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Quantification of microphytobenthos biomass in intertidal sediments: layer-dependent variation of chlorophyll *a* content determined by spectrophotometric and HPLC methods

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Abstract : We assessed the difference between chlorophyll *a* (Chl. *a*) values estimated by spectrophotometer using either 90% aceton or N,N-dimethylformamide (DMF) as an extractant, and by HPLC using DMF, in intertidal sediment samples from the Seto Inland Sea, Japan. This was conducted both at the surface(0–0.5 cm) and subsurface(0.5–2 cm) layers and through the vertical profile of the sediments (each next cm to 10 cm depth). The Chl. *a* content determined using the three different procedures gave similar results at the surface. In contrast, the Chl. *a* content at the subsurface layer was 94.5±13.7 and 70.1±19.4% that found at the surface when spect ophotometrically determined using 90% aceton and DMF, respectively, while it was 47.7 ±16.9% when determined by HPLC. Such difference between procedures was consistent with depth. At the 6–7, 8–9 and 9–10 cm layers, Chl. *a* determined spectrophotometrically varied from 1.1 to 2.1 $\mu g\ g^{-1}$, while it was not detected by HPLC. These results indicate that the more practical spectrophotometric method can be reasonably employed to quantify the living fraction of microphytobenthic biomass in the surface layer of these sediments. At lower layers, only relying on either aceton or DMF extraction of pigments and spectrophotometric determination is likely to lead to an overestimation of the Chl. *a* content up to >50%. This can be related to the progressive increase and interference with depth of photosynthetic degradation products whose reliable estimates can only be obtained by chromatographic quantification.

Key words : *Microphytobenthos, biomass, chlorophyll a, methods, HPLC, spectrophotometer, tidal flat, Seto Inland Sea*

1. Introduction

During the last decade, major interest has arised on the role of intertidal microphytobenthos as a primary carbon source for estuarine food webs and on its implications in the cycling of nutrients (SULLIVAN and MONCREIFF, 1990; DE JONG and DE JONGE, 1995; HEIP *et al.*, 1995; MACINTYRE *et al.*, 1996; GUARINI *et al.*, 1998). It is therefore important that the determination of chlorophyll *a* (Chl. *a*) content in sediments could give a reliable estimate of the living fraction of microphytobenthic biomass. This is particularly critical for the top few mm of sediments where microphytobenthic photosynthetic activity occurs (REVSBECH *et al.*, 1983; KROMKAMP *et al.*, 1998) and primary production is calculated (PINCKNEY and ZINGMARK, 1993; BARRANGUET *et al.*, 1998). The HPLC-technique has been earlier indicated as the most reliable one, particularly in sediment samples containing a mixture of pigments (DAEMEN, 1986). MACINTYRE *et al.*(1996) indicated that there may be negligible to large (40%) error due to interference of degradation products where Chl. *a* is measured by spectrophotometer. Other works showed either good agreement between spectrophotometric and HPLC measurements (PLANTE-CUNY *et al.*, 1993) or a slight overestimation of Chl. *a* content determined by spectrophotometer of 2 to 5% (BARLOW *et al.*, 1990). Such controversy in results obtained using different methods suggests that possible discrepancies should be

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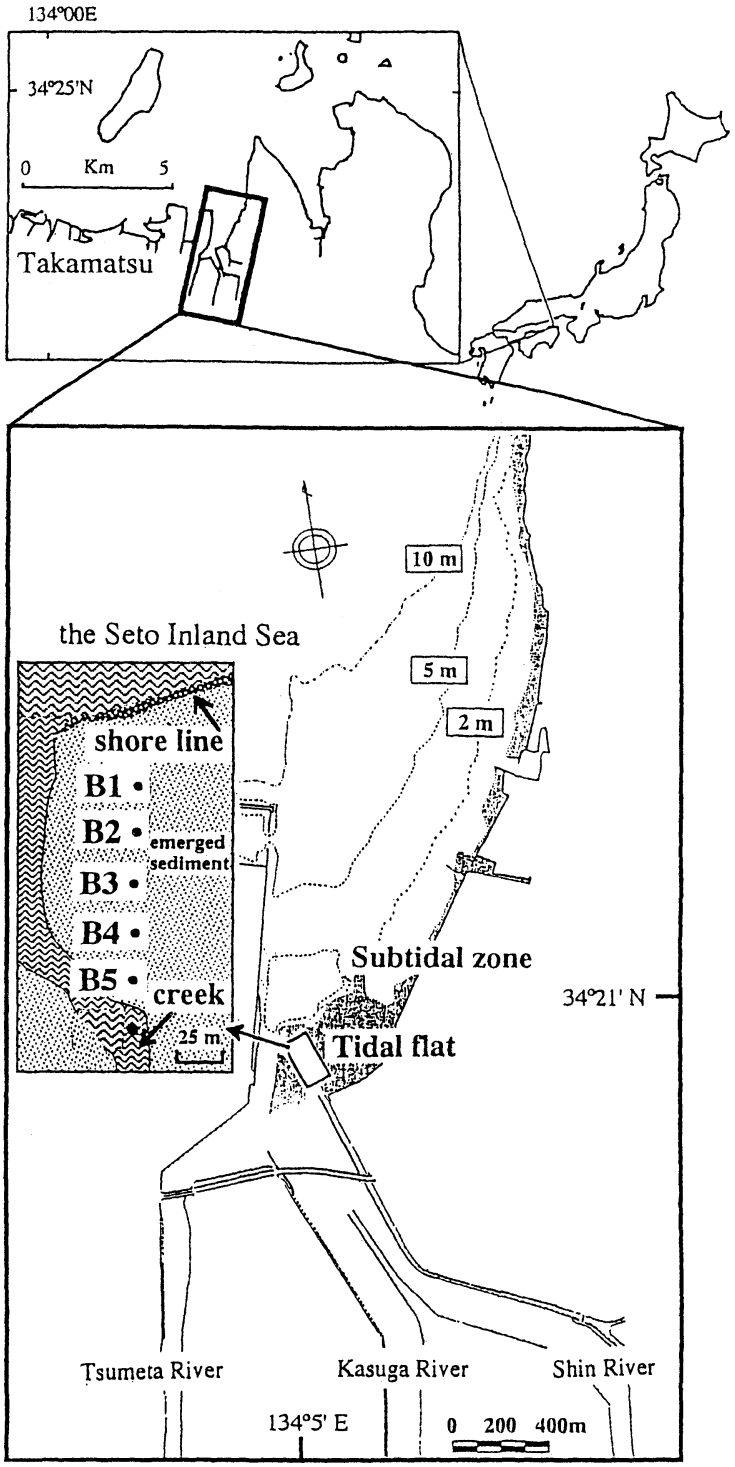

Fig. 1. Study area and location of the sampling stations.

checked in each relevant study area. It can be inferred that the more practical spectrophotometric method can be suitable for long-term and intensive survey schemes, provided that concurrent spectrophotometric and HPLC estimates either show a reasonable approximation or are normalized (DE JONG and DE JONGE, 1995). Until now, an evaluation of possible discrepancies between methods has been restricted to the upper 1 cm layer of sediments (DAEMEN, 1986; BARLOW *et al.*, 1990; PLANTE-CUNY *et al.*, 1993; MACINTYRE *et al.*, 1996). In the present work, we additionally tested whether there can be a layer-dependent variability in the Chl. *a* content determined by spectrophotometer and HPLC, due to the increasing fraction of degradation products with depth (SUN *et al.*, 1994; CARIOU-LEGALL and BLANCHARD, 1995; LUCAS and HOLLIGAN, 1999) which may concurrently cause decreasing reliability of the spectrophotometric method (DAEMEN, 1986; BARLOW *et al.*, 1990). In the spectrophotometric method, we employed two different extractants for Chl. *a*, such as 90% aceton and N,N-dimethylformamide (DMF), the latter also used for HPLC measurements.

2. Materials and methods

2.1. Study area and sampling procedure

We collected the sediment samples for chlorophyll *a* (Chl. *a*) measurements at five stations located on a sandflat of a tidal estuary in the Seto Inland Sea (Fig. 1), where a multidisciplinary project is in progress on the cycling biophilic elements (MONTANI *et al.*, 1998; MAGNI and MONTANI, 1997; 1998; 2000; MAGNI *et al.*, 2000). Sampling occured on 3 different dates, during a spring low tide: October 20 (Stn. B4), November 19 (Stns. B5 to B1) and December 18, 1997 (Stn. B4). On each occasion, we randomly collected emerged sediment samples at 7 to 8 spots of a station, using an acrylic core tube (3 cm i.d.) gently pushed by hand into the sediment. Both the surface (0–0.5 cm) and subsurface (0.5–2 cm) layers were carefully extruded and sliced off the sediment. Sediment samples from the same layer were pooled together and brought to the laboratory within 2 h for further treatment and analysis. Additionally at Stn. B4, we collected sediment samples at lower layers (each next cm to a 10 cm depth), on October 20, 1997.

2.2. Sediment treatment and analysis

In the laboratory, chlorophyll *a* (Chl. *a*) and phytopigment degradation products (i.e. total pheopigments) were extracted from duplicate subsamples of wet sediment (ca. 1 g) using either 90% acetone of N,N-dimethylformamide (DMF). After 24 h of darkness at 4℃, the samples were sonicated for 5 min, centrifuged at 3000 rpm (1000 × g) for 10 min, and extracts were spectrophotometrically analysed for Chl. *a* and pheopigment content. Chl. *a* and pheopigment values were obtained, using either extractants, before and after acidification with 1 N HCl, respectively, according to LORENZEN's (1967) method, as described in PARSONS *et al.* (1984), where the volume of water is substituted by the dry weight (DW) of the sediment expressed in gram. Values were expressed as $\mu g\ g^{-1}$ and corrected for porosity, as measured by the water content. This was obtained after drying duplicate sediment subsamples at 105 ℃ for 20 h. Concurrently on each occasion and station, we determined, from the same sample, the Chl. *a* content by HPLC using DMF as an extractant, according to SUZUKI and ISHIMARU (1990). The apparatus used for the HPLC pigment analysis consisted of 2 pumps (Jasco 880-PU intelligent HPLC pump) driven by a gradient programmer (Shimadzu C-R5A), an injection valve with a 200 μl loop (Rheodine 7125) and a fluorescent detector (Jasco 870-UV intelligent UV-VIS) with a 8 μ l flow cell. Cromatographic separation was carried out with a Toso TSK gel ODS-80 TM, ϕ 4.6 mm, 250 mm column. Absorbance for Chl. *a* was set at 440 nm. The solvents for the HPLC gradient used were as follows: solvent A was methanol: water:ion pair solution (7:2:1, v/v/v); the ion pair solution consisted of 0.75 g tetrabutyl ammonium acetate and 3.5 g of ammonium acetate dissolved in 50 ml redistilled water. Solvent B was methanol and ethylen acetate (4:1, v:v). The flow rate was 0.8 ml min^{-1} and the gradient was linear. The program utilized was 30 min long. It started with 50% of solvents A and B, reached 100% solvent B in 10 min and stayed

with 100% of solvent B until the end. Standard solutions of Chl. *a* were obtained from Wako pure chemical.

3. Results and discussion

The chlorophyll *a* (Chl. *a*) content determined using the three different procedures gave similar results at the surface layer (0–0.5 cm), irrespective ot the station and the sampling occasion, with mean values of 7.0 and 7.2 $\mu g\ g^{-1}$ for the spectrophotometric and HPLC methods, respectively (Table 1). In contrast at the subsurface layer (0.5–2 cm), the mean Chl. *a* varied remarkably from 6.6 ± 0.9 to 3.5 ± 1.3 $\mu g\ g^{-1}$, as determined by spectrophotometer using 90% aceton and by HPLC, respectively (Table 1). At this layer, the former procedure gave Chl. *a* estimates significantly higher than those obtained using both the spectrophotometric and HPLC technique and DMF as an extractant ($p<0.05$ and $p<0.001$, respectively; Table 1).

Accordingly, the Chl. *a* content at the subsurface layer resulted to be 94.5 ± 13.7 and 70.1 ± 19.4% that found at the surface when spectrophotometrically determined using 90% aceton and DMF, respectively, and 47.7 ± 16.9% when determined by HPLC (Table 1). Such difference in Chl. *a* estimates among procedures was consistent through the vertical profile, being remarkably higher those determined spectrophotometrically and extracted by 90% aceton down to the 4–5 cm layer (Fig. 2). At the 6–7, 8–9 and 9–10 cm layers, Chl. *a* determined spectrophotometrically varied from 1.1 to 2.1 $\mu g\ g^{-1}$, while it was not detected by HPLC (Fig. 2).

The study by DAEMEN (1986) indicated that the difference between Chl. *a* values estimated by spectrophotometer or by HPLC in surface sediments samples from the Oosterschelde Estuary (the Netherlands) was not likely to be more than 20% on average. In a subsequent work carried out in intertidal areas of the same estuary, DE JONG and DE JONGE (1995) adopted a conversion factor of 0.7 to make spectrophotometer and HPLC estimates of Chl. *a* in surface sediments comparable, where spectrophotometer value, 0.7=HPLC value. The results of the present study show that in our study area the Chl. *a* in the uppermost layer (0–0.5 cm) of the sediments estimated by the llceoser spectrophotometric method is reasonably similar to that estimated by HPLC. This in a agreement with the results of earlier studies by BARLOW *et al.* (1990) and by PLANTE-CUNY *et al.* (1993). In contrast, at lower layers a strong overestimation occurred when the Chl. *a* content was estimated by spectrophometer, most remarkably using 90% aceton as an extractant.

Table 1. Chlorophyll *a* (Chl. *a*) estimates at the surface (0–0.5%) and subsurface (0.5–2cm) layers of intertidal sediments and the ratio between layers, using the spectrophotometer method and either 90% aceton or N,N-dimethylformamide(DMF) as an extractant, and the HPLC method (DMF as an extractant). ANOVA: single factor; *: $p<0.05$; ***: $p<0.001$; ****: $p<0.0001$; ns; not significant)

Sampling date	Station	Surface (0–0.5cm)			Subsurface (0.5–2cm)			Subsurface/Surface		
		Spectrophotometer		HPLC	Spectrophotometer		HPLC	Spectrophotometer		HPLC
		Aceton ($\mu g\ g^{-1}$)	DMF ($\mu g\ g^{-1}$)	DMF ($\mu g\ g^{-1}$)	Aceton ($\mu g\ g^{-1}$)	DMF ($\mu g\ g^{-1}$)	DMF ($\mu g\ g^{-1}$)	Aceton ratio (%)	DMF ratio (%)	DMF ratio (%)
20 Oct 1997	B4	5.9	5.4	5.8	5.2	2.5	1.0	88.1	46.3	17.2
19 Nov 1997	B5	6.6	7.2	7.0	7.2	4.0	3.4	108.1	55.6	48.6
	B4	6.7	5.7	6.1	7.1	5.7	3.5	106.0	100.0	57.4
	B3	6.9	7.7	8.1	5.4	7.1	5.2	78.3	92.2	64.2
	B2	6.5	6.5	7.3	7.2	4.5	4.5	110.8	69.2	61.6
	B1	8.2	8.3	7.4	6.7	5.4	3.8	81.7	65.1	51.4
18 Dec 1997	B4	8.2	7.9	8.4	7.2	4.9	2.8	87.8	62.0	33.3
	AVG	7.0	7.0	7.2	6.6	4.9	3.5	94.5	70.1	47.7
	SD	0.9	1.1	1.0	0.9	1.4	1.3	13.7	19.4	16.9
Anova: Single Factor										
Spectrophotometer (aceton)				ns			***			****
Spectrophotometer (DMF)			ns			*		*	*	*
		ns		ns	*		ns			

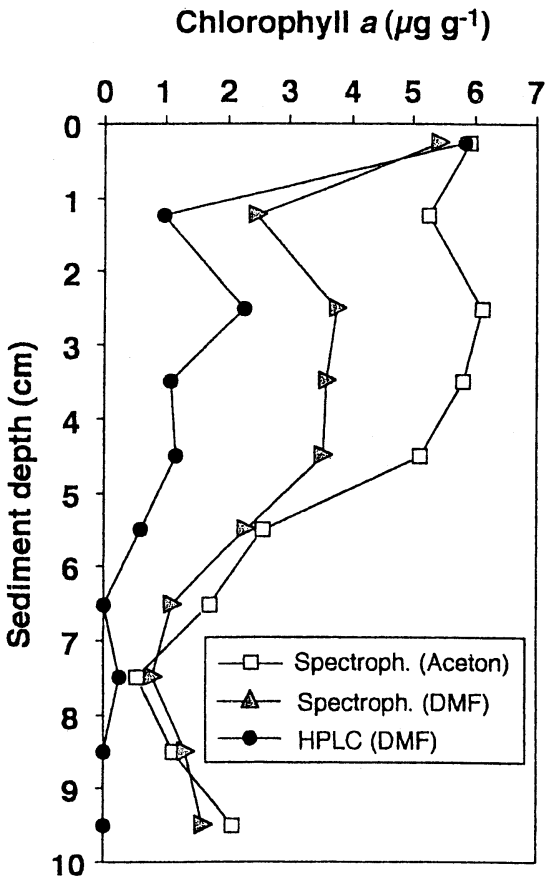

Fig. 2. Vertical distribution of chlorophyll *a* (Chl *a*) content using the spectrophotometer method and either 90% aceton or N,N-dimethyl-form amide (DMF) as an extractant, and the HPLC method (DMF as an extractant)(20 Oct. 1997).

Our HPLC measurements indicated the presence of living microphytobenthos in the non-photosynthetic active layers of the sediments down to 7–8 cm layer (Fig. 2). Several studies indicated that microphytobenthos can growth heterotrophically if light is not available (CADEE and HAGEMAN, 1974; HELLEBUST and LEWIN, 1977; DARLEY *et al.*, 1979; PELETIER, 1979), even surviving for many months (GARGAS and GARGAS, 1982). Vertical migration by epipelic benthic diatoms may be related to the effect of tidal waves and currents (STEVENSON, 1983; KINGSTON, 1999) and tidal cycle (PINCKNEY and ZINGMARK, 1991). Sediment reworking by macrozobenthos, abundant on this flat (MAGNI and MONTANI, 1998; MAGNI *et al.*, 2000), may also represent an important factor of microphytobenthos transport in the sediments (BRANCH and PRINGLE, 1987; ALLER, 1988). However, the decrease of the living fraction of microphytobenthos occurred rapidly with depth (Fig. 2). This was consistent with previous studies conducted in intertidal sediments (BARLOW *et al.*, 1990; SUN *et al.*, 1994; CARIOU-LEGALL and BLANCHARD, 1995; DE JONG and DE JONGE, 1995), suggesting a progressive increase with depth of photosynthetic degradation products (LUCAS and HOLLIGAN, 1999) whose reliable estimates can only be obtained by chromatographic quantification (BARLOW *et al.*, 1990). We conclude that the more practical and rapid spectrophotometric method can be reasonably employed to quantify the living fraction of microphytobenthos in the surface sediments of the flat under investigation. At lower layer, only relying on either aceton or DMF extraction of pigments and spectrophotometric determination is likely to lead to an overestimation of the Chl *a* content up to >50%.

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