tool for the measurement of bacterial secondary production in the aerobic zone of the sediment along with bacterial numbers, morphotype composition, cell volumes, biomass, frequency of dividing cells (FDC), and abiotic variables. Bacterial numbers ranged from 2.5×10^9 to 8.5×10^9 cells ml^{-1} , corresponding to bacterial biomass values of 64 and $288 \mu\text{g C ml}^{-1}$, respectively. Both bacterial production methods yielded similar and reliable results over the whole investigation period, varying between 42 and $2,350 \mu\text{g C L}^{-1} \text{h}^{-1}$ for the thymidine method and between 67 and $2,490 \mu\text{g C L}^{-1} \text{h}^{-1}$ for the leucine method, and also corresponded well with the FDC values. Only during late spring and early summer was the thymidine uptake uncoupled from leucine incorporation. Temperature was found to be of significant importance for the variation of all bacterial parameters. In addition carbon inputs from the water column were apparently crucial stimulators of benthic bacterial growth. The spring phytoplankton bloom together with higher temperatures led to a strong positive response of the bacterial community in the sediment. However, the planktonic algal production was not sufficient to cover the benthic bacterial C-demand and, as derived from mass-balance calculations, decaying macrophytes of the previous season seemed to provide the main energy source until the middle of spring. From the middle of August to the beginning of October, the period of maximal biomass of submerged macrophytes, all bacterial parameters exhibited their annual maximum. The benthic bacterial community was dependent on leachates and decay products of the submerged vegetation during this time. Temperature limitation was most probably the reason for the low biomass and production values observed during autumn and winter.

Modification and decomposition processes of material in sediments are dominated by microorganisms that may be characterized as the driving force for the cycle of elements (Meyer-Reil 1994). The measurement of bacterial production is therefore of crucial importance in understanding the role of sediments for the carbon cycle in aquatic ecosystems. Soon after its introduction by Fuhrman and Azam (1980, 1982) in the early 1980s, the ^3H -thymidine incorporation method became the most widely used approach for determining bacterial secondary production in aquatic environments, due to advantages, such as specificity of bacterial uptake at short incubation times and relatively inexpensive and quick handling. However, many assumptions (conversion factors for cellular carbon, produced cells per incorporated pmol or, for thymidine, content of DNA per cell) and additional investigations (isotope dilution, saturation, and time-course experiments) are needed to lead to reliable bacterial production estimates.

A second approach, dealing with incorporation of labeled substances into bacterial cells is provided by the ^{14}C -leucine incorporation method, developed by Kirchman et al. (1985) and elaborated for routine measurements by Simon and

Azam (1989). Within the last years strong debate has taken place on the consistency of these methods (Chin-Leo and Kirchman 1988, 1990; Kirchman 1992; Servais 1992; Tibbles 1996; Shiah and Ducklow 1997). In many of these investigations big discrepancies were found between production estimates derived from ^3H -thymidine and ^{14}C -leucine incorporation. Such differences were explained as unbalanced growth of the bacterial population that can mainly be caused by two factors, temperature (Tibbles 1996; Shiah and Ducklow 1997) and substrate supply (Chin-Leo and Kirchman 1990). It is now thought that both methods should be used as complementary tools for estimating bacterial secondary production.

For sediment samples the dual label method has been applied only a few times, for a sandy estuarine sediment (Tibbles et al. 1992), a profundal lake sediment (Tuominen 1995; Tuominen et al. 1996), and an alkaline fen (Gsell et al. 1997). As mentioned by Sander and Kalff (1993), studies on freshwater sediment bacterial production other than riverine sites are scarce and to our knowledge no data exist on shallow lakes with dominating submerged vegetation. In highly productive wetlands and shallow lakes a considerable portion of primary production is deposited to the sediment (Austin and Findlay 1989; Boström 1991), representing an important carbon source for benthic microheterotrophs. Here we present a study concerning the silty littoral freshwater sediment of a macrophyte-dominated, temperate oxbow of the river Danube. First, a dual label approach for estimating bacterial production was established, taking care to investi-

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Acknowledgments

This project was financed by a grant from the city of Vienna (MA 45-HY; DI. W. Schiel). Special thanks are due to D. B. Nedwell, M. A. Moran, and two anonymous reviewers for helpful and constructive comments on the first version of the manuscript.

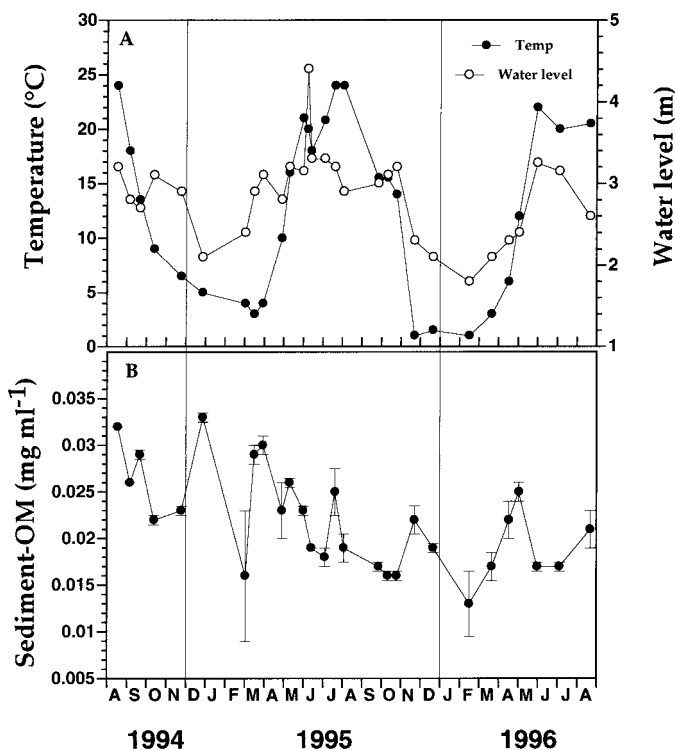


Fig. 1. Variation in temperature, water level (A), and organic matter content (B) during a 2-yr cycle from 1994 to 1996 in the Kühwörter Wasser.

gate the reasons for potential discrepancies between the ³H-thymidine and the ¹⁴C-leucine methods. Second, over a 2-yr period the production estimates were related to other measured bacterial and environmental variables in order to elucidate the role of the benthic bacterial community in the carbon cycle during different seasons.

Materials and methods

Study site and sampling—Soft bottom sediments were sampled in triplicate at one station at the Kühwörter Wasser, an oxbow lake within the backwater system Lobau of the river Danube located at the left downstream bank in Vienna, Austria (Kirschner and Velimirov 1997). The Lobau has been separated from the main stream since the 1870s by a series of embankments, with only one downstream connection remaining at the lowest part. During situations of extreme floods (approximately once every 2–3 yr) water is dammed up through this opening and high sediment loads can be deposited in the oxbow. However, the water level, recorded from a fixed gauge at the lowest end of the Kühwörter Wasser, can vary significantly over the seasons (Fig. 1). The investigated oxbow lake has an average open water area of 0.23 km² and an average water depth of about 1 m. The residence time ranges from several weeks to months during the major part of the year with the exception of flooding periods, occurring mostly in spring after snow-melt. A thick silty sediment layer, with a mean depth of 39 cm (unpubl. data), fills the basin of the Kühwörter Wasser, with

submerged macrophytes (*Myriophyllum spicatum*, *Potamogeton pectinatus*, *Ceratophyllum demersum*, *Najas marina*, *Nitellopsis* sp., etc.) and floating-leaved Nymphaeaceae (*Nuphar lutea*, *Nymphaea alba*) dominating the system. A periodically flooded, emergent macrophyte belt, composed of *Phragmites australis*, *Schoenoplectus lacustris*, and *Typha latifolia* with an approximate area of 0.13 km² surrounds the major part of this oxbow.

In the center of the oxbow, three sediment corers with an inner diameter of 5 cm were carefully introduced by hand into the sediment to a depth of about 15 cm and withdrawn by applying a slow rotary movement in order to prevent resuspension of the upper sediment layer. The sediment cores were transported within 30 min to the laboratory in a cooled box at in situ temperature. In the laboratory, the overlying water was removed carefully and the central part of each sediment core was subsampled with a sterilized 25-ml pipette with a cut-off tip to achieve a bigger aperture. Care was taken to sample only the uppermost oxidized zone (0.5–1 cm thickness) that was defined by its light brown color in contrast to the dark brown layer below. Each subsample was transferred into a sterilized glass vial, and a slurry was produced by mixing the sediment with a stirring magnet. No sterile water was added because of the high water content of the sediment.

Bulk density, dry weight, and organic matter content—For determination of the bulk density of the sediment, a tared vessel of known volume was filled with sediment slurry and weighed. For the determination of dry weight and ash-free dry weight (organic matter content), a known amount of sediment was dispensed on aluminum foil, dried to constant weight at 95°C (dry weight), and burned for 6 h at 460°C. The weight loss upon ignition was considered to represent the ash-free dry weight fraction. All sediment parameters were related to volume (Bird and Duarte 1989).

Dual label incubation and extraction of macromolecular pools—Subsamples (0.25 g) of each of the slurries were put into 1.7-ml screw-cap Eppendorf tubes. During the first months of the investigation period (August 1994 to April 1995), the three slurries were mixed together. The use of a slurry has been tested and shown to have no effect on the measured incorporation rates of labeled substances compared to undisturbed samples (Dobbs et al. 1989; Kaplan et al. 1992). ³H-thymidine (specific activity: 196,000 dpm pmol⁻¹; NEN Research Products) and ¹⁴C-leucine (specific activity: 720 dpm pmol⁻¹; NEN Research Products) were added simultaneously to the sediment samples and incubated at in situ temperature for 1 h. As was shown by Chin-Leo and Kirchman (1988) for aquatic samples, no significant difference was observed between dual-label and single-label experiments for both thymidine and leucine. After the addition of 100 μl 37% formaldehyde, the samples were centrifuged in an Eppendorf centrifuge (5415 C) at 14,000 rpm (15,800 × g) for 10 min. The supernatant was removed carefully with a pipette (Gilson-Pipetman) and the macromolecules were extracted with 1 ml of a 0.6 M NaOH/0.1% sodium dodecyl sulfate (SDS)/25 mM ethylenediaminetetraacetic acid (EDTA) solution (Kaplan et al. 1992; Tibbles et al.

1992) at 100°C in a water bath for 4 h and left to stand overnight at room temperature. Extracting the macromolecules at room temperature, as proposed by Marxsen (1996) for streambed sediments, was tested, but resulted in approximately 80% lower values, as compared to the method described above. After centrifugation at 14,000 rpm for 10 min the supernatant was transferred into a new Eppendorf vial. Hydrochloric acid (100 μ l of 25% v/v) was added for acidification of the extract, along with 100 μ l of a herring-sperm-DNA solution (5 mg L⁻¹; Sigma-Aldrich) as coprecipitant for the labeled DNA (Moriarty and Pollard 1981, 1982) and 65 μ l trichloroacetic acid (TCA, 100%; Sigma-Aldrich) for precipitation of the macromolecules. Addition of a humic extract as coprecipitant for DNA and proteins (Moriarty and Pollard 1981, 1982; Kaplan et al. 1992) was not necessary because of the dark brown color of the sediment extract, indicating a high amount of humic substances. After cooling for at least 30 min on ice (visible formation of a precipitate), the samples were again centrifuged at 14,000 rpm for 10 min. After one washing step with 5% TCA, the supernatant was withdrawn and the pellet, considered to contain both DNA and protein, was used to separate these two macromolecular fractions. TCA (1 ml of 5% v/v) was added and boiled for 30 min to solubilize DNA (Kaplan et al. 1992). After centrifugation (14,000 rpm, 5 min) the supernatant was transferred into 7-ml scintillation vials (Canberra Packard). The pellet was redissolved in 1 ml of 0.6 M NaOH at 100°C in a water bath for 1 h, centrifuged (14,000 rpm, 5 min), and the supernatant containing the protein fraction was transferred into 7-ml scintillation vials. Scintillation cocktail (5 ml, Ultima Gold; Canberra Packard) was added and radioactivity was measured with a dual-label counting program in a Canberra Packard scintillation counter (1900 TR). Counts were automatically corrected for quenching using a stored standard curve and a machine counting efficiency program.

The linearity of the incorporation over time and the appropriate concentrations of the isotopes were checked in preliminary experiments. A constant amount of 3.2 μ M (final concentration) of both ³H-thymidine and ¹⁴C-leucine was added to the sediment slurries and incubation was stopped after 10–120 min to check for linearity of the isotope incorporation. Time course experiments revealed that both ³H-thymidine and ¹⁴C-leucine incorporation were linear for at least 1 h. Saturation experiments were performed on two occasions during summer 1994. Concentrations ranging from 0.05 μ M to 3.2 μ M (final concentration in the sediment) of ³H-thymidine and ¹⁴C-leucine were added to the slurries.

Because no saturation for either substrate at concentrations of 3.2 μ M could be observed (data not shown), isotopic dilution experiments were performed at every sampling date. In addition to a constant amount of ³H-thymidine (0.4 μ M final concentration) and ¹⁴C-leucine (3.2 μ M final concentration), increasing amounts of unlabeled thymidine (0.5–8.0 μ M final concentration) and leucine (4.0–64.0 μ M final concentration) were added. The reciprocal plot method (Pollard and Moriarty 1984) was applied to calculate the extent of the isotope dilution.

Control of efficiency of extraction and separation of DNA and protein—The extraction efficiency of the basic solution and conditions used was examined with two different approaches. In the first approach the sediment was extracted repeatedly under the same conditions described above until no more radioactivity could be found in both the DNA and the protein fraction. In the second approach bacteria were labeled with both ³H-thymidine and ¹⁴C-leucine and mixed to autoclaved sediment that was then extracted as mentioned above. Briefly, 500 ml of water of the Kühwörter Wasser were amended with 0.1 g yeast extract and 0.5 g glucose. As microscopic examinations of a parallel setup confirmed, bacteria reached the log-phase after 14 h and 400 μ Ci (50 nM final concentration) ³H-thymidine plus 10 μ Ci ¹⁴C-leucine (300 nM final concentration) were added. After 24-h aliquots of 8 ml were transferred to 10 ml polycarbonate centrifuge tubes (Sigma, Germany) and centrifuged for 5 min at 6,300 \times g (Sigma 3K30, Germany). The supernatant was decanted and the pellet washed three times with Davies salt medium (DSM), until no radioactivity could be detected in the supernatant. The labeled bacteria were then resuspended in 1 ml DSM and stored at –20°C. To test extraction efficiency, 50- μ l aliquots (eight replicates) of the labeled cell suspension were measured for radioactivity after addition of 1 ml 5% TCA in order to destroy the cells and prevent self-absorption of the radioactivity by the cell wall. Another eight replicates were added to 250 μ l sediment and extracted as described above. With this experiment we could also test for the efficiency of the separation of DNA and proteins with the chosen acid/base approach.

Conversion of incorporation rates to carbon production—The measured ³H-thymidine and ¹⁴C-leucine incorporation rates per sample were normalized to 1 ml of wet sediment and multiplied by the calculated isotope-dilution factor. For the data derived from the saturation experiments in summer 1994, the amount of isotopic dilution was estimated by a kinetic approach assuming Michaelis–Menten kinetics, according to van Looij and Riemann (1993). V_{\max} was considered as true incorporation rate, and the amount of isotopic dilution was calculated by dividing V_{\max} by the incorporation rate measured at the maximum concentration used in the saturation experiments (0.8 μ M ³H-thymidine and ¹⁴C-leucine on 19 August 1994 and 3.2 μ M on 7 September 1994). For ³H-thymidine a conversion factor of 2×10^6 cells (pmol TdR incorporated)⁻¹ was applied (Bell 1990; Chin-Leo and Kirchman 1988), and carbon production was then computed by multiplication with the average cellular carbon content (*see below*). The potential range of errors of the conversion factor for estimating bacterial production is mentioned in the discussion. ¹⁴C-leucine incorporation rates (leu_{inc}) were converted to carbon production (BSP) according to Simon and Azam (1989) by using the following equations:

$$BPP = leu_{inc} \times ID \times (100/7.3) \times 131.2 \quad (1)$$

where BPP = bacterial protein production; ID = the measured isotope dilution, 100/7.3 = 100/mol% of leucine in protein; 131.2 = formula weight of leucine.

$$BSP = BPP \times 0.86 \times 1.18 \quad (2)$$

where 0.86 and 1.18 represent the factors requested for converting bacterial protein production into BSP, taking into account that 86% of the weight of the amino acids is the carbon moiety and that 18% of the amino acids are not detected by high-pressure liquid chromatography (HPLC) (Simon and Azam 1989).

Bacterial cell numbers, cell volumes, biomass, and frequency of dividing cells—Bacterial numbers were determined according to the acridine-orange direct count method (Hobbie et al. 1977). Wet sediment slurry (1 ml) was dispensed into an autoclaved 40-ml plastic vial and mixed with 34 ml of a sterile-filtered formaldehyde solution (4% final concentration). Sodium-tetrapyrophosphate solution (5 ml of 0.01 M final concentration) were added prior to sonication at 40 W (three times, 20 s) using a Branson sonifier 450. According to Ellery and Schleyer (1984), these conditions were previously tested to remove efficiently the bacterial cells from the sediment particles without any measurable reduction of the cell numbers. No significant difference was found between cell counts before and after sonication of triplicate water samples with previously determined bacterial cell density. The samples were then diluted 2,000 times, and 1 ml was mixed with a few drops of an acridine-orange solution (final concentration: 0.01%). After 2 min the mixture was filtered through a black 0.2- μm pore-size filter (Millipore) that had been mounted onto a cellulose-nitrate filter (Sartorius, 0.2 μm pore size) resulting in an even distribution of the cells on the filter. Filters were observed at a magnification of 1,250 \times with a Leitz Diaplan microscope equipped with an HBO 50-W mercury lamp (excitation wavelength 450–490 nm, cutoff filter 515 nm). Bacteria were separated into four classes according to their different morphology: rods, cocci, vibrios, and filamentous bacteria. Cells were operationally defined as rods if their length and width differed by more than 0.1 μm . Bacteria longer than 3 μm and showing a diameter smaller than or equal to 0.5 μm were defined as filamentous forms. Bacteria were sized by an eyepiece micrometer. Fluorescent latex beads with diameters of 0.11, 0.22, 0.6, and 0.88 μm were used for calibration of the sizing procedure (Velimirov and Walenta-Simon 1992). Cell volume estimations were based on the assumption that all bacteria are spheres or cylinders with two hemispherical caps. At least 15 microscopic fields per sample were counted and 160–200 cells were measured (>40 per morphotype). Cellular carbon content in fg C cell⁻¹ (C) was calculated from estimated cell volumes (V; μm^3) assuming the allometric relation $C = 120V^{0.72}$ after Norland (1993). Dividing cells were determined in at least 20 fields per sample by counting only those cells in the initial stages of invagination (Hagström et al. 1979).

Results

Dual-label incubation and extraction of macromolecular pools—The chosen 0.6 M NaOH/0.1% SDS/25 mM EDTA solution was found to be an appropriate medium for the extraction of both DNA and proteins from silty freshwater sediments. This was tested in two ways. Method 1 (repeated extraction of the sediment) showed that after the first ex-

traction step 91.6% (standard deviation [SD] 2.8%) of the incorporated tritium label was found either in the extracted DNA or protein fraction. For the radioactive carbon a recovery of 97% (SD: 1.9%) was achieved after the first extraction step. After three steps no more radioactivity could be measured in either the DNA or the protein fraction. With method 2 (addition of labeled bacteria) an extraction efficiency of 85.7% (SD: 2.6%) was obtained for the tritium label and 95.6% (SD: 3.2%) for the radiocarbon label. Method 2 also indicated the high efficiency of the chosen acid-base approach for separating DNA and proteins. Only 6.3% of the tritium label of the radiolabeled cells occurred in the protein fraction, while only 15.9% of the ¹⁴C-label occurred in the DNA fraction. If the acid-base approach would have been inefficient, higher percentages of the ³H and ¹⁴C should have occurred in the protein and DNA fraction, respectively.

Environmental variables—During the 2-yr cycle temperature varied between 1°C and 24°C (Fig. 1A). In January and February of both years the Kühwörter Wasser was covered with an ice layer. The water level, recorded at the lower end of the Kühwörter Wasser, varied between 1.80 m and 4.40 m (mean: 2.85 m; Fig. 1A). Lowest values usually occurred during winter, while highest levels were recorded in spring during the period of snow melt. Average bulk density of the sediment was 1.097 g ml⁻¹ (1.067–1.136), while the dry matter content varied between 0.09 to 0.20 g per g fresh weight. The organic matter content of the sediment ranged from 13 to 33 mg ml⁻¹ (Fig. 1B) with a mean value of 21 mg ml⁻¹, showing no significant correlation with any of the measured parameters.

Bacterial numbers, cell volumes, and biomass—Bacterial numbers peaked in late summer of all investigated years with maximum densities of 8.51×10^9 cells ml⁻¹ (Fig. 2A). A minimum of 2.49×10^9 cells ml⁻¹ was observed in October 1994, soon after the summer peak. Also in summer 1995 a sharp decline after the summer period occurred in October, but not as strong as 1 yr before. Average cell volumes were highest in late summer for all years with maxima of 0.207 μm^3 cell⁻¹ (Fig. 2B). In 1995, though, maximal average cell volumes of only 0.16 μm^3 were recorded. In addition to the summer maximum, a spring peak could also be observed in both years. During the cold period the mean cell volume was low, varying between 0.11 and 0.12 μm^3 cell⁻¹. Variation of bacterial biomass followed the pattern of bacterial numbers with maximum values up to 288 $\mu\text{g C ml}^{-1}$ in late summer (Fig. 2C). The lowest value of 63.6 $\mu\text{g C ml}^{-1}$ was recorded in October 1994 at the end of a steep decline after the summer maximum. Assuming that 50% of the organic content of the upper sediment layer is carbon, total bacterial carbon biomass amounted to only 1.4% of the organic carbon in the sediment over the investigation period. The highest contributions were found in summer with 2.6%, the lowest during winter with 0.6%.

Bacterial secondary production and frequency of dividing cell (FDC) rates—Bacterial secondary production, estimated with the ³H-thymidine and the ¹⁴C-leucine method, varied much more strongly over the year than did bacterial numbers

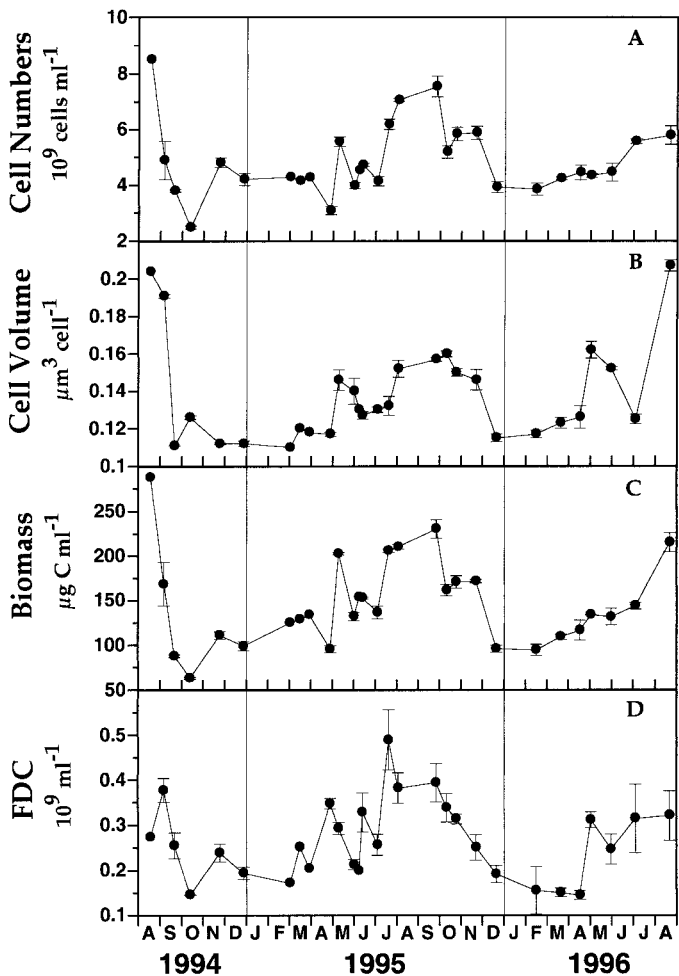


Fig. 2. Bacterial numbers (A), cell volumes (B), biomass (C), and FDC-values (D) during a 2-yr cycle from 1994 to 1996 in the upper sediment centimeter of the Kühwörter Wasser. Values represent mean of three replicates plus one standard deviation.

or biomass (Fig. 3A). The TdR approach yielded values between 42 and 2,350 $\mu\text{g C L}^{-1} \text{h}^{-1}$ with a mean of 676 $\mu\text{g C L}^{-1} \text{h}^{-1}$; with the leucine approach production rates in a range from 67 to 2,490 $\mu\text{g C L}^{-1} \text{h}^{-1}$ were found, with a mean of 892 $\mu\text{g C L}^{-1} \text{h}^{-1}$. Combined data over the whole investigation period showed no statistically significant difference between the two methods (ANOVA, $P > 0.1$). During most of the investigation period the leucine/thymidine ratio varied between 0.7 and 1.4. The average ratio over the investigation period was 1.6, the median was 1.2, showing that the ^{14}C -leucine method led to slightly higher results than the ^3H -thymidine approach. Only during the periods from June to August in 1995 and 1996 were significant differences between the methods recorded (ANOVA, $P < 0.05$ and 0.01, respectively), with leu/thy ratios of up to 5.0. The lowest ratio of 0.4 was recorded on 22 November 1995. However, irrespective of the difference between the two methods, a clear seasonal pattern could be observed for the production rates with two annual maxima, one occurring in spring and a second in summer. Also, a strong significant positive correlation could be found between the two methods, with a

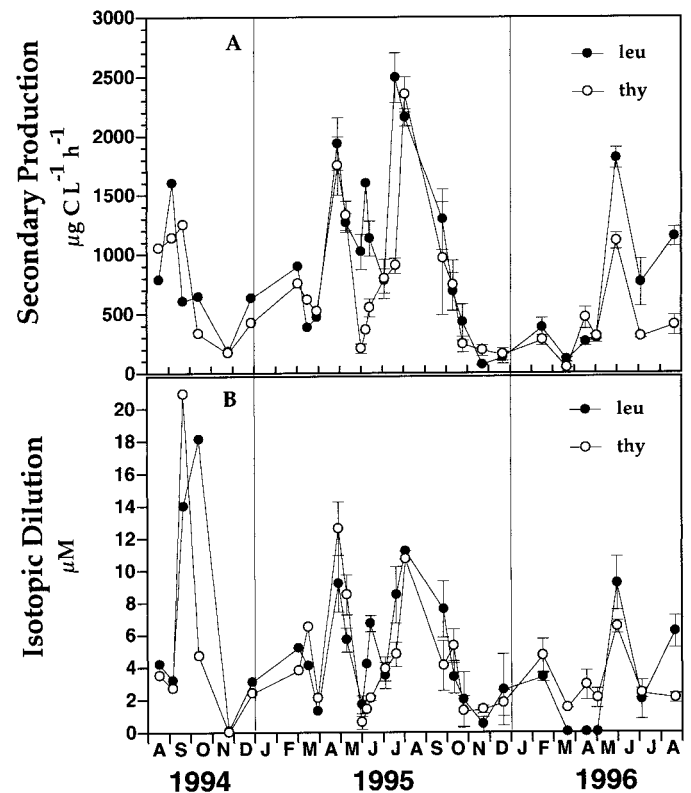


Fig. 3. Bacterial secondary production (A) and calculated isotopic dilution values (B), measured with the ^3H -thymidine (thy) and ^{14}C -leucine (leu) method in the upper sediment centimeter of the Kühwörter Wasser during a 2-yr cycle from 1994 to 1996. Values represent mean of three replicates plus one standard deviation.

Spearman-rank correlation coefficient of $r_s = 0.72$ ($P < 0.001$; $n = 29$), corroborating the high correspondence between the two approaches for measuring sediment bacterial production. The extent of isotope dilution determined with the reciprocal plot method varied widely over the seasons for both isotopes (Fig. 3B). For ^3H -thymidine, between 0 and 20.9 μM (mean: 4.4 μM) extra- and/or intracellular thymidine diluted the added isotope; in the case of ^{14}C -leucine, the values ranged from 0 to 18.1 μM (mean: 4.9 μM). Low values occurred in the cold seasons, while during summer and a short period in spring the isotope dilution was highest. The isotope dilution values were significantly correlated with the production rates, estimated from the incorporation of the respective isotopes ($r = 0.80$ and 0.71 for ^3H -thymidine and ^{14}C -leucine, respectively; $P < 0.001$). The percentage of tritium label found in the DNA fraction was rather low during the whole investigation period (17–49% of the label found in both protein and DNA; mean: 33%; data not shown), indicating that a large amount of the incorporated thymidine was catabolized and used for metabolic pathways other than DNA synthesis, for example protein synthesis. On the other hand, the percentage of radiocarbon occurring in the protein fraction averaged 80% over the whole period (range: 71–95%; SD: 1.9%; data not shown) and was significantly negatively correlated with the percentage of tritium label in the DNA fraction ($r = -0.82$; $P < 0.001$).

The FDC ranged from $0.15 \times 10^6 \text{ ml}^{-1}$ to $0.49 \times 10^6 \text{ ml}^{-1}$ (Fig. 2D). Numbers below $0.20 \times 10^6 \text{ ml}^{-1}$ were observed during the cold periods, while numbers above $0.3 \times 10^6 \text{ ml}^{-1}$ occurred only in summer and during a short period in early spring of both 1995 and 1996. The observed variations corresponded rather well with the measured production rates obtained by the ^3H -thymidine and the ^{14}C -leucine approach. The corresponding Spearman-rank correlation coefficients (r) were 0.54 ($P < 0.01$) and 0.61 ($P < 0.001$) for thymidine and leucine, respectively. When FDC was expressed as percentage of total cell numbers, the correlations were less significant ($r = 0.41$; $P < 0.05$ and $r = 0.43$; $P < 0.05$, respectively). The calculated FDC values varied from 3.2% in August 1994 to 11.3% in April 1995.

Turnover time of the bacterial community, calculated from ^{14}C -leucine and the ^3H -thymidine incorporation rates, ranged from 2 to about 100 d.

Discussion

Correspondence between the thymidine and leucine incorporation method—Our investigations show that, over a 2-yr period, bacterial secondary production estimates derived by the ^3H -thymidine method were not significantly different from the data obtained by simultaneous ^{14}C -leucine incorporation. We observed a strong positive correlation between the two methods ($r = 0.72$; $P < 0.001$) and the production rates were in the same range (Fig. 3).

Only during two periods, one from June to July 1995 and one from June to August 1996, were big differences observed, when the carbon production rates derived from leucine incorporation were up to five times higher than the rates derived from thymidine incorporation. An uncoupling of DNA and protein synthesis has been previously observed by authors working in a variety of aquatic environments. Molar ratios of leucine and thymidine incorporation were reported to range between 6 and 50 for water-column and mesocosm data (Shiah and Ducklow 1997). Chin-Leo and Kirchman (1988, 1990) found that the molar ratio could exceed a value of 75. In our study the ratios varied between 12 and 183 over the whole investigation period (mean: 53; median: 38), being significantly higher than those cited above. Tibbles et al. (1992) also reported much higher ratios for sediment samples (mean values ranging from 51 to 113) than for water samples (mean values ranging from 2 to 39) from an estuarine environment. One of their explanations for this observation was the presence of large populations of sulfate-reducing bacteria that are incapable of incorporating exogenous thymidine. Several anaerobic bacterial species were demonstrated to be unable to utilize exogenous thymidine (Gilmour et al. 1990; Winding 1992). However, other explanations are also conceivable. First, conversion factors may vary significantly over the seasons, as was observed for water-column bacteria in another oxbow of the river Danube (Wihlidal et al. in prep.). Second, unbalanced growth might occur during certain periods over the year when cells increase strongly in volume, as was the case in the sediment of the Kühwörter Wasser during summer. However, high Leu/TdR ratios do not necessarily imply the occurrence of

unbalanced growth, because despite high Leu/TdR ratios, we came to nearly equal carbon production estimates for both methods, when incorporation rates were converted to bacterial carbon production using conversion factors that were the same for sediment (Fallon and Boylen 1990; Wellsbury et al. 1996) and for water samples (Chin-Leo and Kirchman 1988; Simon and Azam 1989; Kirschner and Velimirov 1997). The reason for this is simply that, when TdR-incorporation rates are converted to C-production, the cell volume of the bacteria is used in the calculations (*see Materials and methods*), while leucine-derived C-production rates are cell volume independent (Simon and Azam 1989). Cell volumes of sediment bacteria in the Kühwörter Wasser are two to three times bigger (Fig. 2B) than water column bacteria (Kirschner and Velimirov 1997) thus leading to relatively higher C-production estimates for sediment bacteria, when estimated with the ^3H -thymidine method. This implies that in sediment environments, under the assumption of a rather constant DNA amount per cell irrespective of the cell volume, bacteria have to produce relatively more protein in comparison to DNA to produce the same amount of cells as do smaller water-column bacteria. During the two observed periods of uncoupling between the ^3H -thymidine and the ^{14}C -leucine method, however, it is also possible that an oxygen deficiency occurred even in the uppermost millimeters of the sediment, thus leading to lower thymidine uptake rates. Such a decrease of thymidine incorporation rates during summer was also observed for mesotrophic Lake Erken, when redox potential was low (Goedkoop and Törnblom 1996).

Reliability of the proposed dual-label design—We applied two independent methods to check the efficiency of our extraction medium, both of which yielded similar results. However, the extraction protocol yielded a lower extraction efficiency in coastal sandy marine sediments, as tested in a preliminary series in the bay of Calvi (unpubl. data), and the protocol of Craven and Karl (1984) for carbonate sands was recommended for sandy ecosystems. Because of the great variability over the seasons, even at time intervals of 2 weeks, isotopic dilution should be measured at every sampling event for both ^3H -thymidine and ^{14}C -leucine. Adding radioactive substances at concentrations high enough to prevent isotope dilution in organic-rich silty sediments is possible but is rather limited by the high costs. Similarly, high saturation values as in the Kühwörter Wasser were found by Marxsen (1996), where ^{14}C -leucine incorporation rates saturated above $20 \mu\text{M}$ in a sandy stream bed. Also Kairesalo et al. (1995) reported values of $>15 \mu\text{M}$ for lake sediments. Another problem encountered when high substrate concentrations are added is the possible danger of enhancement of bacterial growth. In fact, during some sample periods this was possibly observed for the thymidine incorporation rates of the dual-label assays (Fig. 4). For these samples only the first data points could be used for calculating the isotopic dilution. Integrating all points and assuming that the fitting of a straight line is appropriate for the calculation of the dilution values (Pollard and Moriarty 1984), the result would lead to unrealistic ID and production values more than 10 times higher than the production rates obtained by the leucine method. Because there are no reports of in situ gener-

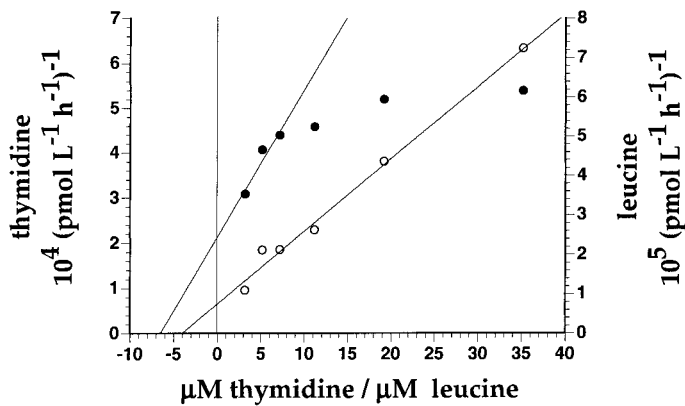


Fig. 4. Example for an isotopic dilution plot, where simultaneous incorporation of increasing concentrations of leucine (open triangles) and thymidine (filled circles) led to enhanced bacterial thymidine uptake. Regression curve of thymidine was calculated only from the first three data points. 3.2 μM concentrations of ^3H -thymidine and ^{14}C -leucine were diluted with increasing amounts of non-radioactive thymidine and leucine, ranging from 0 μM to 32 μM .

ation times of sediment bacteria as low as what would be estimated from a straight line fit of all points (2–6 h at 10–14°C), we excluded the exceedingly high isotopic dilution values along with the associated estimates of DNA synthesis. In addition, regression analyses were performed by combination of all possible adjacent points and comparing the respective r^2 values (Kaplan et al. 1992). The best fit was always obtained for the three or four lowest concentrations. The leucine incorporation rates, in contrast, were never affected.

A low and variable proportion of the tritium label (mean: 33%) was recovered in the DNA fraction, indicating that the added thymidine was used to a large extent by bacteria as a carbon and/or nitrogen source. The values were much lower than those found for bacterioplankton in the Kühwörter Wasser (Kirschner and Velimirov 1997). For a coastal marine sediment, Carman et al. (1988) reported only 2% incorporation of label originating from added thymidine into the DNA fraction. This indicates the necessity of the isolation of DNA from total TCA-insoluble material. Much higher and more constant percentages (i.e., $80 \pm 1.9\%$) of the incorporated ^{14}C -label were found in the protein fraction. For further investigations in this ecosystem a constant factor of 0.8 could be applied when measuring ^{14}C radioactivity in the TCA-insoluble material alone.

Another indication of the reliability of the dual label approach is the strong positive correlation of the estimated FDC values with the ^3H -thymidine ($r = 0.54$; $P < 0.01$) and ^{14}C -leucine ($r = 0.61$; $P < 0.001$) production estimates. A strong correlation with the ^3H -thymidine method was also observed by Wellsbury et al. (1996) for estuarine and freshwater sediments. However, FDC values varied much less over the year (coefficient of variation [CV] = 32.1%) than did the thymidine (CV = 78.1%) or leucine incorporation rates (CV = 73.1%). Converting FDC values to bacterial carbon production is difficult and often leads to much higher production estimates than the incorporation methods (Fallon

and Boylen 1990; Boon 1991). It was also found that FDC conversion factors are dependent on temperature (Hagström and Larsson 1984). Therefore it is possible that small differences in the FDC values reflect big differences in actual growth rates in response to varying temperature or substrate supply and would lead to a bigger CV once FDC values are converted to production estimates.

Ecological considerations—Temperature seems to be one of the most important ecological factors influencing the bacterial community in the sediment of the Kühwörter Wasser. Temperature was significantly positively correlated with nearly all investigated bacterial parameters and explained 59% of the variation of the ^{14}C -leucine-derived production rates. Sander and Kalff (1993), summarizing results from a variety of different ecosystems, reported that temperature was the most powerful predictor of bacterial growth rates in both freshwater and marine sediments, with r^2 values ranging from 0.48 to 0.58. In their study, organic content was found to be a second important factor controlling bacterial secondary production in freshwater sediments. This was not obvious in our data, probably because the range of the organic matter content in our study was small, while in Sander and Kalff (1993) organic matter concentrations ranged over three orders of magnitude. Only 25% of the variation in bacterial production estimates derived from ^3H -thymidine incorporation were explained by temperature primarily due to a poor relationship with temperature during the two periods of uncoupling between ^{14}C -leucine and ^3H -thymidine incorporation (Fig. 3A). Temperature explained only 35% of the variation of FDC values, indicating that other important factors are also responsible for the variation of bacterial growth. Because the Kühwörter Wasser has no direct upstream connection to the main stream, high waters are delayed by 2 weeks in comparison to their occurrence in the Danube. Thus, water level is not expected to have a significant influence on the sediment bacterial community, which could be corroborated by our findings (Fig. 1). Only during situations of extreme floods (approximately once every 2 yr) is water dammed up through a downstream connection at the lowest part of the Lobau and are high sediment loads deposited in the oxbow lake.

A highly significant and positive relationship between the ID and the production values was observed for both methods ($P < 0.001$ for all cases). One possible interpretation might be that during periods with increased extracellular leucine and thymidine sources, bacterial growth is enhanced, reflecting larger pools of readily utilizable organic matter. In fact, high ID and production values (Fig. 3) occurred only during periods when increased organic matter flux to the sediment was expected, i.e., the period of the phytoplanktonic spring bloom and the period during and shortly after the intense macrophyte development (Kirschner and Velimirov 1997). Increase in benthic bacterial production during late spring following the sedimentation of a spring diatom bloom in eutrophic and mesotrophic lakes was observed by Johnson et al. (1989), Boström (1991), and Goedkoop and Johnson (1996). As Törnblom and Boström (1995) pointed out, bacterial sediment communities are able to respond quickly to sedimentation events, even at low temperatures. However, in

the Kühwörter Wasser an increase in organic matter content of the sediment that coincided with periods of strong phytoplankton and macrophyte development was observed in spring 1995 and 1996 and in summer 1994, but not during summer 1995. On the other hand additional peaks in organic matter content were registered in late autumn of 1994 and 1995, most probably due to the settling of macrophytes to the lake bottom (pers. obs.), although high bacterial production rates may have been prevented by low temperature. In addition, high organic matter content does not necessarily mean a high availability of the material for bacterial utilization (Nedwell 1987), and thus there may be low growth rates during periods with high organic matter input.

Variation of bacterial numbers and biomass followed changes in bacterial production rates with a short lag time (Fig. 2). Maximal density and biomass were observed in spring and summer shortly after the peaks in secondary production. Interestingly, the bacterial cell volumes also showed maximal values when bacterial numbers were high. In a series of Canadian lakes, water-column variables such as chlorophyll *a* (representing autochthonous organic matter) were found to be of minor importance for benthic bacterial biomass (Schallenberg and Kalff 1993), while macrozoobenthic biomass, water content, and the hydraulic flushing rate (representing the input of allochthonous organic matter) together were shown to explain a major part of the variation. Organic matter cycling in benthic freshwater habitats may therefore occur mainly between allochthonous detritus, microbes, and macrozoobenthos (Schallenberg and Kalff 1993; fig. 8). However, the application of their model was developed for larger lakes with an extensive drainage basin area and a high hydraulic flushing rate and may not apply for the Kühwörter Wasser. In this oxbow lake the annual autochthonic production of submerged (Kirschner et al. in prep.) and emergent (Kirschner and Velimirov, in prep.) macrophytes is substantial and inputs of allochthonous material are mainly confined to autumnal leaf fall of the surrounding trees or, as mentioned above, to the periodical occurrence of extreme floods. Microbenthic primary production is certainly also a possible important carbon source for the sediment bacterial community in the Kühwörter Wasser, although during the development of the intense submerged macrophyte vegetation (May–November; Kirschner et al. 1999) this factor probably plays a role only at sites where no light limitation occurs at the lake bottom. Top-down control of bacteria in freshwater sediments seems to be due mostly to macrozoobenthic (Schallenberg and Kalff 1993) or meiozoobenthic organisms (Goedkoop and Johnson 1996) ingesting high amounts of bacteria associated with sediment particles. Flagellates (Starink et al. 1996), ciliates (Epstein and Shiaris 1996; Kemp 1988), and viruses (Maranger and Bird 1996) have also been shown to have significant impacts on benthic bacterial populations. However, it is not known, to what extent these factors contribute to bacterial mortality in sediments.

Interecosystem comparison of sediment bacterial parameters—Data from the Kühwörter Wasser sediment are well within the range of data reported from other freshwater environments. Bacterial numbers varied from 0.66×10^9 to 25×10^9 cells ml^{-1} in several freshwater habitats (Schallenberg

and Kalff 1993) and bacterial biomass range from 1.0×10^9 (alkaline fen: Gsell et al. 1997) to 151×10^9 (hypertrophic lake: Bell and Ahlgren 1987) cells (g dry weight) $^{-1}$. On an areal basis, the bacterial biomass of the Kühwörter Wasser was $640\text{--}2,880$ mg C m^{-2} for the upper cm that also agrees well with the areal calculations from other sediments (Findlay et al. 1986; Austin and Findlay 1989). The ratio of bacterial biomass carbon to total sediment organic carbon ranged from 0.9 to 20.3% (mean: 5.2%) in several freshwater sediments (Schallenberg and Kalff 1993), and a mean of 8% was found in a profundal sediment of a Swedish eutrophic lake (Boström 1991). Assuming a carbon content of 50% of the organic matter, the calculated values for the Kühwörter Wasser (0.6–2.6%) were in the lower range of these data.

In silty sediments of a freshwater marsh, dominated by different emergent and floating-leaved macrophytes, production rates determined via thymidine incorporation ranged from 0.001 to $0.94 \mu\text{g C ml}^{-1} \text{h}^{-1}$, with no significant difference between habitats (Austin and Findlay 1989). While these values are lower than our findings ($0.04\text{--}2.49 \mu\text{g C ml}^{-1} \text{h}^{-1}$), applying our conversion factors to the raw data of the previous study results in nearly identical bacterial production rates. Doubling times of the bacterial sediment population in the Kühwörter Wasser ranged from 2 to 108 d, considerably higher than in the water column, where values from 7 hours to 3.5 d were observed over a 1-yr cycle (Kirschner and Velimirov 1997). Similar values, ranging from 1 (Bott and Kaplan 1985) to 687 d (Austin and Findlay 1989) were also found for sediments of other freshwater habitats. Sander and Kalff (1993) speculated that the reason for the long doubling times may be due to a lower bacterial loss rate in sediments thus leading to higher cell numbers, or alternatively due to possible artifacts, associated with applying the ^3H -thymidine method to sediments. The good agreement of the ^3H -thymidine and ^{14}C -leucine method in this argues against the second explanation for the Kühwörter Wasser. Another reason for this observation, proposed by Novitsky (1987) and Törnblom and Boström (1995) might be that sediment bacterial populations consist of a larger percentage of dormant or empty bacterial cells than do water-column populations. In addition, sediment organic matter might be more refractory than organic matter in the overlying water, where bacteria can utilize freshly produced and exuded dissolved organic matter, although this argument may be more relevant only for deep-water sediments (Suess 1980; Sander and Kalff 1993).

Carbon budget for the Kühwörter Wasser—To assess the magnitude of the carbon flow through the bacterial compartment in the sediment of the Kühwörter Wasser, we adopted a number of conservative assumptions. For calculating the bacterial carbon demand from production rates, a constant bacterial growth efficiency of 31% (Kristiansen et al. 1992) was applied throughout the year. Growth efficiency values in the literature for sediment bacteria range from 10% to 40% (Bell and Ahlgren 1987; Törnblom 1996; Goedkoop et al. 1997) with the highest values recorded during spring periods (Törnblom 1996). Although the value of 31% was established for bacterioplankton, it fits well into the range of values reported for sediments and was used previously for

Table 1. Bacterial secondary production (BSP) and carbon demand (BCD), expressed as $\text{g C m}^{-2} \text{ month}^{-1}$, in the upper centimeters of sediment. Values represent monthly averages of all values recorded during the investigation period from 1994 to 1996.

Month	^{14}C -leucine		^3H -thymidine	
	BSP*	BCD†	BSP‡	BCD‡
January	2.7	8.6	2.0 (1.0–2.8)	6.4 (3.2–9.0)
February	2.5	8.1	1.8 (0.9–2.5)	5.9 (3.0–8.3)
March	3.5	11.2	3.6 (1.8–5.0)	11.5 (5.8–16.1)
April	7.9	25.4	8.0 (4.0–11.2)	25.6 (12.8–35.8)
May	8.3	26.9	6.8 (3.4–9.5)	21.8 (10.9–30.5)
June	9.0	29.0	2.7 (1.4–3.8)	8.6 (4.3–12.0)
July	10.0	32.2	4.9 (2.5–6.9)	15.9 (8.0–22.3)
August	10.1	32.7	9.4 (4.7–13.2)	30.4 (15.2–42.6)
September	8.4	27.1	8.0 (4.0–11.2)	25.9 (13.0–36.3)
October	4.3	14.0	3.2 (1.6–4.5)	10.5 (5.3–14.6)
November	0.9	2.9	1.3 (0.7–1.8)	4.1 (2.1–5.7)
December	2.8	9.0	2.1 (1.1–2.9)	6.9 (3.5–9.7)

* Production rates calculated after Simon and Azam (1989).

† Calculated assuming a growth efficiency of 31% (Kristiansen et al. 1992).

‡ Production rates calculated assuming a conversion factor of 2.0×10^6 cells pmol^{-1} , values in parentheses calculated using a factor of 1.0×10^6 and 2.8×10^6 cells pmol^{-1} (see text for details).

the bacterioplankton of the Kühwörter Wasser (Kirschner and Velimirov 1997).

Production and carbon demand rates were calculated per m^2 and per month for both years (Table 1). For the calculations derived from TdR data, the conversion factor of 2.0×10^6 was applied for two reasons. First, this factor had already been used by other authors for sediment bacteria (e.g., Fallon and Boylen 1990; Wellsbury et al. 1996), and second, this value is close to the mean of the range from 1.0×10^6 (a factor that was also used by other authors for sediment bacteria; e.g., Austin and Findlay 1989; Gsell et al. 1997) to 2.8×10^6 (a factor applied for the water-column bacteria of the Kühwörter Wasser; Mathias et al. 1995; Kirschner and Velimirov 1997). From March to May, the period of the phytoplankton spring bloom (Kirschner and Velimirov 1997) and the bacterial secondary production peak (Fig. 3A), the benthic bacterial C demand amounted to 63.5 g C m^{-2} (Leu) and 59.0 (TdR), respectively. As calculated by Kirschner and Velimirov (1997), about $115 \mu\text{g C L}^{-1} \text{ d}^{-1}$ of the phytoplankton primary production in the Kühwörter Wasser are left over for the detritus pool, corresponding to $115 \text{ mg C m}^{-2} \text{ d}^{-1}$ (average depth of the Kühwörter Wasser = 1 m) or to 10.6 g C m^{-2} for the whole period from March to May. Assuming that this amount becomes completely available for the superficial benthic bacterial community by sedimentation, a deficit of 48.4 – 52.9 g C m^{-2} needs to be covered to balance the bacterial carbon demand. This could originate either from benthic algae or from degrading submerged macrophytes of the previous season, settled to the lake bottom. The average standing stock of submerged macrophytes in August and September 1995 was 105 g C m^{-2} (Kirschner et al. 1999). If this standing stock begins to be degraded by both planktonic and benthic bacteria in October, the material left over at the end of February can be calculated after subtraction of the measured bacterial C-demand rates for the

water column (Kirschner and Velimirov 1997: table 3) and for the sediment (this paper: Table 1) during this period. C demand of water column bacteria (reduced for the carbon covered by planktonic primary production) amounted to 6.3 – 7.8 g C m^{-2} , while C demand of the benthic bacteria made up between 33.8 (TdR) and 42.6 g C m^{-2} (Leu). Therefore between 54.6 to 64.9 g C m^{-2} of undegraded macrophyte material is still available to the bacterial community. This value fits well with the deficit value of 48.2 – 52.9 g C m^{-2} for the benthic bacterial C demand during the period from March to May, providing strong evidence for the hypothesis that bacteria live on the detritus of the submerged macrophytes until June of the following year. In fact, degradation studies of the different dominant macrophytes in the Kühwörter Wasser revealed that complete degradation occurs by summer of the following year (Kirschner et al. in prep.).

During the summer maximum of benthic bacterial secondary production rates, no planktonic primary production is available to the benthic microbial community. Even the bacteria in the water column are dependent on other C sources for 23.4 – 33.4 g C m^{-2} for the period from July to September (Kirschner and Velimirov 1997). Together with the benthic bacterial carbon demand, an additional subsidy of 95.6 – 125.4 g C m^{-2} is required by the microbial community in the Kühwörter Wasser. This subsidy might come from either autochthonic and allochthonic sources, i.e., benthic algae, exudates, and decomposition products of macrophytes and their epiphytic organisms, as well as terrestrial organic matter like leaves from the surrounding trees.

Conclusion

The dual-label design for measuring bacterial secondary production in silty freshwater sediments provided consistent results over a 2-yr investigation period. Periods of uncoupling between the thymidine and the leucine method occurred in late spring and early summer and suggested an underestimate of the production rates by the thymidine method during that time. A comparison of the leucine and thymidine incorporation rates with FDC values revealed highly significant positive correlations, supporting the reliability of the dual-label protocol. In addition, the calculated carbon flux through the bacterial compartment based on the results of the dual-label method corresponded well to the availability of different C sources in this shallow oxbow lake over the seasons. We therefore recommend the dual-label protocol described here for measuring bacterial secondary production in sediments of other ecosystems, provided that crucial parameters such as isotopic dilution, extraction efficiency, and the appropriate conversion factor for the environment are regularly determined.

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Received: 30 December 1998

Accepted: 21 July 1999

Amended: 6 August 1999