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Different filtration treatments and centrifugation in measuring bacterial production in brackish waters

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We tested the filtration method routinely used in pelagic bacterial production measurements against some new, modified and extended methods. We used both ^{14}C-leucine and ^{3}H-thymidine in comparison of two filter types, two modifications of the centrifugation method and the effect of filtering samples first through GF/F-filters (for ^{14}C-leucine only). Tests were run for natural brackish water samples and batch cultures from both pelagic and coastal waters. All seasons were covered in the sampling. The centrifugation methods gave systematically lower results than the filtration methods. Also the use of polycarbonate filters resulted in lower values in measuring ^{3}H-thymidine incorporation compared to the use of cellulose-nitrate filters. Combining the routine cellulose-nitrate filtration with filtering the incubated sample first through a combusted GF/F-filter did not affect the final results. This procedure can therefore be used to get information about the total carbon content of the samples. These results point out that the choice of the post-incubation treatment procedure has a substantial effect on the final bacterial production estimate.

Introduction

Bacterial production measurements are nowadays regarded an obvious part of any study quantifying the carbon flow in an aquatic ecosystem. The most widely accepted methods so far have been the ^{3}H-thymidine incorporation method, first introduced by Fuhrman and Azam (1980), and the ^{3}H-leucine incorporation method, developed a few years later by Kirchman *et al.* (1985). The former method is based on measuring DNA-production, the latter on measuring protein synthesis. Since introduction both methods have been subject to continuous modifi-

cations (reviewed by Robarts and Zohary (1993) for the thymidine method, *see* e.g., Chin-Leo and Kirchman (1988), Wicks and Robarts (1988), Simon and Azam (1989), Kirschner and Velimirov (1999) for the development of the leucine method). These methods are based on empirical conversion factors used to calculate the bacterial production from the measured isotope labelling of DNA or protein, respectively. Ideally the conversion factors are defined every time the methods are used, but as this is very time consuming, most often values taken from literature are used. It is crucial to get accurate incorporation rate estimates, as inaccuracies multiply in conversions.

Routine measurements today consist of incubating samples with radioisotope followed by filtration on either cellulose-nitrate or polycarbonate filters, then extracting the samples with ice-cold TCA before measuring the incorporated label trapped on filters in a scintillation counter. In 1992, Smith and Azam suggested using centrifugation instead of filtration in sample processing, resulting in lower cost per sample as well as reduced amount of radioactive waste. We used this method parallel to the traditional filtration method in various experiments carried out between 1995 and 1999. We used both ^{3}H-thymidine and ^{14}C-leucine to ascertain that the method could be used with both isotopes as stated by Smith and Azam (1992). At the same time we compared two different filter types (cellulose-nitrate and polycarbonate). In some experiments during 1998 and 1999 we filtered the incubated samples first through GF/F or combusted GF/F filters and then through cellulose-nitrate or polycarbonate filters. GF/F filters were then combusted in order to get an estimate of the total carbon content of the sample together with the amount of leucine incorporation.

Material and methods

Water for the experiments was collected either from pelagic surface waters of the Gulf of Finland, Baltic Sea (research cruises onboard R/V Aranda and R/V Victor Bujnickij) or from coastal waters near Helsinki, Finland. All seasons were covered in sampling (Tables 1 and 2). Water samples were either used immediately in experiments or a nutrient enriched, predator free batch culture was prepared. A batch culture always consisted of 1800 ml double GF/F (Whatman) filtered seawater and a seawater inoculum of 200 ml that had been filtered through a 0.8

Table 1. Experiments with ^{14}C-leucine. Statistically significant (* = $P < 0.05$) differences found when compared with the reference treatment (CN) by the Dunnett's *t*-test after a one-way ANOVA for each experiment separately. For treatment acronymes *see* Fig. 1. Values in boldface when $P > 0.05$ (no Dunnett's *t*-test run). Origin: P = pelagic, C = coastal. Sample: N = natural, B = batch. nd = no data, – = insignificant difference.

Exp.	Date	Origin	Sample	*P*-value	Treatments						
					PC	C1	CT	GF-CN	GF-PC	CGF-CN	CGF-PC
1	May 1995	P	N	0.0043	nd	*	nd				
2	May 1995	P	B	0.0480	–	–	*				
3	Jun. 1995	C	N	0.0483	–	–	*				
4	Jun. 1995	C	B	**0.0679**							
5	Jun. 1995	C	N	**0.4227**							
6	Jun. 1995	C	B	0.0016	*	–	–				
7	Jun. 1995	C	B	0.0040	–	*	*				
8	Jun. 1995	C	B	0.0001	–	*	*				
9	Jun. 1995	C	B	0.0074	–	–	*				
10	Jun. 1998	C	N	**0.1182**	nd	nd	nd	nd	nd		nd
11	Jun. 1998	C	B	0.0001	nd	nd	nd	nd	nd	*	nd
12	Dec. 1998	P	B	0.0020	–	–	*	–	–	–	–
13	Dec. 1998	P	B	**0.1740**							
14	May 1999	P	N	0.0001	–	*	*	–	–	–	–

μm pore size polycarbonate filter (Nuclepore, Poretics). The GF/F filters allow fast removal of larger particles (algae, zooplankton) as well as most of the bacteria (Lee *et al.* 1995) while filtering through a 0.8 μm pore size polycarbonate filter is a more gentle way to produce predator-free water without breaking the bacterial cells. Batches were enriched with 5.7 μM N, 0.65 μM P and 16.7 μM C. The absence of small flagellates in filtrates was checked by epifluorescence microscopy. Bacterial growth in batches was monitored by acridine orange cell counts (Hobbie *et al.* 1977) and experiments were conducted when logarithmic growth was achieved.

All incubations were carried out in sterile vessels (either combusted, autoclaved or, in case of microcentrifuge tubes, acid-washed) at ambient temperatures. High incubation concentrations of ^{14}C-leucine (130 to 270 nM; Amersham CFB-183) and ^{3}H-thymidine (20 to 70 nM; Amersham TRK-637) were used to ensure reliable counts. The ambient saturation concentrations reported in the Gulf of Finland and northern Baltic Proper area are around 5 nM for thymidine and 40 to 80 nM for leucine (Heinänen and Kuparinen 1992, Heinänen 1993). Therefore even the fast growing batch cultures most likely were saturated with the isotope concentrations used. The incubation time varied from 1 to 3 hours depending on the incubation temperature. Blanks were killed with either 37% formalin or 50% TCA before adding the isotope, and the same chemicals were used to terminate the incubations accordingly. After termination all samples were kept in ice-cold conditions until further processing within one hour. Samples were run either in 3 or 6 and blanks in 1 or 3 replicates. Eight different treatments were tested (Fig. 1).

Filtration treatments

The two commonly used filter types, cellulose-nitrate (CN; Ø 25 mm, pore size 0.2 μm, Sartorius) and polycarbonate (PC; Ø 25 mm, pore size 0.2 μm, Nuclepore or Poretics) filters, were tested. The results from the treatment routinely used in our laboratory, filtration on the cellulose-nitrate filter, were considered as reference results with which the results of all the other treatments were compared.

Cellulose-nitrate filters were briefly soaked in ice-cold 5% TCA before use to avoid non-washable labelling of the filters (Kairesalo and Saukkonen 1990). Otherwise both filters were treated the same way in processing. Extraction of the macromolecules was carried out on filters (Riemann 1984, Børsheim 1990). Subsamples of 5 to 10 ml were taken from large incubation vessels and filtered in ice-cold conditions (vacuum never exceeding 16.5 kPa). Filtration funnels were flushed with 2 ml of ice-cold 5% TCA and removed. Cells on filters were then extracted five times with 1 ml of ice-cold 5% TCA. Filters were placed in scintillation vials, 10 ml of scintillation liquid (Insta-Gel Plus, Packard) was added and samples were left to stand overnight to dissolve the cellulose-nitrate filter. The next day the samples were shaken vigorously to ensure homogeneity before counting in a scintillation counter (Wallac RackBeta 1217). (Fig. 1: CN and PC).

The samples filtered on GF/F filters were extracted the same way as the other filters mentioned above. They were combusted in a carbon analyser (Junitek Oxidizer) and a commercial amine cocktail (Lumasorb II, Lumac LSC B.V.) was used for trapping of $^{14}CO_2$. 12 ml of scintil-

Table 2. Experiments with ^{3}H-thymidine. Statistically significant (* = $P < 0.05$) differences found when compared to the reference treatment (CN) by the Dunnett's *t*-test after a one-way ANOVA for each experiment separately. For treatment acronymes *see* Fig. 1. Origin: P = pelagic, C = coastal. Sample: N = natural, B = batch. – = insignificant difference.

Exp.	Date	Origin	Sample	*P*-value	Treatments		
					PC	C1	CT
15	Jun. 1995	C	B	0.0001	–	*	*
16	Jun. 1995	C	B	0.0001	*	*	*
17	Jul. 1995	P	N	0.0017	–	*	*
18	Jul. 1995	P	N	0.0003	*	*	*
19	Jul. 1995	P	N	0.0001	*	*	*
20	Aug. 1995	C	B	0.0002	*	*	*
21	Aug. 1995	C	B	0.0001	–	*	*
22	Sep. 1995	C	B	0.0042	–	*	*
23	Feb. 1996	P	B	0.0001	*	*	*
24	Feb. 1996	P	B	0.0001	*	*	*

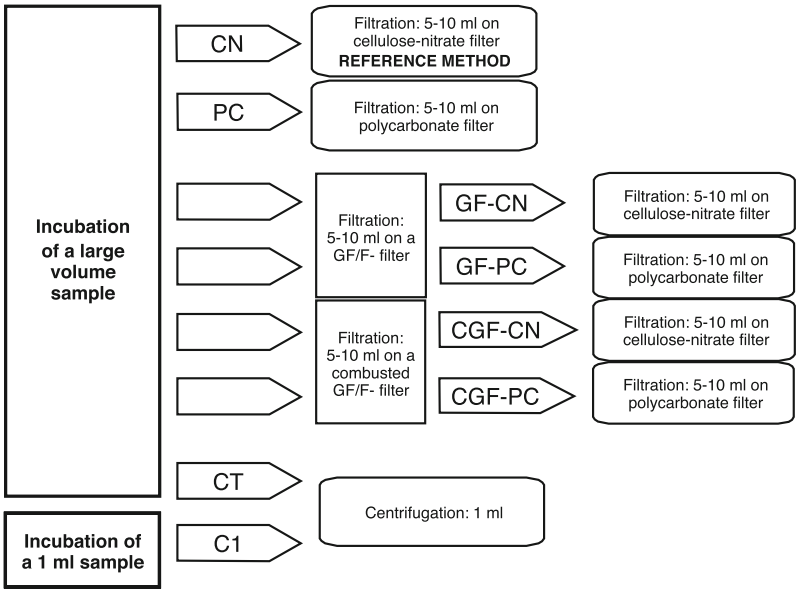

Fig. 1. Flow chart of the eight treatments tested. *See* text for details.

lation cocktail (Carboluma, Lumac LSC B.V.) was added and samples were counted in a scintillation counter. The "leftover" filtrates now consisted of seawater, the small cells that passed the GF/F filter and the TCA that was used in extraction of the cells trapped on the GF/F filters. The filtrates were further filtered on cellulose-nitrate or polycarbonate filters and these were then treated as mentioned above. The dpm-counts of the combusted GF/F filters were added to the dpm-values of the corresponding cellulose-nitrate or polycarbonate filters to get the total amount of isotope incorporation in the samples. (Fig. 1: GF-CN and GF-PC).

At low carbon concentrations the GF/F filters must be combusted prior to use in order to remove any background carbon on them. Combusting the GF/F filters, however, makes them fragile, and results in plenty of glass fibres in the filtrate (checked microscopically). Therefore it was also tested whether using combusted instead of non-combusted GF/F filters has any effect on the final incorporation rate estimate. (Fig. 1: CGF-CN and CGF-PC).

Centrifugation treatments

Samples (1 ml) were incubated in microcentrifuge tubes. 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). No difference in results was found between these two speed options of the centrifuge. When using the higher speed, however, it was sometimes impossible to maintain the low temperature, and therefore the lower speed was mainly used. 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 last removal of supernatants the microcentrifuge tubes were cut in three pieces, put in glass scintillation vials and 10 ml of scintillation cocktail (Insta-Gel Plus, Packard) was added. Cutting the microcentrifuge tubes in pieces ensured that they were fully covered by the scintillation cocktail, therefore making sure that all the radioactivity in the tubes was dissolved in the cocktail. The samples were counted the next day in a scintillation counter. (Fig. 1: C1).

To see whether the incubation volume would have an effect on the results, 1 ml subsamples 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 samples incubated in microcentrifuge tubes. (Fig. 1: CT).

Data processing

Experiments were performed in all stages of annual plankton succession and 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 counts. In order to see the differences between treatments the dpm data was scaled. Each dpm count was divided by the average dpm count of the reference method (filtering on cellulose-nitrate filter) of that experiment and constant 1 was subtracted. This gave a result matrix in which all the data varied from –0.699 to 0.379. 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 nested analysis of variance (differences resulting from different treatments nesting within different experiments) for the whole data set and with one-way analysis of variance for each experiment separately. Different treatments were compared with the reference treatment by Dunnett's *t*-test when appropriate. ^{14}C-leucine and ^{3}H-thymidine incorporation results were analysed separately from each other.

Results

^{14}C-leucine incorporation

Filtration treatments

Using different types of filters did not have a systematic effect on incorporation rates (Fig. 2). Nested ANOVA revealed no significant differences between cellulose-nitrate and polycarbonate filters (CN and PC; Table 3). In the one-way ANOVA run for each experiment separately, only once (out of 11 experiments) did results from the polycarbonate filters differ significantly from those of the cellulose-nitrate filters (Table 1).

Filtering the samples first through GF/F-filters did not affect the final incorporation rate estimate either. Once (out of 5 experiments) a significant difference was found when using combusted GF/F-filters followed by filtering on cellulose-nitrate filters (CGF-CN; Table 1). Most of the radioactivity of the samples (58% to 95%; after blank subtraction 67% to 100%) was always recovered from the GF/F-filters. Dpm-counts from the GF/F filters ranged from 1297 to 83 360 in different experiments with blanks from 2% to 20% of the corresponding samples. Replicates were good (CV% from 0.27% to 22.6%). Blanks from re-filtering the filtrate were substantially high, constituting nearly 60% (polycarbonate filters) and 90% (cellulose-nitrate filters) of the sample activity found on corresponding re-filtered samples.

Centrifugation treatments

Results from the centrifugation treatments were (with only four exceptions) lower than those from the reference treatment (C1 and CT; Fig. 2), and nested ANOVA showed significant differences between treatments (Table 3). When analysed with one-way ANOVA separately for each experiment, a significant difference was only noticed 4 times out of 12 when samples were incubated in microcentrifuge tubes. 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). Significant differences were related neither to the season of the sampling nor to the sample being natural or from a batch culture. There was no relationship between the ratio of blank dpm/sample dpm and the significant differences found.

To find a reason for the systematically lower results of the centrifugation treatments compared to the filtration, the supernatants, respectively the filtrates were in one experiment (Exp. 14, May 1999) collected and their radioactivity measured in a scintillation counter. Adding these 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.

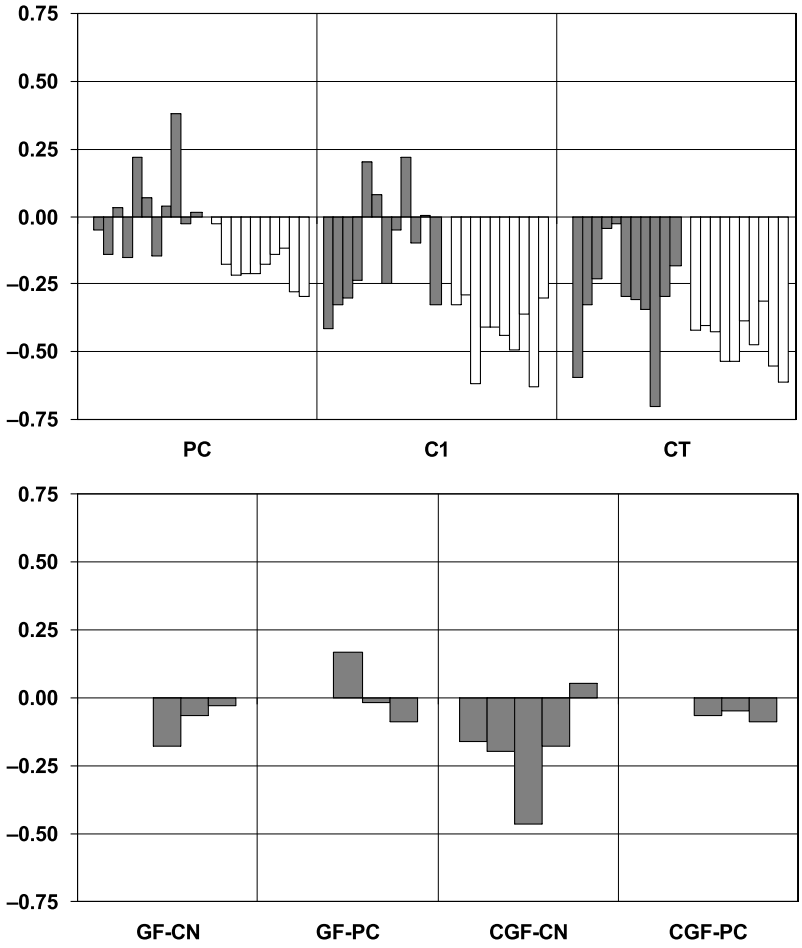

Fig. 2. Averages of the scaled results from all experiments. The negative bars show values lower than those achieved with the reference method whereas the positive bars show values higher than those achieved with it (*see* text for details). Bars shown in the same order as the experiments. No gaps drawn when no data. Shaded bars = experiments with ^{14}C-leucine, white bars = experiments with ^{3}H-thymidine.

Table 3. Statistically significant (* = $P < 0.05$) differences found when compared to the reference treatment (CN) by the Dunnett's *t*-test after a nested ANOVA for the whole data set. For treatment acronymes *see* Fig. 1. (nd = no data, – = insignificant difference).

Treatment	^{14}C-leucine	^{3}H-thymidine
CN	ref.	ref.
PC	–	*
GF-CN	–	nd
GF-PC	–	nd
CGF-CN	*	nd
CGF-PC	–	nd
C1	*	*
CT	*	*

^{3}H-thymidine incorporation

Filtration treatments

Using polycarbonate filters instead of cellulose-nitrate filters with ^{3}H-thymidine made a surprisingly large difference in results (PC; Fig. 2 and Table 3). In all cases the results were lower than with the reference treatment, and in 6 cases out of 10 experiments the difference was significant (Table 2). No relationship between significant differences and season, sample type or the ratio of blank/sample were found.

Centrifugation treatments

The centrifugation method failed to produce comparable results when used in ^{3}H-thymidine incorporation measurements. All 10 experiments showed significantly lower results with centrifugation treatment compared to the reference treatment both when incubating samples in microcentrifuge tubes and when transferring subsamples to the tubes (C1 and CT; Fig. 2, Tables 2 and 3).

Discussion

Filtration treatments

The filters we tested were both of pore size 0.2 μm. Blanks were always lower when using polycarbonate filters. Even so, the results in most cases when using leucine and every time when using thymidine were higher when measured with the cellulose-nitrate filters than with the polycarbonate filters. Hollibaugh and Wong (1992) as well as Kirchman (1992) came up with similar results even as the mixed cellulose ester filters (Millipore) they used were of pore size 0.45 μm and the polycarbonate filters (Nuclepore and Poretics) of pore size 0.2 μm. Higher counts from thicker, matrix-type cellulose-nitrate filters could be an artefact of unincorporated isotope binding within the filter. However, re-filtering experiments by Hollibaugh and Wong (1992) argue against this explanation. They re-filtered the filtrate of a polycarbonate filter through a mixed cellulose filter to see whether some smaller particles had passed through the pores of the polycarbonate filters, a suggestion that has come up in earlier experiments [reviewed by Li (1990)]. They were unable to recover enough isotope to account for the difference between parallel filtrations on the two filter types, concluding 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.

Filtering the samples through GF/F and then on membrane filters did not affect the final incorporation results. Most of the sample radioactivity (58% to 95%; after blank subtraction 67% to 100%) was always found on the GF/F filters. This was expected, as the glass fibre matrix is known to retain particles much smaller than the nominal pore size (0.7 μm) of the filter (Lee *et al.* 1995). In many experiments we also used batch cultures in which the cell size is larger than in natural samples. One must anyhow bear in mind that the smallest bacteria pass through the glass fibre filter, which makes the carbon measurement an underestimate. Lee *et al*. (1995) tested the natural bacterioplankton retention capacity of the GF/F filters and found out that 35% to 43% of the cells, corresponding to 22% to 38% of the biomass passed through the filter. They also draw attention to the fact that no studies on the retention capacity of the combusted GF/F filters have been published. It would therefore be advisable to count and size the cells before and after GF/F filtering to get an estimate of the fraction that passed through the filter.

Re-filtering the filtrate on cellulose-nitrate and polycarbonate filters increased the total activity only a little as the blanks of the re-filtered samples were high (60% to 90% of the corresponding samples). A possible reason for this is that while filtering the sample through GF/F filters some glass fibres break free from the matrix, ending up in the filtrate. Glass fibres then cover the membrane filter, making washing with TCA inefficient. This may be the case especially when using precombusted filters as these are more fragile and release more fibres in the filtrate (checked microscopically). Glass fibres may also bind isotope abiotically, but as this happens to the same extent in both blanks and true samples, it should not affect the final results.

Centrifugation treatments

The results we got using the centrifugation treatment were nearly constantly (19 times out of 23) lower compared to the reference treatment (filtering on cellulose-nitrate filters). The standard deviation between the replicates was always at the same level, often even lower compared to the other treatments (CV% 1.9% to 34.2%). In the original treatment by Smith and Azam (1992) they added the scintillation liquid straight into the microcentrifuge tubes and put these into scintillation vials to be counted. 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 and Azam (1992) washed their samples only once (either with 5% TCA or with 80% ethanol) after the first precipitation with TCA. In our preliminary tests we found out that three washes with 5% TCA were needed to remove unincorporated isotope from our samples. On the other hand, we also washed the filters more extensively than they did (2 ml + 5 × 1 ml compared with 2 × 1 ml). We did not test the effect of extracting the samples with 80% ethanol, but this step would have been expected to affect the samples 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 it 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 minute centrifuging at 15 988 × g or 25 848 × g is not enough to make the pellet stick to the tube while the supernatant is removed. Recently, Kirschner and Velimirov (1999) stated that for freshwater samples a NaCl addition was needed as a co-precipitant in order to get reliable results when using the centrifugation treatment. The sample water we used in our experiments was brackish with low salinity (5‰ to 7‰), and therefore adding a co-precipitant could have proved useful as well. Kirschner and Velimirov (1999) got dependable results only when using 3.5% NaCl (final concentration), which is comparable to the salinity of the oceans. Kirschner and Velimirov (1999) also pointed out that using a co-precipitant results in a visible pellet, making withdrawal of the sample by accident less likely. They did, however, find out that the precipitated proteins within the pellets need to be redissolved by boiling in NaOH for 20 minutes prior to adding the scintillation cocktail. Co-precipitation with NaCl, therefore, adds an extra step in the centrifugation treatment procedure.

In addition to the above mentioned problems we also faced the label loss when using the centrifugation method. Not only was some incorporated isotope lost in 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 in 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, we have not come up with any explanation to this phenomenon.

Considering all these uncertainties we cannot recommend centrifugation as the method of choice for bacterial production measurements in the brackish Baltic Sea, neither do we trust the polycarbonate filters to give results comparable to those of the cellulose-nitrate filters, especially when ^{3}H-thymidine is used. Filtering the samples through combusted GF/F filters prior to the routine cellulose-nitrate filtration, and combusting the GF/F filters then in a carbon analyser, gives important information about total carbon content of the samples without biasing the bacterial production estimate. This procedure is especially advantageous when the sample volume is small, giving no possibility to do carbon measurements separately, or when dealing with unique, non-replicable samples such as marine snow aggregates.

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