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Different fixatives and chloridric acid concentrations in microphytobenthic primary production estimates using radiolabeled carbon: their use and misuse

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Abstract

In literature, different fixatives have been used to stop microphytobenthic photosynthetic activity estimated using the ^{14}C technique, and different concentrations of HCl have been used to remove the excess labeled bicarbonate. To standardize these two steps of the ^{14}C method, we designed a series of experiments using sublittoral muddy sediments colonized by microphytobenthos. The first aim was to identify which fixative among formaldehyde, glutaraldehyde, and HCl was most effective in arresting photosynthetic activity. Formaldehyde increases cell membrane permeability leading to a loss of assimilated carbon because an underestimate of disintegrations per minute (DPM) of up to 64% was obtained when compared with HCl values. Glutaraldehyde led to an overestimate of DPM due to its autofluorescence. We propose that the best way to stop photosynthetic activity is by using HCl. The second aim of the article was to define which HCl concentration from 0.1N to 5N was sufficient to remove the excess labeled bicarbonate. The residual inorganic ^{14}C led to an overestimate of DPM values when mild acids were added. None of the acid treatments completely removed the inorganic labeled carbon because of the sediment matrix effect. However, a further pH decrease, caused by too strong acid normalities, could digest the organic carbon pool. Besides dark estimates, blank production estimates should be assessed before each primary production experiment to consider the sediment matrix effect.

There is not yet a standard method for measuring primary production in the sediment. Most studies quantifying benthic microalgal production have used some variant of the light-dark chamber method and measurements of either dissolved oxygen flux or uptake of ^{14}C-labeled substratum (Cahoon 1999). Nondestructive methods as different oxygen-sensing techniques (Glud et al. 2000) and variable fluorescence techniques as Pulse Amplitude Modulated fluorescence (Jesus et al. 2005) are now widely used to determine high-resolution distribution of microphytobenthic photosynthesis in time and space. However, microelectrodes are fragile and require delicate instrumentation that complicates in situ studies while fluorescence techniques still offer only relative photosynthetic rate measurements. Extensive replication is needed to quantify the spatial variability when estimates are scaled up to larger areas (Migné et al. 2004).

Among the ^{14}C methods applied to the sediment matrix, the slurry technique is still widely used. Its main advantage is measuring primary production with good spatial resolution. Moreover, it can be used on any type of sediment, it is easy to apply, and no special incubation equipment is needed (Jönsson 1991). This method may not be as accurate as some innovative techniques, because it gives only a potential estimate of the primary production rates (Barranguet et al. 1998) since the existing gradients in the sediment are destroyed (Kromkamp and Forster 2006). However, if no nutrients are limiting, the measured potential photosynthetic rates might reflect real rates (Barranguet et al. 1998).

In literature different fixatives are used to stop the photosynthetic activity of benthic microalgae. Van Raalte et al. (1976) arrested the uptake of ^{14}C with 3% formalin; similarly, Sullivan and Moncreiff (1988) added 4% buffered formalin to stop isotope uptake. MacIntyre and Cullen (1995) killed the benthic assemblage with 50 µL of borate-buffered formalin.

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Table 1. Summary of primary production fixatives and acidification methods in the sediment. FOR = formaldehyde; GTA = glutaraldehyde.

Fixative	Final conc. or volume added	HCl	Sample volume	Reference
—	—	100 µL conc.	2.5 mL	Barranguet et al. 1998
—	—	100 µL conc.	2.5 mL	Barranguet and Kromkamp 2000
FOR	50 µL	250 µL, 6N	1 mL	Blanchard et al. 1996
FOR	50 µL	250 µL, 6N	1 mL	Blanchard et al. 1997
—	—	0.2N	3 mL	Guarini et al. 2002
FOR	50 µL	0.25 mL, 6N	1 mL	MacIntyre and Cullen 1995
—	—	5 drops, 0.1 M	n.a.	Miles and Sundbäck 2000
GTA	5%	concentrated	n.a.	Perkins et al. 2001
GTA	5%	concentrated	n.a.	Perkins et al. 2002
—	—	0.2 mL, 0.5N, filtered	12 mL	Roux et al. 2002
GTA	1%	0.5 mL, 10%	n.a.	Smith and Underwood 1998
GTA	1%	50 µL conc.	0.5 mL	Smith and Underwood 2000
FOR	4%	50 mL, 2%	125 mL	Sullivan and Moncreiff 1988
FOR	3%	n.a.	12.5 mL ca.	Van Raalte et al. 1976

n.a., not available.

Also Blanchard et al. (1996, 1997) stopped all biological activity after ^{14}C incubation by the addition of 50 µL of buffered formalin (Table 1).

Smith and Underwood (1998) used glutaraldehyde (1% vol/vol) which was added to labeled cores after the assimilation period. Also in the experiment performed on a culture the same concentration of glutaraldehyde was added to prevent further assimilation or reallocation of ^{14}C (Smith and Underwood 2000). Perkins et al. (2001, 2002) increased the concentration of glutaraldehyde to 5% to terminate microphytobenthic primary production.

Other authors stop carbon incorporation by directly adding HCl at different concentrations. Barranguet et al. (1998) and Barranguet and Kromkamp (2000) used 100 µL concentrated HCl; Miles and Sundbäck (2000) added 5 drops of 0.1M HCl, whereas Guarini et al. (2002) stopped the photosynthetic activity by the addition of 0.2N HCl (sample volume: 3 mL). Adding the same acid volume (and normality) to different sample volumes, the final acid concentration can vary considerably. We found that in literature very different concentrations of HCl are used to remove the nonincorporated ^{14}C. Some authors used concentrated HCl, without mentioning the added quantity or molarity (Perkins et al. 2001, 2002). Other authors specified the quantity but not its molarity (Barranguet et al. 1998; Barranguet and Kromkamp 2000; Smith and Underwood 1998). In some experiments, extremely high concentrations of HCl were used (MacIntyre and Cullen 1995; Blanchard et al. 1996, 1997) whereas in others low quantities and/or molarities were added (Miles and Sundbäck 2000; Guarini et al. 2002).

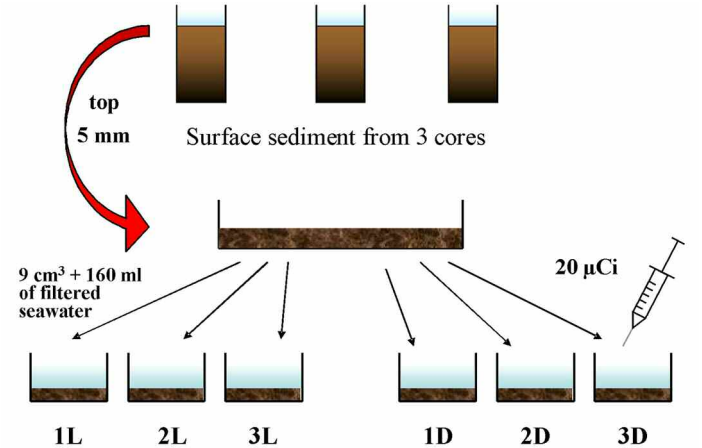
From a review of the earlier and recent literature, we found that different methods were used to stop the photosynthetic activity, and that highly variable concentrations of HCl were added to remove the excess labeled bicarbonate which was not assimilated by the microalgae. To standardize these two steps of the ^{14}C method, we designed a series of experiments using sublittoral sediments rich in microphytobenthos. The first aim of the experiments was to identify which fixation method among formaldehyde, glutaraldehyde, and HCl was the most effective in arresting photosynthetic activity in a sediment slurry. The second aim was to define which HCl concentration, with an equal volume, among 0.1N, 0.2N, 0.5N, 1N, 2N, and 5N was sufficient to remove the excess labeled bicarbonate (when no other fixative was used) but not so strong to digest the organic carbon pool.

Materials and procedures

Sediment sampling—Sediment sampling was performed on 29 Jun 2006, 26 Jul 2006, and 31 Aug 2006 at a coastal station (17 m depth) of the Gulf of Trieste (northern Adriatic Sea). During each sampling, three virtual undisturbed muddy sediment cores (<10% of sand and a detrital component, Cibic et al. 2007) were collected about 200 m offshore using a KC Haps Bottom Corer with polycarbonate corer tubes (13.3 i.d. with a sample area of 127 cm^2). At the time of sampling, temperature and PAR irradiance were recorded by a Profiling Natural Fluorometer (PNF-300A Biospherical Instruments), 50 cm above the bottom. The sediment cores were immediately transferred to the laboratory and placed in an 80-L aquarium, which was subsequently filled with 50 L seawater collected in situ at 15 m depth. The three cores were completely submerged taking care to avoid sediment resuspension. Flushing by an air pump kept the water at oxygen saturation point. A light:dark cycle of 15:9 h in June and July, and of 14:10 hours in August was applied. Sediment cores were kept at their in situ temperature (18.5°C in June 20.1°C in July and 21.0°C in August) and in situ light

conditions (150 µmol photons s^{-1} m^{-2} in June 125 µmol photons s^{-1} m^{-2} in July and 90 µmol photons s^{-1} m^{-2} in August).

Sediment experimental design—The sediment cores were maintained at in situ conditions for a few days before the experiment commenced. This was necessary to obtain a well-developed microphytobenthic community in the surface and subsurface layers. In fact, only the first millimeters of sediment collected from the three cores were used to perform the experiment. The collected sediment was placed in a high-wall Petri dish (i.d. 19 cm) and gently homogenized using a glass rod. Six aliquots of 9 cm^3 were sucked up with a syringe and transferred to 6 high-wall Petri dishes (i.d. 11 cm). To each replicate, 160 mL filtered (0.2 µm) bottom seawater was added together with 20 µCi (0.74 MBq) of $NaH^{14}CO_3$ (DHI, Denmark) and gently homogenized using a glass rod (Fig. 1). Three of these replicates were incubated at in situ temperature and light conditions, whereas the other three were wrapped in aluminum foil to maintain them in darkness. All replicates were than incubated under in situ conditions for 45 min. After incubation from each Petri dish, 18 aliquots of 9 mL were withdrawn and placed into scintillation glass vials. The 18 vials were divided in 3 series of six subsamples; each of these



1L	2L	3L	1D	2D	3D
1L HCl 0.1N	2L HCl 0.1N	3L HCl 0.1N	1D HCl 0.1N	2D HCl 0.1N	3D HCl 0.1N
1L HCl 0.2N	2L HCl 0.2N	3L HCl 0.2N	1D HCl 0.2N	2D HCl 0.2N	3D HCl 0.2N
1L HCl 0.5N	2L HCl 0.5N	3L HCl 0.5N	1D HCl 0.5N	2D HCl 0.5N	3D HCl 0.5N
1L HCl 1N	2L HCl 1N	3L HCl 1N	1D HCl 1N	2D HCl 1N	3D HCl 1N
1L HCl 2N	2L HCl 2N	3L HCl 2N	1D HCl 2N	2D HCl 2N	3D HCl 2N
1L HCl 5N	2L HCl 5N	3L HCl 5N	1D HCl 5N	2D HCl 5N	3D HCl 5N
1L GTA 0.1N	2L GTA 0.1N	3L GTA 0.1N	1D GTA 0.1N	2D GTA 0.1N	3D GTA 0.1N
1L GTA 0.2N	2L GTA 0.2N	3L GTA 0.2N	1D GTA 0.2N	2D GTA 0.2N	3D GTA 0.2N
1L GTA 0.5N	2L GTA 0.5N	3L GTA 0.5N	1D GTA 0.5N	2D GTA 0.5N	3D GTA 0.5N
1L GTA 1N	2L GTA 1N	3L GTA 1N	1D GTA 1N	2D GTA 1N	3D GTA 1N
1L GTA 2N	2L GTA 2N	3L GTA 2N	1D GTA 2N	2D GTA 2N	3D GTA 2N
1L GTA 5N	2L GTA 5N	3L GTA 5N	1D GTA 5N	2D GTA 5N	3D GTA 5N
1L FOR 0.1N	2L FOR 0.1N	3L FOR 0.1N	1D FOR 0.1N	2D FOR 0.1N	3D FOR 0.1N
1L FOR 0.2N	2L FOR 0.2N	3L FOR 0.2N	1D FOR 0.2N	2D FOR 0.2N	3D FOR 0.2N
1L FOR 0.5N	2L FOR 0.5N	3L FOR 0.5N	1D FOR 0.5N	2D FOR 0.5N	3D FOR 0.5N
1L FOR 1N	2L FOR 1N	3L FOR 1N	1D FOR 1N	2D FOR 1N	3D FOR 1N
1L FOR 2N	2L FOR 2N	3L FOR 2N	1D FOR 2N	2D FOR 2N	3D FOR 2N
1L FOR 5N	2L FOR 5N	3L FOR 5N	1D FOR 5N	2D FOR 5N	3D FOR 5N

Fig. 1. Sediment experimental design from sampling to acidification. 1L, 2L, 3L = light replicates; 1D, 2D, 3D = dark replicates. GTA = glutaraldehyde; FOR = formaldehyde. For details, *see* text.

3 series was treated differently as follows:

a) not fixed but directly treated with HCl;
b) fixed with 0.4 mL 4% final concentration of formaldehyde buffered solution $CaMg(CO_3)_2$ (Carlo Erba) filtered on 0.2 µL filters;
c) fixed with 0.4 mL 1% final concentration of glutaraldehyde (Carlo Erba).

To each final sample series obtained from each Petri dish, 200 µL HCl at progressively higher concentrations (0.1N-0.2N-0.5N-1N-2N-5N which correspond to final HCl concentrations of 0.002N-0.004N-0.01N-0.02N-0.04N-0.11N, respectively) was added, and samples were left under a fume hood overnight to remove the labeled bicarbonate, which had not been assimilated by the microalgae. Ten milliliters of the Scintillation cocktail Ultima Gold XR (Perkin Elmer) were added to the samples that were placed in 50 mL plastic conical vials. They were centrifuged at 3500*g* for 10 min, the supernatant was removed and analyzed on a QuantaSmart TRI-CARB 2900 TR Liquid Scintillation Analyzer (Packard BioScience) including quenching correction, obtained using internal standards. The pellet was suspended in 10 mL scintillation liquid, vortexed until the solution was homogeneous and centrifuged again. This procedure was repeated three times. In a previous experiment, we observed that a series of three extractions was needed to recover 90% of the photosynthesized labeled C from our samples. This percentage was determined adding a known ^{14}C activity to an autoclaved sediment slurry on which a series of extractions was performed. The sum of the first three extractions, expressed as DPM values, was necessary to extract approximately 90% of the added labeled C. The remaining 10% of the added ^{14}C was strongly bound to the sediment matrix and difficult to remove, therefore it was left in the sediment pellet (Cibic et al. 2008). Following the same protocol, DPM resulting from the 3 extractions were summed. DPM counts were carried out twice on QuantaSmart TRI-CARB 2900 TR Liquid Scintillation Analyzer. Afterward, DPM readings were also measured in duplicate using a Beckman LS 6000 TA Counter system (Beckman Coulter). We had noted that when using glutaraldehyde (GTA) as a fixative, consecutive counts of the same sample had given extremely variable DPM values. Therefore, different counts were performed to verify the DPM stability also when other fixatives were used. For the same reason, two different analyzers were used to verify the instability of DPM counts referred to samples fixed with GTA.

Statistical analysis—DPM were normalized to the value at 5N HCl (no aldehydes used to stop the photosynthetic activity) before performing the statistical analysis. A data matrix was constructed with HCl normalities as variables and treatments (with all light and dark replicates of the three monthly experiments) as samples. Trends between different fixatives were analyzed with Primer5 v 5.2.9 (Plymouth Marine Laboratory) program using multidimensional scaling (MDS) analysis based on Bray-Curtis similarity coefficient for all pairs of

treatments. Variation in trends between different fixatives was subsequently tested for significance with an ANOSIM (analysis of similarity) using the same software. ANOSIM tests a priori–defined groups (subgroupings based on treatments) against random groups in ordinate space. The R_{ANOSIM} statistic values, generated by ANOSIM in PRIMER, are a relative measure of separation of the a priori–defined groups. A zero (0) indicates that there is no difference among groups, whereas a one (1) indicates that all samples within groups are more similar to one another than any samples from different groups.

Significant differences in acid normalities, when no other fixative was used, were also tested. The DPM data failed statistical tests for normality and therefore, a Kruskal-Wallis test (non parametric one-way ANOVA by ranks) was performed. Subsequently, a pairwise Wilcoxon test was applied to detect significant differences between pairs of experiments considering the six acid concentrations.

Glutaraldehyde autofluorescence estimate in sediment samples—We estimated DPM values obtained from 3 replicates of sediment resuspension with the only addition of glutaraldehyde, i.e., without labeling, to verify if the obtained values were comparable to sample background counts or higher due to glutaraldehyde autofluorescence.

Removal of surplus ^{14}C—To assess if the acids were strong enough to remove inorganic C, we performed another experiment. A sediment resuspension was prepared, maintaining both the same proportion between seawater and fresh sediment and the same labeled carbon activity as already described. Fifteen aliquots were withdrawn and placed into scintillation glass vials. 200 µL of HCl at 3 different concentrations (0.1N-2N-5N) were immediately (with no incubation period) added to 3 samples series, while 3 replicates were left without acid treatment. Finally, 3 replicates of sediment resuspension were left without labeled ^{14}C and acid to test the sample background count. All samples were left in a fume hood overnight.

Sediment matrix effect—To assess the sediment matrix effect on the pH value, a litmus test was performed on 9 mL-aliquots of sediment slurry, adding the same acid series. In addition, to verify how much the volume of the sediment sample affects the pH value, we reduced the sediment volume to 5 mL keeping the same acid concentrations as in the previous experiments (i.e., 200 µL HCl at progressively higher concentrations, 0.1N-0.2N-0.5N-1N-2N-5N, which correspond to final HCl concentrations of 0.004N-0.008N-0.02N-0.04N-0.08N-0.19N, respectively).

Assessment

Different fixatives and HCl concentrations in microphytobenthic primary production—The two duplicate measurements obtained on a single sample using the two scintillation counters were very similar, and therefore, we used the mean value of all four counts, for all three fixatives.

HCl—DPM dynamics of the first experiment greatly differed from those of the second and third experiments (Fig. 2). In detail, DPM values, especially those ranging from 0.2 to 2N, in the experiment performed on 4 July were higher than those obtained from the other two experiments. Moreover, on 4 July dark values of the second biological replicate (2D) were much higher than 1D and 3D. In the second and third experiment, a clear decreasing trend from 0.1N to 5N was evidenced. ANOVA test performed on the six acid normalities showed that the pairs of groups were significantly different except for the pair 0.2N-0.5N and the pair 1N-2N. Overall, the mean of the standard deviation percentage was 8% in all three experiments.

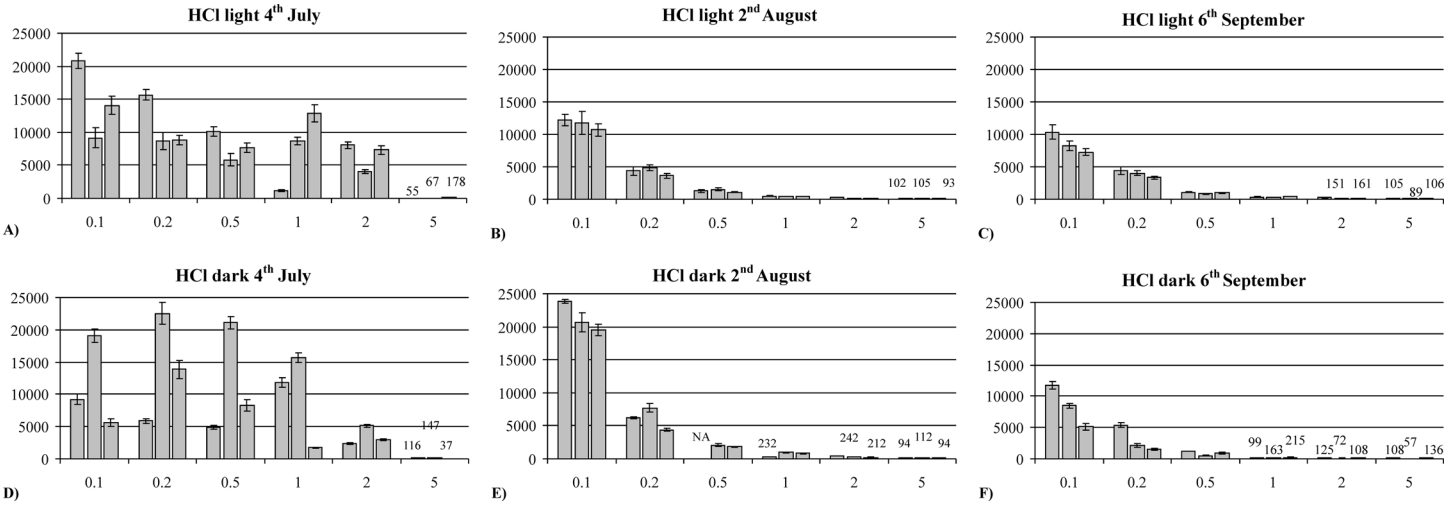


Fig. 2. DPM of light and dark replicates resulted from the three experiments, carried out using only chloridric acid as fixative. Each bar represents the mean of four scintillation counts (2 from the Quanta-Smart and 2 from the Beckman scintillation analyzer) for each biological replicate. Data are normalized to the mean light or dark value (calculated from the three biological replicates) at 5N HCl (when no aldehydes were used to stop the photosynthetic activity).

Glutaraldehyde—When glutaraldehyde was used as fixative (Fig. 3) very high standard deviations from the four counts were obtained, especially in the second and third experiments (up to 129%). Unlike the first experiment with HCl, where there was no clear dynamics, in the first experiment with glutaraldehyde a decreasing trend was observed both for light and dark values. On the contrary, in the second and particularly in the third experiment, there was only a slight difference in DPM values going from 0.1N to 2N. Moreover, on 2 Aug and 6 Sep, dark values acidified with HCl 5N led to high DPM values, comparable with those where lower HCl concentrations were used.

Formaldehyde—The three experiments where formaldehyde was used as fixative led to comparable results when HCl was used (Fig. 4). Overall, standard deviations obtained from the four scintillation counts were quite low (varying from 5% to 7%). The first experiment did not highlight any trend among the six HCl concentrations used to remove the excess-labeled C. In contrast, the second and third experiment showed a clear decreasing variation from 0.1N to 5N. In both experiments, there was a drop in DPM values going from 0.1N to 2N.

Trends between HCl and GTA treatments and between formaldehyde (FOR) and GTA treatments differed significantly (R_{ANOSIM} = 0.43; P = 0.001, R_{ANOSIM} = 0.43; P = 0.001, respec-

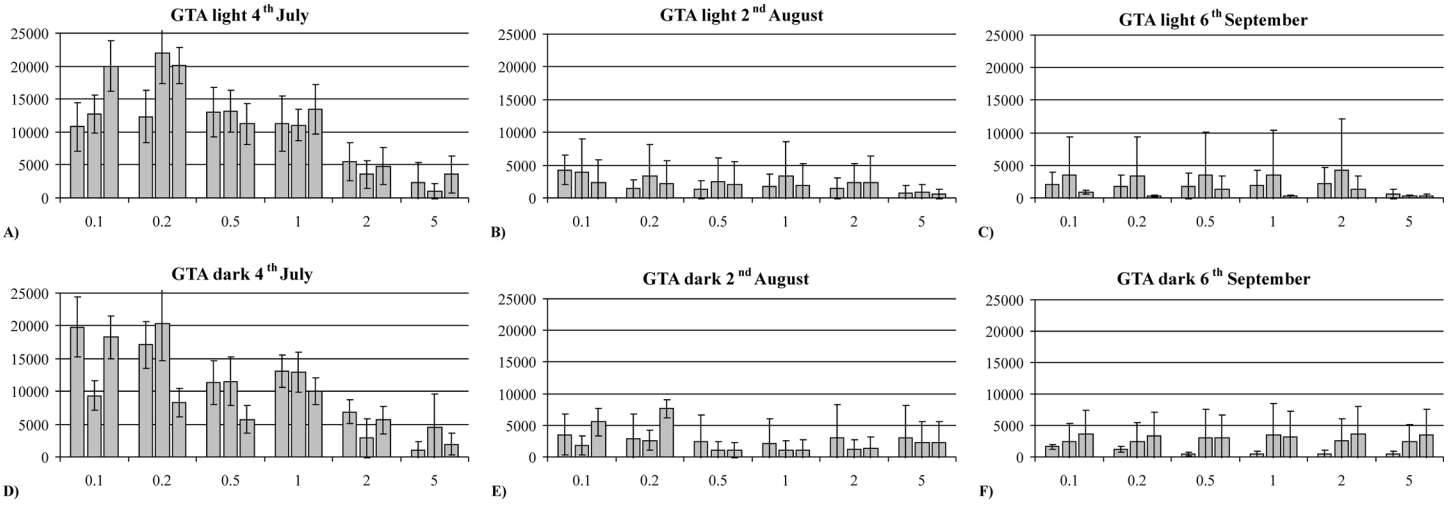


Fig. 3. Disintegrations per minute (DPM) of light and dark replicates resulted from the three experiments, carried out when glutaraldehyde was used as fixative. Each bar represents the mean of four scintillation counts (2 from the Quanta-Smart and 2 from the Beckman scintillation analyzer) for each biological replicate. Data are normalized to the mean light or dark value (calculated from the three biological replicates) at 5N HCl (when no aldehydes were used to stop the photosynthetic activity).

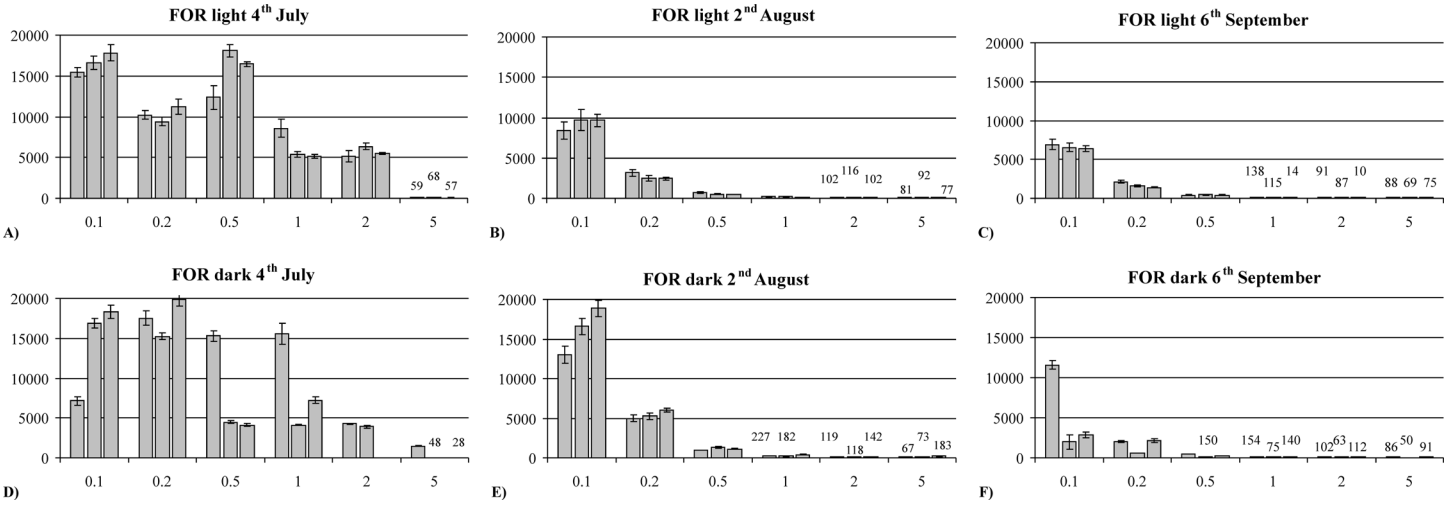


Fig. 4. DPM of light and dark replicates resulted from the three experiments, carried out when formaldehyde was used as fixative. Each bar represents the mean of four scintillation counts (2 from the Quanta-Smart and 2 from the Beckman scintillation analyzer) for each biological replicate. Data are normalized to the mean light or dark value (calculated from the three biological replicates) at 5N HCl (when no aldehydes were used to stop the photosynthetic activity).

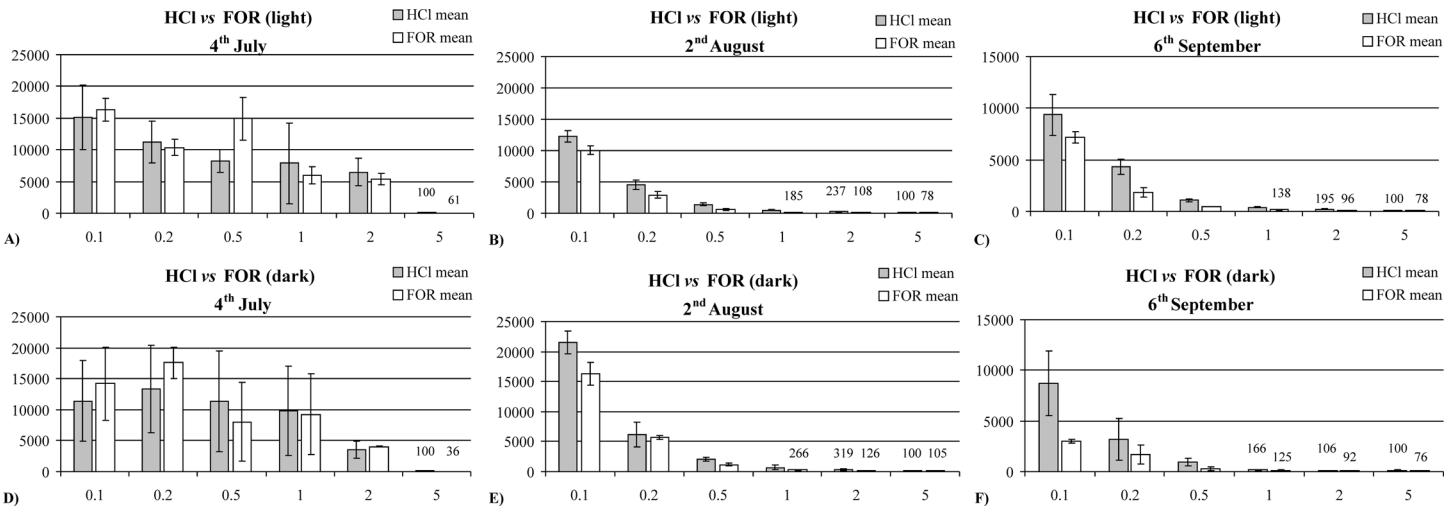


Fig. 5. Comparison between DPM values of samples stopped with HCl and fixed with formaldehyde. Each bar represents the mean of the three biological replicates (1L, 2L, 3L or 1D, 2D, 3D) considering only the first counts. Data are normalized to the mean light or dark value (calculated from the three biological replicates) at 5N HCl (when no aldehydes were used to stop the photosynthetic activity).The outliers 1DFOR5 and 3DFOR2 of the first experiment, 1DHCl0.5 of the second experiment, and 1DFOR0.1 of the third experiment are excluded from the calculation of the mean value because they greatly differed from the other two biological replicates.

tively) in the comparisons among 5N HCl normalized DPM. Trends between HCl and FOR treatments did not differ significantly. For this reason, we excluded GTA data from further processing. A direct comparison between HCl and formaldehyde was made.

While in the first experiment, it was not possible to distinguish any dynamics between the two fixatives, in the last two experiments formaldehyde values were distinctively lower both for light and dark replicates (Fig. 5). For example, both in the second and third experiment, light values fixed with formaldehyde were on average 43.7% lower than those fixed with HCl. This decrease ranged from 18.5% of the 0.1N treatment in the second experiment to 63.6% of the 1N treatment in the last experiment. Interestingly, the difference between the two treatments, expressed as percentage, was very similar in the last two experiments. In the 0.5N treatment, the percentages of difference in the second and third treatment were identical (56.9%); in the 1N treatment, these percentages were also very alike: 62.6% in the second and 63.6% in the third experiments; in the 2N treatment they were similar: 54.5% in the second and 50.5% in the third one; finally, in the 5N treatment 21.7% in the second and 22.1% in the last experiment.

Removal of surplus ^{14}C—Although, we did not use all the 6 acid concentrations added in the first 3 experiments, the 3 acid concentrations added in this experiment highlighted a clear decreasing trend going from no acid treatment to 5N HCl treatment. The difference in DPM normalized values between one treatment and the next one was of about one order of magnitude (Fig. 6). Even the strongest HCl treatment did not remove all the inorganic ^{14}C, in view of the fact that the background count was 3% of the 5N treatment.

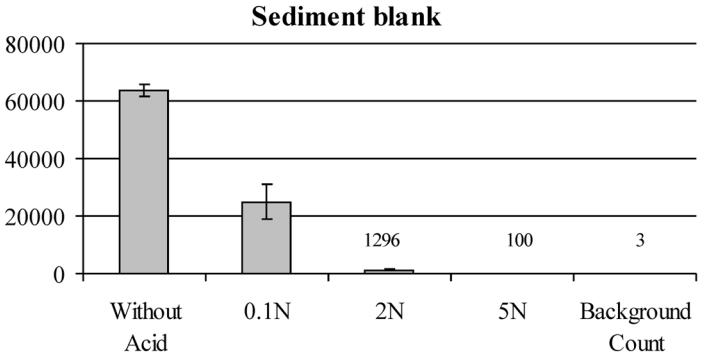


Fig. 6. DPM of blank production estimates treated with different acid concentrations (0.1N, 2N, and 5N), without acid and the background count. On the *y* axis, data are expressed as percentages of the mean value at 5N HCl (no aldehydes).

Estimating the activity of the residual pellet, we obtained low normalized DPM values (12 for the 5N treatment; 24 for the 2N treatment, compared with the values shown in Fig. 6). Our extraction method with three centrifugations allowed us to recover almost all the labeled assimilated carbon, despite benthic diatoms being strongly attached to the sediment grains.

Effects of HCl acid on the sediment slurry—The litmus test performed on the sediment slurry showed that the pH value did not change much from 0.1N to 1N treatments (varying from pH 8 to pH 7), while a brusque drop was observed going from 2N to 5N treatment (Table 2). Only nearly halving the sediment slurry volume let us obtain a very low pH and a normalized value (3.3 DPM) comparable to the normalized background count.

Table 2. Litmus test performed on the sediment using two different volumes (5 and 9 mL) in order to test the pH value at increasing acid normalities.

		pH value						
		Without acid	0.1N	0.2N	0.5N	1N	2N	5N
Sediment	5 mL	8	8	7	7	6	2-3	1
	9 mL	8	8	7-8	7-8	7	5	1-2

Discussion

It is known from literature that glutaraldehyde can induce autofluorescence in cells fixed for fluorescence microscopy (Collins and Goldsmith 1981). Nevertheless, glutaraldehyde is occasionally used as fixative in primary production experiments (Table 1). The high standard deviations among both replicates and counts obtained in our experiments led us to consider glutaraldehyde as an unstable fixative. Actually, when glutaraldehyde was used as fixative, generally higher DPM values, especially those coupled with HCl normalities ranging from 0.5 to 5N, were obtained than those from the other two treatments. The glutaraldehyde autofluorescence was also confirmed by our DPM estimates in sediment samples without labeling, since DPM counts in those samples were much higher than the sediment background counts. This magnification effect was observed only in the first extraction (2292 ± 1239 DPM for glutaraldehyde addition against < 30 DPM for the background count, data not normalized), even magnified in the second reading (18032 ± 6515 DPM, data not normalized). Most certainly, glutaraldehyde was removed together with all the seawater and collected in the first extraction. The subsequent two extractions were performed on the pellet, resuspended in the scintillation cocktail, giving DPM values comparable with the background counts. It seems that glutaraldehyde autofluorescence occurs in water solutions only. In fact, when glutaraldehyde-fixed samples were filtered and primary production was estimated from filters, these shortcomings due to glutaraldehyde autofluorescence did not occur. Using this method, all the water soluble fixative was eliminated from the filter and collected; only the filtrated fraction, containing the major part of the fixative, showed high DPM values due to glutaraldehyde autofluorescence (data not shown).

In the last two experiments, DPM of samples containing formaldehyde were distinctively lower than those without any aldehydes. We infer that some ^{14}C leakage through the cellular membrane may occur after fixation with formaldehyde. Aldehydes react readily with proteins, and the cell membrane becomes immediately highly permeable (Gorman et al. 1980). Both Strickland and Parsons (1968) and Marshall et al. (1973) warned that when formaldehyde is used to stop photosynthesis, a loss of some (unspecified amount) of label can take place. Subsequently, also Silver and Davoll (1978) found that formaldehyde always causes a significant loss of activity in treated populations when compared with the controls.

Since HCl is added to remove the excess-labeled C, it is important to know the pH of the acidified sample. In fact, if HCl is not sufficiently concentrated, the pH will not reach the value that is necessary to shift the equilibrium toward the CO_2 fraction, and consequently, the removal of the labeled bicarbonate from the system. In water samples, only a pH < 5 ensures a complete shift toward the CO_2 fraction (Libes 1992). We observed that adding progressively higher concentrations of HCl allowed us to remove increasing amounts of labeled inorganic carbon. However, the comparison with the background count (3% of the 5N treatment) revealed that even the strongest HCl treatment did not remove all the ^{14}C. We suppose that the carbonate content in the sediment of the Gulf of Trieste, approximately 30% (Ogorelec et al. 1991), and the high organic matter content in our muddy sediments, caused a buffer effect, limiting the pH lowering.

We believe that the sediment type is of paramount importance. When the percentage of mud is particularly high, it is likely that the sediment contains also higher amounts of humic, fulvic acids, and humin, which enhance the buffer effect evidenced in our experiments. This sediment type is typical of estuarine and coastal areas characterized by low hydrodynamism and high sedimentation rates. Moreover, in carbonate sediments, the buffer effect should be even of greater importance, and therefore, it is likely that a very high acid concentration is needed to reduce the pH. A series of experiments performed on sediments with increasing carbonate contents should be designed to define the right volume and concentration of HCl necessary to be added to remove the exceeding ^{14}C.

Since some inorganic carbon cannot be easily removed from the sediment matrix, therefore representing a sort of "labeled sediment background," it should be subtracted from the incubated primary production estimates. These blank values should be comparable with the dark ones, since the latter, not being exposed to light during incubation, should contain only inorganic ^{14}C. However, these conditions are not always met. Especially in summer, when the microalgae are exposed to relatively high irradiance, the residual energy within their photosynthetic apparatus, permit them to continue the assimilation of ^{14}C also in the darkness for some time (Fouilland et al. 2001). Therefore, it can occur that the dark values are slightly higher than the blank ones, because in the dark values both the labeled sediment background and the residual energy effect are included. Consequently, subtracting the dark value from the light one both effects are removed.

Performing a calculation of the difference between light

and dark mean values, we observed that this difference, especially in the third experiment, was not constant, but it increased with decreasing acid concentration, leading to DPM values up to 2 orders of magnitude between the 0.1N and 5N treatments (134792 and 1329 DPM, respectively, data not normalized). This difference, which is usually applied in primary production estimates, should be evaluated only on samples that were acidified with a relatively strong acid; otherwise, there is the risk of overestimating the primary production rate due to the residual inorganic ^{14}C (neither uptaken by the microalgae nor removed by the acid). Therefore, in addition to dark estimates, blank production estimates should be assessed before each primary production experiment to test which acid concentration is going to be effective.

Primary production estimate in the sediments derives from Steemann-Nielsen (1952) labeling method that was initially designed for water samples. Since Grøntved (1960) first adapted this protocol to assess the primary production rate in the sediment matrix, other authors contributed to modify the methodology in several ways. From this "reworking" of the original method many different protocols, which are still in use, had been originated. However, they are hardly comparable. The steps of the protocols, which are more diversified among the reviewed literature, other than added ^{14}C activity and incubation time, are the type of fixative to stop the photosynthetic activity and the HCl concentration to remove the exceeding ^{14}C. Although our results suggest that the use of aldehydes as fixatives to stop the photosynthetic activity in sediment samples lead to erroneous estimates of DPM values, the reinterpretation of existing data are unfeasible. Commonly in the literature primary production is expressed as a rate, and the formula including DPM values is rarely reported by the authors. Without the actual DPM values, it is not possible to reinterpret the published data. Moreover, the protocol descriptions do not always give detailed information, e.g., fixative and sample volumes, HCl concentration, etc., to allow a DPM re-estimation.

Conclusions and recommendations

The first aim of this study was to verify the best fixative in arresting the photosynthetic activity. Aldehydes do stop photosynthetic activity, but they present several crucial problems compromising their use in primary production. Formaldehyde increases cell membrane permeability, leading to a loss of assimilated carbon, because an underestimate of DPM values of up to 64% was obtained when compared with HCl values. Vice versa, glutaraldehyde led to an overestimate of DPM values due to its autofluorescence. When HCl was used as fixative, none of these problems were encountered.

The second aim of this study was to define which HCl concentration, with an equal volume, among 0.1N, 0.2N, 0.5N, 1N, 2N, and 5N was sufficient to remove the excess-labeled bicarbonate. The HCl concentration up to 2N did not remove all the inorganic ^{14}C, as shown both indirectly in the litmus test and directly in the sediment blank experiment. Even 5N treatment did not completely eliminate the inorganic-labeled carbon because of the sediment matrix effect. In fact, only a further decrease of the pH led to DPM values comparable to background counts. But, this further pH decrease, caused by a very strong acid normality (final HCl concentration of 0.2N) or, in our case, halving sample volumes, probably led to a collapse of the cells and a consequent digestion of the organic carbon pool.

Therefore:

* we advise for using directly HCl to stop primary production experiments;
* we do not recommend the use of the aldehydes which were tested in these experiments;
* we recommend the performance of a blank production estimate before each experiment to assess the buffer effect of the sediment matrix. Our data were obtained using muddy sediment, with a carbonate content of about 30%; it is likely that sediments with different carbonate contents may lead to diverse results.

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