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Phytopigment and DNA determinations in long-time formalin-preserved trap samples

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ABSTRACT: This study was specifically designed for sediment trap researchers and oceanographers working with organic material which is fixed *in situ* with formalin and analysed several months after collection. The effects of long-term formalin storage on phytopigment and DNA determinations were tested on seawater, algal cultures and trap material. DNA determinations in formalin-preserved samples were hampered by the use of specific flurochromes (i.e. Thiazole Orange and Hoechst). In contrast, the use of DABA (diaminobenzoic acid) reagent allowed these problems to be overcome, making DNA determination feasible in formalin-preserved samples without any interference. DNA concentrations measured in all samples did not change significantly with long-time formalin storage (up to 9 mo), indicating that, independently from the quantity and the source of genetic material, non significant DNA losses occurred. In contrast, chloropigment concentrations in formalin-preserved samples significantly decreased. The decreasing temporal pattern of chlorophyll *a* (chl *a*) concentrations in formalin-preserved algal culture and seawater samples was similar, suggesting that chl *a* degradation is independent from the initial pigment concentration. A possible explanation for the strong decrease in chl *a* concentrations is changes in its molecular structure. The recovery of total chloroplastic pigments in trap samples was higher than the recovery of chl *a* observed in algal cultures and seawater samples. Such discrepancy appears to be due to the different degradation rates of the phaeopigments after their fixation in the sediment traps.

KEY WORDS: Formalin · Trap samples · DNA · Phytopigments

INTRODUCTION

Vertical fluxes of settling particles are generally assumed to be largely dependent upon primary production and are characterised by large seasonal fluctuations (Suess 1980, Honjo et al. 1988). Such changes coupled with an often unpredictable interannual variability of particulate fluxes in the ocean require long and intensive trap samplings to obtain detailed quantitative information (Honjo & Manganini 1993). Given the lability of the organic material to microbial degradation within sediment traps, treatments of the accumulating particles with various preservatives are necessary, especially during long-term deployments (Gardner et al. 1983). The effectiveness of poisons such as formalin for halting microbial activity in sediment trap collection is now well established (Knauer et al. 1984, Lee et al. 1992). The most common parameters investigated during these studies are total mass and organic carbon fluxes, which are not significantly affected by the use of formalin (Hedges et al. 1993). However, bacterial and phytoplankton density have been found to strongly decrease in long-time formalin-preserved seawater samples (Booth 1987, Turley & Hughes 1992). Therefore, it is likely that a certain loss of some biochemical compounds of organic matter will occur.

Information on the effects of preservatives (such as formalin) on some biochemical components of particu-

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late organic matter is extremely scarce. Wakeham et al. (1993) demonstrated that the use of certain preservatives showed no significant differences on the determination of proteins, carbohydrates and lipids when compared to non-treated samples. Conversely, amino acids and pigments showed a high variability between treatments, but were not correlated with either the treatment used or the number of swimmers collected. Moreover, due to the lack of control samples, they could not reach any conclusions about the effects of preservatives on amino acid and pigment determination. Saiz et al. (1998) pointed out that nucleic acid determination by specific fluorochromes (i.e. Thiazole Orange) is hampered in formalin-fixed zooplankton samples.

The present study was designed: (1) to define a protocol suitable to measure DNA concentrations in formalin-fixed samples; (2) to provide information on the effects of formalin on phytopigment and DNA concentrations in long-time-course experiments carried out on sediment trap material.

MATERIALS AND METHODS

In order to provide a wider spectrum of responses from different biological samples, experiments were carried out on natural seawater samples (as representative of the water column where particles are produced) and on algal cultures (as representative of the phytoplankton component, the major factor responsible for the production of settling particles).

DNA quantification in formalin-fixed and untreated samples. Algal samples of *Isochrysis* sp. and *Tetraselmis* sp. provided by CCAP (Culture Collection of Algae and Protozoa, Scotland) were grown on f/2 media (Guillard 1975). Cultures were kept in a temperature-controlled room at 20 ± 1°C with a 12:12 h light:dark cycle. Samples for DNA analysis were collected during the exponential phase of growth and processed as follows: aliquots of algal samples were filtered onto Whatman GF/F (nominal pore size 0.45 µm, estimated pore size about 0.7 µm) and GS Millipore filters (pore size 0.2 µm); other aliquots were fixed with prefiltered (0.2 µm) buffered formalin (4% final concentration) and filtered after 24 h on the same filters previously utilised.

Ten litres of surface water samples collected in June 1997 in the Adriatic Sea were prefiltered through 200 µm mesh net and divided into two 5 l aliquots: one aliquot was fixed with prefiltered buffered formalin and the other divided into subsamples of 500 ml and immediately filtered onto GS filters. After 24 h, fixed seawater samples were processed in the same way described for untreated material.

Long-term effects of formalin on phytopigment and DNA concentrations. Ten litres of surface water collected in the Northern Adriatic Sea in May 1998 was prefiltered through 200 µm mesh net and fixed with formalin. Immediately after fixation (t_0), subaliquots of 500 ml were filtered onto Whatman GF/F for phytopigment determination and onto GS Millipore filters for nucleic acid determination. For time-course DNA and phytopigment determination, samples were processed after 7, 14, 30, 60, 120 and 150 d.

One hundred milliliters of *Isochrysis* sp. algal culture were fixed with formalin and subsamples of 5 ml were immediately processed as described for seawater samples. Time-course DNA and phytopigment measurements on algal culture were carried out with the same time interval utilised for seawater samples.

Sediment traps were deployed in December 1997 and in January 1998 in the Gulf of Gaeta (Thyrrenhian Sea, 20 m depth). The area was selected because it is characterised by a strong biodeposition derived from the presence of large farm plants located in the Gulf. This guaranteed the collection of large amounts of organic materials over a period of only 24 h (i.e. duration of the deployments). Samples of trap collection were not treated *in situ* with any preservative. After recovery, 100 ml of sediment trap material were fixed with formalin and subaliquots of 7 ml collected at each sampling time were filtered onto GS and GF/F filters for DNA and phytopigment analysis. For DNA and phytopigment determination, trap material was sampled immediately after formalin addition and after 150 and 270 d.

All biological samples utilised during time-course phytopigment and DNA analysis were preserved with prefiltered buffered formalin and kept in the dark at 4 to 6°C. All samples were analysed immediately after filtration according to the methods described below.

DNA determination. Jones et al. (1995) demonstrated, comparing 3 different methods (spectrophotometric using diphenylamine assay; fluorometric using Hoechst dye; and fluorometric using diaminobenzoic acid, DABA) for particulate DNA determination on algal cultures and seawater samples, that diphenylamine and DABA yielded similar DNA concentrations whereas Hoechst-DNA yields were significantly lower. Saiz et al. (1998) reported that the use of specific fluorochromes for nucleic acid determination (such as Thiazole Orange) is precluded in zooplankton samples treated with formalin.

Two different fluorometric methods, selected for their sensitivity, were compared here: one utilises selective fluorochromes for total nucleic acid (Thiazole Orange, Lee et al. 1986) and DNA (Hoechst 33258, Paul & Myers 1982) determinations and the other utilises DABA for DNA quantification (Holm-Hansen et al. 1968).

Particulate DNA determination on samples filtered onto GF/F was carried out according to Berdalet & Dortch (1991). DNA was extracted from the filters in 5 ml of TRIS-Ca^{2+} buffer by sonication (Branson 2200 Sonifier, 60 W, 47 kHz) for 30 s (Paul & Myers 1982). All solutions and materials were kept at 2 to 4°C throughout the extraction procedure. Two stains were utilised for nucleic acid fluorescence determination: Thiazole Orange was used to evaluate total nucleic acid fluorescence (at 511 nm excitation and 533 nm emission wavelengths) and Hoechst 33258 was used for the measurement of DNA fluorescence (at 360 nm excitation and 460 nm emission wavelengths).

Particulate DNA determination on samples filtered onto GS has been carried out according to Bailiff & Karl (1991). The filters were extracted in 100% acetone for 1 h at −20°C, they were centrifuged and the supernatants discarded. The pellets were newly resuspended in cold acetone (100%, −20°C) and extracted for 30 min at −20°C until the filters were completely dissolved (usually 2 or 3 separate rinses were required). The samples were then washed once with 90% acetone (4°C), once with 10% TCA (4°C) and twice with 95% ethanol (4°C), and the resulting pellet was dried for 1 h at 60°C. Particulate DNA was measured fluorometrically using DABA reagent (at 405 nm excitation and 520 nm emission wavelengths). DNA concentrations were calculated using calibration curves obtained from standard solutions of calf-thymus DNA (standard solutions: 0.2, 0.4, 0.8, 1.0, 2.0, 4.0 and 5.0 µg DNA ml^{-1}).

Phytopigment determination. Pigments were extracted in 90% acetone at 4°C in the dark for 12 h. Samples were then centrifuged and the supernatant filtered through a 0.5 µm Millex-SR filters. Chloropigments were identified and quantified by means of reverse phase HPLC (Mantoura & Llewellyn 1983). The HPLC system consisted of a Beckman 126 model pump, a Rheodyne model 7125 injector, a Beckman 166 model UV-Vis Detector and a reverse phase chromatographic C_{18} column (25 cm × 4.6 mm i.d., 5 µm particle size). The mobile phase consisted of Solvents A (acetone/methanol 10/90 v/v) and B (acetone/methanol 40/60 v/v) pumped at a flow rate of 1 ml min^{-1}. Pigments were eluted using Solvent A for 10 min, followed by a linear gradient from A to B over 5 min and an isocratic hold of B at 100% for 10 min. Pigment detection was at 436 nm for chlorophylls and 405 nm for phaeophytins (Wright et al. 1991) and the pigments were identified by comparing their retention time with those of standard solutions of commercial chlorophyll (chl) *a* and *b* and phaeophytin *a* and *b* obtained by acidification (1 M HCl) of the corresponding chlorophylls (Mantoura & Llewellyn 1983). The identified pigments in the samples were then converted into concentrations from calibration curves obtained from the relationships between peak surface of standards and their relative concentrations.

RESULTS

Particulate DNA determinations in fixed and untreated samples

The fluorescence response of nucleic acids extracted from fixed and unfixed algal cultures and stained with Thiazole Orange (TO) and Hoechst (HO) is illustrated in Fig. 1a–d. TO and HO provided a linear fluorescence response with increasing chl *a* concentration (expressed as ml of algal cultures) only on unfixed samples but gave a very low fluorescence response close to background signal in fixed algal samples, without any increase with increasing pigment concentrations. By contrast, DNA concentrations obtained by DABA on both fixed and untreated algal cultures provided linear response with increasing sample volumes (Fig. 1e,f). Moreover, the comparison between DNA concentrations obtained with DABA in fixed and untreated samples of both seawater and algal cultures did not show significant differences ($t = -1.468$, $p = 0.11$; $t = 0.745$, $p = 0.25$; and $t = 1.183$ $p = 0.15$ for *Tetraselmis* sp., *Isochrysis* sp. and seawater samples respectively; Fig. 1g).

Effect of long-term formalin storage on DNA and phytopigment concentrations

The effects of formalin on DNA concentrations in time-course experiments are reported in Fig. 2a–c. DNA concentrations determined in all biological samples did not change significantly with time (ANOVA, $F = 0.094$, 0.432, 2.62 and 0.726, for seawater, *Isochrysis* sp., and trap materials collected in December 1997 and January 1998, respectively). The average DNA concentration during the course of the entire experiment (i.e. 150 d) was 6.9 ± 0.14 and 0.9 ± 0.12 µg ml^{-1} for seawater and algal culture, respectively. DNA concentrations obtained from trap material collected in December 1997 and January 1998 were on average 52.7 ± 5.9 and 16.4 ± 7.6 µg l^{-1}.

In this study, only time-course formalin effects on chl *a* concentrations of *Isochrysis* sp. algal culture and seawater samples were considered because HPLC chloropigment analysis of the t_0 samples revealed very low peak areas for the other pigments checked, close to the noise signal (data not shown). By contrast, in the sediment trap material, although chl *a* was the dominant pigment, chl *b*, phaeophytin *a* and *b* were clearly detectable (Fig. 3).

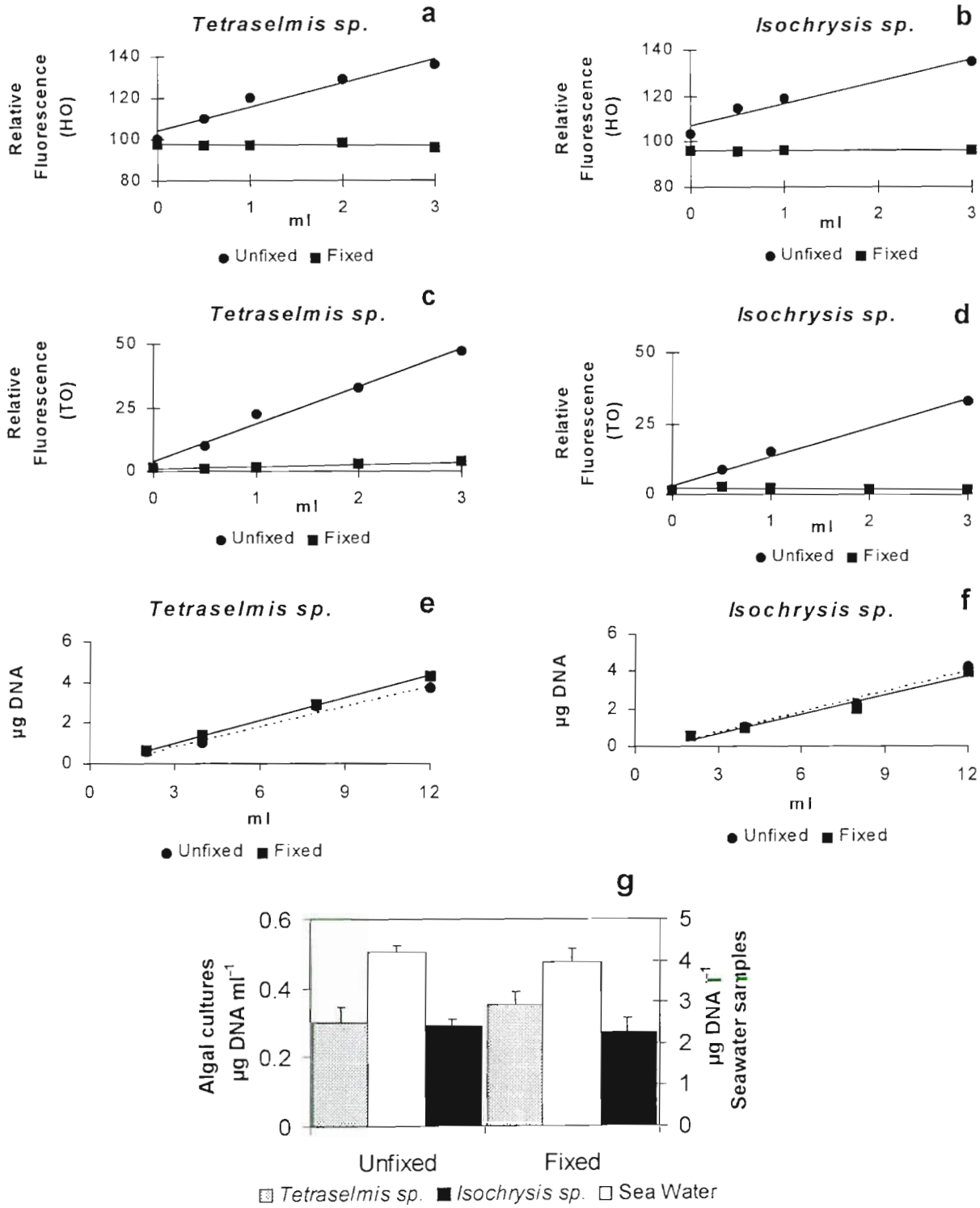

Fig. 1. Comparison of fluorometric responses of nucleic acids extracted from formalin-fixed and untreated algal cultures. (a) Hoechst (HO) fluorescence of fixed and unfixed *Tetraselmis* sp.; (b) HO fluorescence of fixed and unfixed *Isochrysis* sp.; (c) Thiazole Orange (TO) fluorescence of fixed and unfixed *Tetraselmis* sp. and (d) TO fluorescence of fixed and unfixed *Isochrysis* sp.; (e) DNA concentrations measured by DABA in relation to increasing volumes of fixed and unfixed *Tetraselmis* sp.; (f) DNA concentrations measured by DABA in relation to increasing volumes of fixed and unfixed *Isochrysis* sp.; (g) Comparison of DNA concentrations determined with DABA in *Isochrysis* sp. and *Tetraselmis* sp. algal cultures and sea water samples fixed and unfixed with formalin. Standard deviations (n = 3) are reported

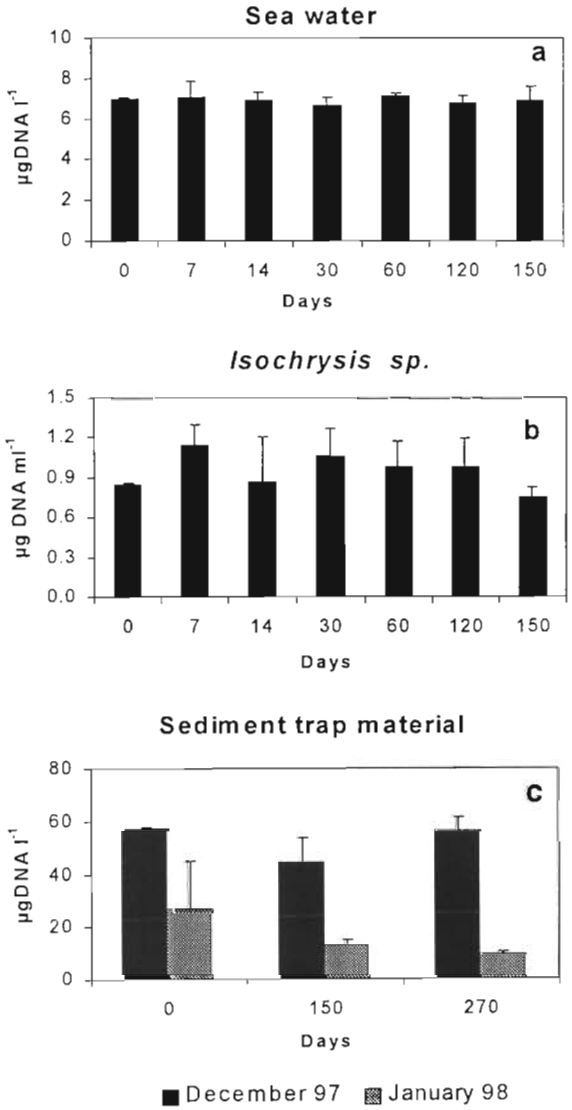

Fig. 2. Temporal pattern of DNA concentrations measured with DABA in formalin-preserved samples: (a) Seawater; (b) *Isochrysis* sp. algal cultures; (c) sediment trap material collected in December 1997 and January 1998. Standard deviations are reported. Analyses were carried out on 3 replicates except for trap material, which was analysed in 4 replicates

Chl *a* concentrations in all samples drastically decreased with time. In only 7 d of formalin storage, chl *a* concentrations decreased from 105.8 to 34.8 ng l^{-1} and from 0.7 to 0.35 µg ml^{-1} in seawater and algal culture, respectively. After 5 mo, chl *a* concentrations recovered in both samples were only about 7% of the initial amount (Fig. 4a,b).

Total chloroplastic pigment concentrations (CPE, here defined as the sum of chl *a* and *b*, and phaeophytin *a* and *b*) in sediment trap material, collected in December 1997 and January 1998, strongly decreased with increasing time of formalin storage (Fig. 4c). After

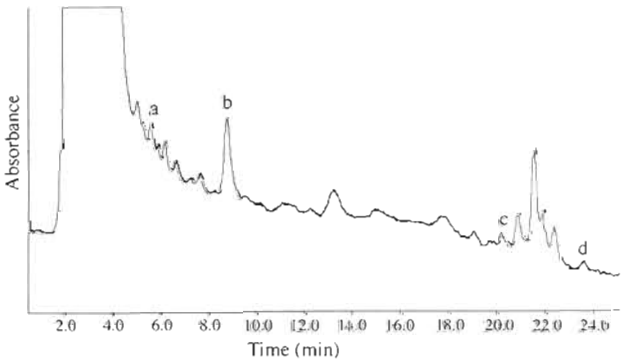

Fig. 3. HPLC chromatogram of pigments extracted from trap material collected in December 1997. (a) chl *b*; (b) chl *a*; (c) phaeophytin *b* and (d) phaeophytin *a*

150 d chloroplastic pigment concentrations decreased for about 70 and 80% in the trap samples of December 1997 and January 1998, respectively.

DISCUSSION

As far as DNA determination is concerned, Saiz et al. (1998) pointed out that nucleic acid concentrations using Thiazole Orange cannot be determined in formalin-fixed zooplankton samples. In agreement with these findings, we have demonstrated that particulate DNA determinations in formalin-preserved phytoplankton samples (i.e. algal cultures) are precluded using both Thiazole Orange and Hoechst. This result is probably due to the creation of specific 'formalin-nucleic acids' interactions that would hamper the formation of the complex fluorochromes-nucleic acids. By contrast, the use of DABA reagent allows to overcome these problems, making DNA determination feasible in formalin-preserved samples without any interference. Therefore, the use of DABA in formalin-fixed samples is recommended for DNA quantification. Since formalin might significantly affect the concentrations of some labile organic compounds over a longer storage period (i.e. months), we tested the effects of long-term formalin preservation of trap samples on DNA and phytopigment concentrations.

DNA concentrations measured in both seawater samples and algal cultures did not display significant differences after 5 mo of formalin preservation. Similarly, DNA concentrations of trap samples determined on a larger time scale (i.e. 9 mo) did not change significantly with time. These results indicate that, independently from the quantity and the source of genetic material (i.e. algal culture, seawater and trap material), non significant DNA losses occurred. Therefore, estimates of DNA fluxes from sediment traps are possible

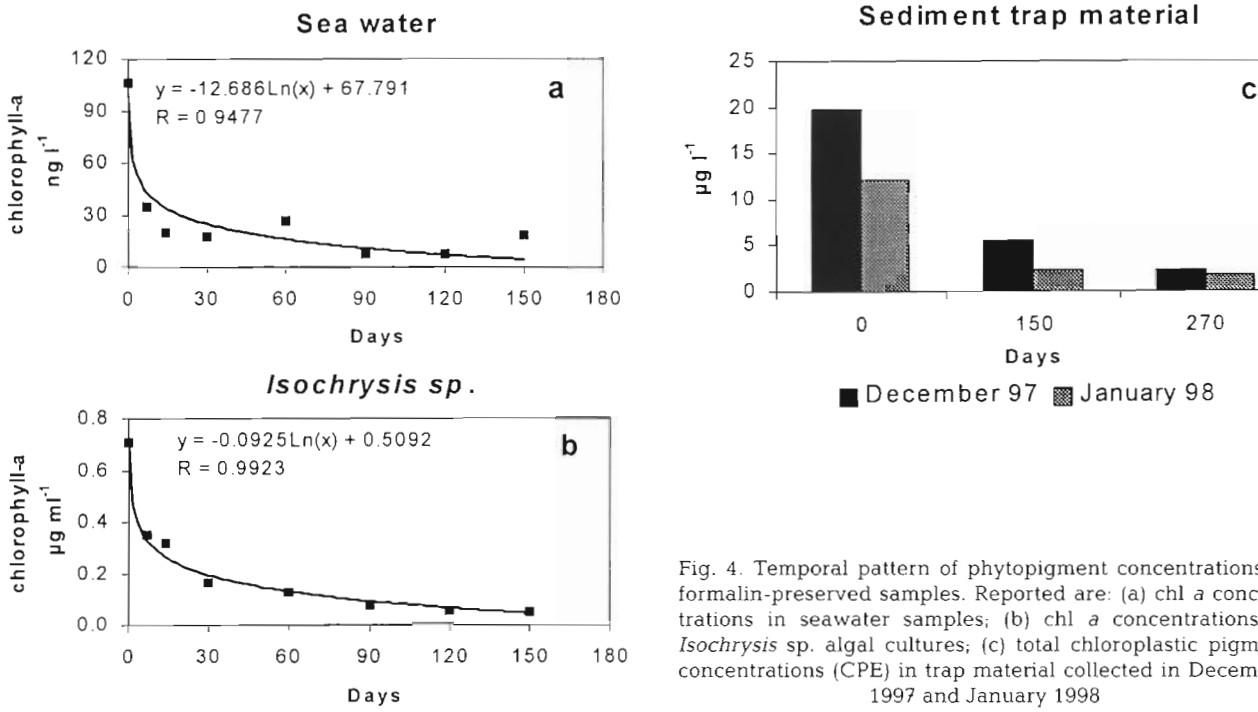

Fig. 4. Temporal pattern of phytopigment concentrations in formalin-preserved samples. Reported are: (a) chl *a* concentrations in seawater samples; (b) chl *a* concentrations in *Isochrysis* sp. algal cultures; (c) total chloroplastic pigment concentrations (CPE) in trap material collected in December 1997 and January 1998

and can indeed be carried out without any significant DNA loss even 9 mo after sediment trap deployment. By contrast, chloropigment concentrations in formalin-preserved samples strongly decreased. The percentage decrease of chl *a* after 7 d of formalin storage was similar in seawater sample and algal culture (67 and 50% of the initial concentrations, respectively). Moreover, 5 mo after formalin addition chl *a* yield accounted for 7% of the initial amount in both samples.

Water samples and algal culture contained different initial amounts of chl *a*. As the loss rates of chl *a* (and consequently the percentage of recovery after 5 mo) were similar in water samples and algal cultures, we might conclude that pigment degradation due to formalin storage is independent of the initial pigment concentration and source.

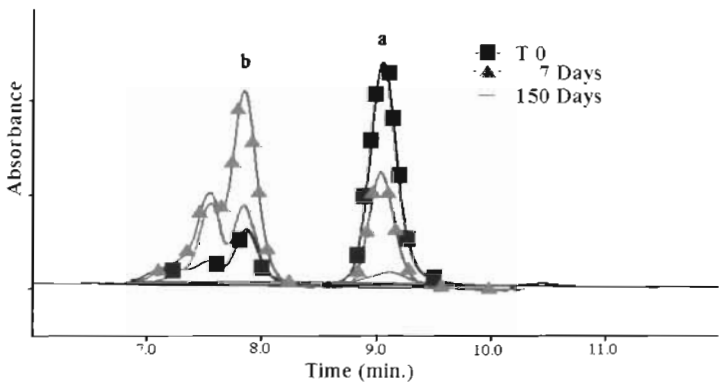

Fig. 5. HPLC chromatogram of pigments of *Isochrysis* sp. algal cultures analysed immediately after formalin fixation (t_0), after 7 d and after 150 d. (a) chl *a*; (b) unidentified peak

A possible explanation for the strong decrease in chl *a* concentrations seems to be due to changes in its molecular structure (Fig 5). In the early phase of formalin storage (i.e. 7 d), the chl *a* peak decreased with the concomitant increase of an unidentified peak that was eluted about 1.2 min before. However, both peaks drastically dropped with time, indicating that formalin induced changes might progressively affect also the molecular structure of this unidentified molecule. The significant pigment loss during the time-course experiment was confirmed by HPLC analysis of trap material. However, the recovery of total chloroplastic pigments (CPE) after 5 mo was higher (19 and 28% in trap samples collected in January 1998 and December 1997, respectively) than the recovery of chl *a* observed in algal cultures and seawater samples. Such discrepancy may be due to the different degradation rates of the pigments included in CPE.

Chl *a* and *b* and their phaeophytins underwent different degradation rates: after 5 mo of formalin storage, chl *a* and *b* in trap samples were reduced by 90% while their phaeophytins were reduced by 70%.

These results indicate that the chl *a* concentrations associated with phytoplankton settling into sediment traps decay during long-term trap deployment due to the presence of

formalin *in situ* (i.e. in the collection cups). Chl *a* concentrations can also be determined after long-term formalin storage in the traps but the initial amount of chl *a* collected can be estimated only after correction using the equations reported in this study, which are based on the time lag between *in situ* cup collection and laboratory analysis (expressed in days). By contrast, more detailed information of decay rates of other pigments is needed to provide accurate corrections of the actual losses of chloropigment concentrations.

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