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## **Submersion time, depth, substrate type and sampling method as variation sources of marine periphyton**

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**Abstract**

Periphyton is an additional food source in African and Asian brackish and freshwater fish ponds. The present study was a preliminary assessment of periphyton development on artificial substrates in temperate marine ponds. The effects of submersion time, substrate type, water depth, and total or partial sampling methods on the quantity and quality of periphyton collected, were evaluated. Four types of substrate (W: wooden poles, S: smooth fiber-glass strips, m: mosquito screen (1mm-mesh) and M: garden netting (5mm-mesh)) were deployed in a marine pond, and periphyton was collected 15 and 30 days later. The total amount of periphyton per substrate unit was collected as one sample or as 5 sub-samples. Results showed that (i) periphyton biomass in a marine pond increased between day 15 and day 30, (ii) more periphyton was collected on mosquito screen than on wooden poles, fiberglass strips and garden netting, (iii) periphyton biomass increased with submersion depth, (iv) sub-sampling leads to an underestimate compared to whole unit sampling, and (v) a correction of periphyton weight must be carried out considering the dissolved inorganic salts present in periphyton samples from marine and brackish ponds. Whole substrate unit sampling using a tube and stopper is recommended to avoid underestimation of periphyton development. Finally, the autotrophic fraction in the periphyton communities was very low compared to periphyton developed on biodegradable substrates in fertilized tropical ponds. Studies on fertilization and use of biodegraded substrates (i.e. long-time submerged wood) are recommended to further optimize periphyton development in temperate marine ponds.

Key words: periphyton, fouling, artificial substrate, marine pond, aquaculture

## 1. Introduction

Periphyton refers to the entire complex of attached aquatic biota on submerged substrates, including associated non-attached organisms and detritus (van Dam et al., 2002). This assemblage comprises bacteria, fungi, protozoa, phyto and zoo-plankton, benthic organisms and detritus (Azim et al., 2005). It can be used as additional food in aquatic production systems. Aquaculture based on periphyton was originally derived from traditional fishing methods known in Africa as Acadja (Welcomme, 1972) and in Asia as Kathas and Samarahs (Van Dam et al., 2002). Artificial substrates are added into aquatic system to enhance the food availability. This semi-extensive aquaculture system is well known to increase the production of fish (Ramesh et al., 1999; Umesh et al., 1999; Azim et al. 2001a). Although widely tested in freshwater fish culture (Azim et al., 2005), the use of periphyton in brackish or marine waters (van Dam et al., 2002; Huchette and Beveridge, 2005; Khatoon et al., 2007) is limited to shrimp (Bratvold and Browdy, 2001; Moss and Moss, 2004; Arnold et al., 2006) and abalone cultures (Kawamura et al., 2005).

Variation of periphyton quantity and quality depends on a range of factors such as (i) submersion time (Azim and Aseada, 2005), (ii) substrate type (Ramesh et al., 1999; Keshavanath et al., 2001; Azim et al., 2002a), and (iii) light intensity and quality (Kirk 1994; Goldsborough et al., 2005). The latter is strongly influenced by the depth of the substrates (Asaeda and Son 2000). Thus, Azim et al. (2001a, 2003b) waited minimum 2 weeks to allow periphyton to develop on the substrates before stocking fishes. Keshavanath et al. (2001) observed that fish production based on periphyton depends on artificial substrate type and preferred to use bamboo rather than PVC pipes or sugarcane bagasse bundles when culturing masheer (*Tor khudree*) fingerlings. Azim et al. (2001b, 2002a, 2004a) and Keshavanath et al. (2001) pooled several sub-samples of periphyton collected at equally spaced depths along vertical substrates to analyse the composition of periphyton. This pooled sample was

considered by these authors to represent the mean composition of periphyton developed on substrate, going from the photic zone close to the surface to the aphotic zone above the bottom.

The potential contributions of semi-extensive aquaculture to environmental protection and restoration of coastal areas have been clearly recognised within EU policy. The SEACASE program (Sustainable extensive and semi-extensive coastal aquaculture system in Southern Europe) was started in 2007 to develop sustainable extensive and semi-extensive coastal aquaculture systems in Southern Europe (Conceição et al., 2007). The present SEACASE study is a preliminary assessment of the feasibility to grow periphyton on artificial substrates in temperate marine ponds. The effects of submersion time, substrate type, water depth, and total or partial sampling methods, on the quantity and quality of periphyton collected, were evaluated. The goals of this study were to identify (i) the best periphyton substrate type and (ii) a methodology of periphyton sampling for further studies on periphyton-based marine aquaculture.

## **2. Materials and methods**

### *2. 1. Experimental site and design*

The experiment was carried out from 9 May till 6 June 2007 in a 200 m<sup>2</sup> marine pond in the IFREMER-L' Houmeau experimental facilities, located on the Atlantic coast of France, near La Rochelle. Four types of substrates were used for this experiment (Fig. 1): (i) 2.5 cm wide square wooden poles (fir tree: W), and 5 cm wide strips of (ii) smooth fiber-glass (S), mosquito screen (1mm-mesh; m) and (iv) garden netting (5mm-mesh; M). The mean submersion depth of the substrates ( $\pm$  SE) was  $76.3 \pm 2.7$  cm after 15 days of submersion

whereas it was  $66.3 \pm 4.6$  cm after 30 submersion days. The mean submerged surface area ( $\pm$  S.E.) was  $713 \pm 9.3$  cm<sup>2</sup> and equal for each substrate type. Eleven poles or strips (called units) of each substrate type were deployed in the marine pond. The units were put 20 cm apart from the closest other units in 4 parallel rows with 11 units each within a 1.0 m x 2.4 m plot, randomly assigning the different unit types to the available locations. The different strip types were suspended in the water column from iron bars fixed on a horizontal wooden frame standing slightly above the surface on poles driven in the bottom, while the pole units were standing in the sediment, under the iron bar.

*Total sampling: Influence of substrate type and submersion time*

On sampling days, four units of each substrate type were randomly collected. All the periphyton on each unit was collected. Sampling was done 15 (23 May 2007; T<sub>15d</sub>) and 30 days after submersion (4-5 June 2007; T<sub>30d</sub>). Collected units were not placed back. In total, 32 units were collected (4 units/type/date x 4 types x 2 dates).

*Sub-sampling: Influence of substrate type and submersion depth*

At the end of the experiment (T<sub>30d</sub>), the remaining 3 units of each substrate type (W, S, m, M) were sampled in a random order. The submerged area of each unit was divided in five 15-cm-segments starting from the bottom (Fig. 2a: 1: 0-15 cm, 2: 15-30 cm, 3: 30-45 cm, 4: 45-60 cm, 5: 60-75 cm). Each 15-cm sub-sample (i.e. 1 to 5; Fig. 2a) was completely cleaned. The order of the segment cleaning was randomly assigned for each unit. Each sub-sample was next separately stored. In total, 60 samples were collected (3 units/type x 4 types x 5 sub-samples/unit).

*Total vs. sub-sampling: comparison of both sampling methods*

Each 15-cm sub-sample was analysed separately. The average periphyton composition on each unit was calculated in two ways (Fig. 2b):

1. Per unit, the data of the five 15-cm sub-samples (1 to 5) were added together, to represent the whole surface area (S-5), and
2. Per unit, the top (1: 0-15 cm), middle (3: 30-45 cm) and bottom (5: 60-75 cm) sub-samples were added together, and extrapolated to the total unit area (S-3).

These data were compared with the results of the whole unit samples (T) collected on the same day (30d). 28 data were thus used for each set of comparison ((4 units/types x 4 types) + (S-3 or S-5 sampling method 3 units/type \* 4 types)).

Three units (one W, m and M) were incorrectly treated and could not be included in the data set. It explains why the total degree of freedom was lower than expected (Tables 1 through 3).

*2.2. Sampling and storage*

At  $T_{15d}$  and  $T_{30d}$ , water temperature ( $^{\circ}\text{C}$ ), salinity, pH were measured with a multi-parameter probe (HI9828 HANNA) at the water top 15 cm of three sites in the pond, at 5:00 PM. Mean water temperature, salinity and pH ( $\pm$  SE) were  $24.4 \pm 0.76$   $^{\circ}\text{C}$ ,  $32.2 \pm 0.14$  ppt and  $8.1 \pm 0.07$  at  $T_{15d}$  vs.  $26.3 \pm 0.93$   $^{\circ}\text{C}$ ,  $32.9 \pm 0.14$  ppt and  $8.2 \pm 0.03$  at  $T_{30d}$ . Mean oxygen concentration ( $\pm$  SE) was at  $6.4 \pm 0.1$   $\text{mg.L}^{-1}$  ( $92.8 \pm 2.2$  %)  $T_{15d}$  and  $6.9 \pm 0.1$   $\text{mg.L}^{-1}$  ( $102.7 \pm 2.7$ %) at  $T_{30d}$ . The water samples were collected immediately after the probe recording. Means of suspended matter ( $\pm$  SE) and particulate organic matter were  $13.7 \pm 1.04$   $\text{mg.L}^{-1}$  and  $1.9 \pm 0.2$   $\text{mg.L}^{-1}$ , respectively, at  $T_{15d}$ . The suspended matter was composed of  $85.9 \pm 0.4$  % of

inorganic matter. At  $T_{30d}$ , mean Chl *a* was  $5.3 \pm 0.3 \mu\text{g}\cdot\text{L}^{-1}$ . Chlorophyll pigments included  $15.2 \pm 1.2$  percent of Phaeophytin *a*.

### *Periphyton*

The order and the location of collected units were randomly assigned. Each unit was sampled by putting a PVC tube (diameter of 6 cm x 110 cm of length) over it and closing it with a 100  $\mu\text{m}$ -meshed stopper to avoid periphyton loss. The length of the submerged part of the collected substrate was measured in order to calculate the exact substrate area with periphyton ( $\text{cm}^2$ ). Each unit was carefully and completely cleaned with fingers and a toothbrush into a plastic flask with a fixed volume of 0.7  $\mu\text{m}$ -filtered sea-water (200 ml for total unit samples and 40 ml for 15-cm samples). All material from the inner part of the net of meshed substrates was removed. Each sample was next sub-sampled using a Motoda box-splitter (Motoda, 1959): 1/8 part was stored in a dark box at  $-20^\circ\text{C}$  for Chl*a* analysis, 7/16 parts were stored with 4% formalin for taxonomic analysis, and 7/16 parts were used for periphyton weight analyses, putting it directly in pre-weighted box at  $60^\circ\text{C}$ .

### 2.3. *Sample analyses*

#### *Dry weight and Ash free dry weight*

Periphyton samples were dried at  $60^\circ\text{C}$  for 72h, weighed (DW: dry weight), and burned for 4h at  $450^\circ\text{C}$  to calculate the ash-free dry weight (AFDW; Byers et al., 1978). DW, AFDW and the weight of ash (ASH) were measured to the nearest  $10^{-5}$  g with an AE240 Mettler Toledo Balance. As filtered sea-water was used to clean units, “salt correction” was applied on



periphyton weight. The effects of added filtered seawater (7/16 of 200ml or 40 ml according to the type of sampling) on DW, ASH and AFDW of periphyton were determined considering the salinity of the cleaning water and the corresponding calibration curves ( $DW (g.L^{-1}) = 1.17 \text{ Salinity} (R^2=0.99)$ ,  $ASH (g.L^{-1}) = 0.94 \text{ Salinity} (R^2=0.99)$ , and  $AFDW (g.L^{-1}) = 0.23 \text{ Salinity} (R^2=0.96)$ ). These equations were established using based on DW, AFDW and ASH content of three replicates of 0.7  $\mu\text{m}$ -filtered water in which the salinity was either 0, 10.7, 20.4, 28, 28.7, 36.37, 36.42, 38.03, 38.12, 40.17 or 40.38 ppt. Sea-water (28 to 40) was collected in marine ponds. Water in which the salinity ranged from 10 to 20 corresponded to diluted sea water by Milli-Q water (0 ppt).

Values were reported to the total sample volume (200 or 40 ml) and to the total length of the unit. DW, ASH and AFDW were thus expressed in  $\text{mg.cm}^{-2}$ .

#### *Chlorophyll a and Phaeophytin a*

Chlorophyll *a* (Chl *a*) and phaeophytin *a* (Phaeo *a*) observed in periphyton were determined with a Turner TD 700 fluorometer after 12 hours of acetone extraction at 4°C in the dark without and with acidification. Nine ml of 100% acetone were added to 1 ml-periphyton as could performed Azim's team (M.C.J. Verdegem, Pers. Com.). Chl *a* and Phaeo *a* data were reported to the total sample volume (200 or 40 ml) and to the total length of the unit. Values were expressed in  $\mu\text{g.cm}^{-2}$  for periphyton. The ratio of phaeophytin vs. sum of chlorophyll pigments was also calculated as  $(\text{Phaeo } a) \cdot (\text{Phaeo } a + \text{Chl } a)^{-1}$  and expressed in % (% Phaeo *a*). The autotrophic index (AI) was calculated as:  $\text{AFDW} (\text{mg.cm}^{-2}) / \text{Chl } a (\mu\text{g.cm}^{-2}) * 1000 \mu\text{g/mg}$  (APHA 1992).

#### *2.4. Statistical analyses*

The assumptions of normality and homoscedasticity were evaluated using Shapiro-Wilk (Shapiro and Wilk, 1965) and Brown-Forsythe (Brown and Forsythe, 1974) tests, respectively. When required, data were transformed to satisfy both assumptions. ANOVAs were next performed to test the influence of (i) submersion time (TIME), (ii) substrate type (TYPE), (iii) submersion depth (DEPTH), (iv) sampling method (SAMPLING), and (v) their interactions on periphyton DW, AFDW, Chl *a*, Phaeo *a*, %Phaeo *a* and AI. Tukey's HSD (honestly significant differences) pairwise multiple comparison tests were used to identify the differences when a source of variation was significant ( $P < 0.05$ ).

### 3. Results

#### 3.1. Total sampling: Influence of submersion time and substrate type

According to the ANOVA results (Table 1), dry weight, ash free dry weight, chlorophyll *a* and phaeophytin *a* varied significantly among submersion time (TIME; Table 1). Means were greater at T<sub>30d</sub> than at T<sub>15d</sub>. Mean AFDW and Phaeo *a* were more than twice higher at T<sub>30d</sub> than at T<sub>15d</sub> (DW: 6.3 mg.cm<sup>-2</sup> vs. 2.3 mg.cm<sup>-2</sup> and Phaeo *a*: 0.2 µg.cm<sup>-2</sup> vs. 0.1 µg.cm<sup>-2</sup>; Fig. 3a, b).

Substrate type (TYPE) significantly affected the amount of periphyton collected in terms of DW, AFDW, Chl *a* and Phaeo *a* (Table 1). Tukey HSD tests revealed that means of DW, AFDW, Chl *a* and Phaeo *a* were larger on mosquito screen (m) than on the other substrate types (M, S or W; Fig. 3c, d). Mean periphyton DW and total chlorophyll pigment varied between 3.4 and 6.4 mg.cm<sup>-2</sup> (Fig. 3c) and between 0.5 and 1 µg.cm<sup>-2</sup>, respectively, among substrate types (Fig. 3d).

The interaction of both factors (TYPE x TIME) was a significant variation source of % Phaeo *a* (Table 1). Relatively more Phaeo *a* was present on mosquito screen at T<sub>30d</sub> (m-T<sub>30d</sub>: 27.7%) than at T<sub>15d</sub> (m-T<sub>15d</sub>: 16.7%). In contrast, mean % Phaeo *a* did not significantly differ over time on wooden poles (W), fiber-glass (S) and garden netting (M). Means ( $\pm$  SE) were respectively  $30.2 \pm 0.9$  %;  $28.2 \pm 1.1$  % and  $18.7 \pm 1.6$  %. At T<sub>15d</sub>, a higher % Phaeo *a* was observed on smooth substrates (W, S) than on meshed substrates (m, M). At T<sub>30d</sub>, the % Phaeo *a* observed on wooden poles (W) was higher than on garden netting (M).

The autotrophic index was significantly different for the factors TYPE and TIME, and showed a significant interaction (Table 1). The mean AI observed on wooden poles (W) was more than 6 times lower at T<sub>15d</sub> ( $1554 \pm 410$ ) than at T<sub>30d</sub> ( $9449 \pm 1479$ ). In contrast, the mean AI observed on the other substrates (S, m, M) did not vary over time.

### 3.2. Sub-sampling: Influence of substrate type and submersion depth

Chl *a*, % Phaeo *a* and the AI were significantly different among substrate type (Table 2). HSD tests showed that at T<sub>30d</sub>, the Chl *a* mean was greater on meshed substrates and fiberglass than on wooden poles (m, M, S:  $0.6 \pm 0.12 \mu\text{g.cm}^{-2} > \text{W: } 0.26 \pm 0.08 \mu\text{g.cm}^{-2}$ ). At T<sub>30d</sub>, % Phaeo *a* varied such as  $\text{W} > \text{S}, \text{M} \geq \text{M}, \text{m}$ . The AI mean was almost three times higher on wooden poles (W:  $2815 \pm 816$ ) than on the other substrates (S, M, m:  $939 \pm 299$ ).

Periphyton DW, Chl *a* and Phaeo *a* significantly changed with depth (DEPTH; Table 2). More periphyton was collected at 60-75 cm depth than at 0-15 cm depth (Fig. 4). Respectively 2 and 12 times more DW and total chlorophyll *a* was collected in the bottom 15 cm than at the top 15 cm. Mean differences between sampling depths of DW and Phaeo *a* were not statistically significant whereas means seemed to increase between 15 and 60 cm depth. In contrast, Chl *a* increased gradually with depth (Fig. 4b). The mean AI ( $\pm$  SE) was

more than three times larger in the top 15 cm ( $3406 \pm 1002$ ) than between 15 and 60 cm ( $900 \pm 260$ ). The % Phaeo *a* did not vary with depth whatever the type substrate (Table 2) and was  $25.7 \pm 2.05\%$ .

### *3.3. Total vs. sub-sampling: comparison of both sampling methods*

#### *Five sub-sampling*

The mean DW, AFDW, Chl *a* and Phaeo *a* differed significantly between sampling methods (SAMPLING (S-5 vs. T); Table 3). Means were higher with the total sampling (T) than the S-5 sub-sampling method whatever the substrate type (Fig. 5a, b). It was particularly right for periphyton quantity rather than quality. DW and AFDW determined through S-5 sampling were 2 and 8 times, respectively lower than T means (Fig. 5a) whereas the mean of chlorophyll pigment obtained with S-5 sampling corresponded to 82.6% of means obtained with total sampling T (Fig. 5b).

#### *Three sub-sampling*

Sampling was a significant source of variation for DW and AFDW (SAMPLING (S-3 vs. T); Table 3). More DW and AFDW were measured with total sampling (T) than with the S-3 sub-sampling method (Fig. 5a). As S-5 means, S-3 means of DW and AFDW were 2 and 8 times, respectively, lower than the T means (Fig. 5a). In contrast, Chl *a* and Phaeo *a* means did not significantly differ between S-3 and T (Fig. 5b).

## **4. Discussion**

#### *4.1. Marine periphyton and its variation sources*

##### *Marine periphyton*

A thin mat of matter was observed on all the immersed surface of the different types of substrate after 15 days of submersion. The inorganic fraction of periphyton (ASH) could originate from trapping of suspended inorganic particles. The latter would be favoured during resuspension caused by wind driven turbulence or people working around units during sampling. The organic matter (AFDW) fraction originated from the accumulation of detritus, bacteria, fungi, flora and fauna on substrates. The presence of photosynthetic pigments (Chl *a* and Phaeo *a*) could indicate flora colonization of artificial substrates. The presence of phaeophytin *a* indicated that the flora observed was partly degraded (15 to 30 %). The mean autotrophic index ranged between 250 (60-75 cm section of mosquito screen at T<sub>30d</sub>) and 9450 (wooden poles at T<sub>30d</sub>). These high values indicate that the periphyton contained mainly heterotrophic organisms and dead organic matter, as specified by Huchette et al. (2000) for an AI above 200. *In situ* observations showed that periphyton was also composed of detritus and small-sized organisms as harpacticoid copepods (Richard et al., unpublished data).

##### *Submersion time*

A significant increase of periphyton DW, AFDW and photosynthetic pigments was shown on all substrate types. According to periphyton colonization models (Hoagland et al., 1982; Steinman, 1996), AFDW and Chl *a* levels increase exponentially until a biomass peak. Organisms at the base of the biofilm become light and nutrient limited, eventually die and detach from the substrate (Hansson et al., 1992; Asaeda et al., 2000; Keshavanath et al.,

2001a; Azim and Aseada, 2005). In this investigation, periphyton was still in its accretion phase on day 30 on all substrate types. As noted Eding et al. (2006), biofilm establishment seems to be slower in marine than in freshwater. It would be better to wait a minimum of 4 weeks rather than 2 as Azim et al. (2001a, 2003b) did in freshwater, before introducing fish in marine periphyton-based ponds.

### *Substrate type*

DW, AFDW, Chl *a*, Phaeo *a* and %Phaeo *a* varied according to substrate type. Keshavanath et al. (2001) showed that biodegradable substrates could be more efficient than synthetic substrates (eg. Bamboo vs. PVC tubes) because of the nutrient leaching that occurred at the substrate-water interface (van Dam et al., 2002). In the same way, Anderson and Underwood (1994) reported higher recruitment by epifauna on plywood than on fibreglass or aluminium substrates in an estuary. In contrast, periphyton biomass was not larger on natural (i.e. wooden poles) than on fibreglass strips in this study. 30 days-submersion time might have been too short to permit to a significant nutrient leaching at the interface of wooden poles. Nevertheless, the periphyton grown on wooden poles contained relative more phaeophytin (higher % Phaeo *a*) and non autotrophic matter (higher AI) than the other substrates. The observed increase in AI could have originated from uptake of decomposition products from the wood.

More dry matter and Chl *a* were found on meshed substrates (mosquito and garden meshes) than on smooth substrates (i.e. wood and fiber-glass). The meshes might favour the trapping of particles, in contrast to smooth surfaces. Moreover, higher circulation of water and nutrients across the meshed substrates could stimulate periphyton growth and explain this result.

The substrate type could also influence the nature of the heterotrophic associated community. Richard et al. (2007) observed that mesh substrates, as aquaculture pens, offered appropriate structures for infauna, as *Corophium* sp. whereas newly submerged smooth substrate, favoured epifauna recruitment. In this study, some polychaete tubes were observed on mosquito screen, but not on smooth substrates. The results of this study indicate that more and qualitatively better periphyton grew on mosquito screen than on the other substrates.

### *Submersion depth*

The light intensity and its spectral composition change with depth, influencing the quality and type of flora (Boston and Hill, 1991; Hansson, 1992; Kirk, 1994), as periphyton (Goldsborough et al., 2005). In contrast to the observations of Azim et al. (2002a), periphyton DW and chlorophyll pigments increased with depth in this investigation. A decrease of the 10 cm-water level at  $T_{30d}$  could explain why less periphyton was collected on the 0-15 cm part of substrates than on the deeper parts. Nevertheless, the lower chlorophyll pigment concentration observed on the 15-60 cm part of substrates compared to the deeper part (60-75 cm) could be due to a photo-inhibition processes, as Hansson (1992) suggested when periphyton Chl *a* was negatively correlated with light. Unfortunately, light incidence was not measured during this study.

Maximal periphyton biomass could be observed where the combination of light and nutrient are optimal (Hansson et al., 2002). In this way, periphyton observed on the deeper part of substrates could have the advantage over the one observed on the surface part by benefiting from nutrient released at the water-sediment interface. Moreover it could benefit from trapping suspended sediment and microphytobenthos present at the bottom of the pond.

## 4.2. Comparisons

### *Sampling method*

The sum of 3 samples taken between 0-15 cm, 30-45 cm and 60-75 cm (S-3 method) led to comparable Chl *a* and Phaeo *a* means with the ones obtained with total sampling (T). That was not the case with S-5 method. Nevertheless, the DW and AFDW of the periphyton collected with both sub-sampling methods (S-5 and S-3) were significantly lower than with total sampling. The sub-sampling, especially the one of mesh substrates, necessitated extra handling for cutting before periphyton collection. Each handling event results in losses, making both the S-5 and S-3 methods less accurate than whole unit sampling. Total sampling was easier and more periphyton was collected. In further studies, the total sampling method will be preferred to sub-sampling one.

In this investigation, the use of 200 ml of filtered salt-water for unit cleaning induced over-estimation of periphyton weight. The DW, the ASH and the AFDW added when cleaning 750 cm<sup>2</sup>-periphyton substrate with 200 ml of 0.7 µm seawater of 32.55 ppt were respectively 10.1, 8.1 and 2 mg.cm<sup>-2</sup>. These values are very important compared to the real periphyton weight (Table 4), especially for DW and ASH. Without the salt correction, ASH would be more than 6 times greater than the real values (with correction) at T<sub>15d</sub> and 3 times at T<sub>30d</sub>. Analysis of three blanks of cleaning water should be envisaged at each sampling date in subsequent studies. To avoid the salt correction, the use of milliQ water could be envisaged in case where the determination of periphyton weight would be the only analysis to carry out on the sampled unit. The periphyton fauna and flora could be analysed from other units cleaned with filtered seawater to avoid osmotic shock of the living cells.



*Periphyton in other aquatic systems*

Absolute values which described the quantity and the quality of periphyton developed on our substrates deployed in marine water were different with the one observed mainly in freshwater by others authors (Table 4). The mean DW observed on our substrates reached 8.8 mg.cm<sup>-2</sup> on mosquito screen m at T<sub>30d</sub> (Table 4). This is relatively high since 10 studies out of 13 found a DW < 5mg.cm<sup>-2</sup>. Maximal mean organic periphyton (AFDW) observed in this study (4.5 mg.cm<sup>-2</sup>) was greater than means observed by others authors which generally did not exceed 1 mg.cm<sup>-2</sup> with the exception of Azim et al., 2002b (Table 4). In contrast to this investigation, in most of the cited studies, periphyton substrates are simply removed from the water causing probably a lot of loosely attached to be lost and could explain lower mean of AFDW. The use of a tube with a stopper for substrate sampling is recommended to avoid underestimation of periphyton development.

High autotrophic index of this investigation (Table 4) was induced by greater AFDW but also by very low chlorophyll *a* concentration observed on substrates (0.4 to 0.6 µg.cm<sup>-2</sup> ; Fig. 3b). Numerous studies observed Chl *a* levels above 10-15 µg.cm<sup>-2</sup> (Azim et al., 2001b,c, 2002a, 2003a; Keshavanath et al., 2001; Table 4). Low periphyton concentration could originate partly from the use of inert substrate (Huchette et al. 2000; Azim et al. 2003b; Liboriussen and Jeppesen, 2006; This study: Table 4) rather than nutrient-leaching substrate (Azim et al., 2001b, 2002a, 2002b). Nevertheless, others factor could influence the primary productivity, such as temperature, light and nutrient availability (Liboriussen and Jeppesen, 2005; Vermaat et al., 2005). The high densities of periphyton recorded by Azim et al. (2001b, 2002a, 2002b) and Keshavanath et al. (2001) were observed in tropical ponds in Bangladesh and India with more light and higher temperatures than in temperate ponds in France, in the Netherlands (Azim et al., 2003b) or Denmark (Liboriussen and Jeppesen, 2006). It is the same in water

column where mean Chl *a* was above 200  $\mu\text{g.L}^{-1}$  in Bengali fresh ponds (Azim et al. 2002b), whereas it was 5  $\mu\text{g.L}^{-1}$  in our temperate marine pond. Productivity in freshwater is generally higher than in marine water. However, the ponds were fertilized with urea, manure, food in most studies listed in Table 4 whereas our pond was not fertilized. Azim et al. (2001c, 2003a) showed that periphyton biomass increased with increasing fertilization rate up to a maximum. Thus, in future studies, as part of EU policy of environmental protection and restoration of coastal areas, fertilized effluents of intensive farms could be used to maximise periphyton production and the associated production of herbivorous fishes.

The present investigation showed that (i) periphyton biomass in a marine pond increased between day 15 and day 30, (ii) more periphyton was collected on mosquito screen than on wooden poles, fiberglass strips or garden netting, (iii) periphyton biomass increased with water depth submersion, (iv) sub-sampling methods underestimated periphyton development compared to whole unit sampling, and (v) a correction of periphyton biomass must be carried out for the dissolved inorganic salts present in marine or brackish systems using blank weight of cleaning salt filtered water. The use of a tube with stopper for substrate sampling will reduce periphyton sampling losses. Finally, the autotrophic fraction in the periphyton communities was very low compared to periphyton developed on biodegradable substrates used in fish cultures in fertilized tropical ponds. Thus, pond fertilization and use of biodegraded substrates (i.e. long-time submerged wood) should be envisaged in further studies on periphyton-based marine aquaculture in temperate regions.

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**Tables**

Table 1

Results of analyses of variance (ANOVAs) testing the effect of substrate type (TYPE: W: wooden poles, S: fiber-glass strip, m: mosquito screen, M: garden netting), submersion time (TIME: T<sub>15d</sub>, T<sub>30d</sub>) and their interactions on periphyton dry weight (DW), ash free dry weight (AFDW), Chlorophyll *a* (Chl *a*), Phaeophytin (Phaeo *a*), % Phaeo *a* (Phaeo *a*.(Chl *a* + Phaeo *a*)<sup>-1</sup>) and autotrophic index (AI: AFDW.Chl *a*<sup>-1</sup>) observed on collected substrates. df: degrees of freedom, MS: mean square, F: Fischer, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001

Variation source	df	MS	F	P	MS	F	P	MS	F	P	
			<b>log DW</b>			<b>log (AFDW + 1)</b>			<b>AI</b>		
TYPE	3	0.473	6.71	0.0022 **	0.552	6.79	0.0021 **	2E+07	8.48	0.0006 ***	
TIME	1	7.521	106.71	< 0.0001 ***	6.244	76.86	< 0.0001 ***	1E+08	32.82	< 0.0001 ***	
TYPE x TIME	3	0.197	2.79	0.0645	0.119	1.46	0.2529	2E+07	6.00	0.0038 **	
Error	22	0.070			0.081			3E+06			
			<b>Chl <i>a</i></b>			<b>Phaeo <i>a</i></b>			<b>% Phaeo <i>a</i></b>		
TYPE	3	0.157	15.89	< 0.0001 ***	0.013	6.46	0.0025 **	217.81	20.98	< 0.0001 ***	
TIME	1	0.202	20.43	0.0002 ***	0.105	52.08	< 0.0001 ***	119.05	11.47	0.0025 **	
TYPE x TIME	3	0.028	2.82	0.0615	0.004	1.86	0.1643	53.40	5.14	0.0072 **	
Error	23	0.010			0.002			10.38			

Table 2

Results of ANOVAs testing the effect of substrate type (TYPE: W: wooden poles, S: fiber-glass strips, m: mosquito screen, M: garden netting), submersion depth (DEPTH: 1: 0-15 cm; 2: 15-30 cm; 3: 30-45 cm, 4: 45-60 cm, 5: 60-75 cm) and their interactions on Periphyton dry weight (DW), ash free dry weight (AFDW), chlorophyll (Chl *a*), phaeophytin (Phaeo *a*) and % Phaeo *a* (Phaeo *a*.(Chl *a* + Phaeo *a*)<sup>-1</sup>) and autotrophic index (AI: AFDW.Chl *a*<sup>-1</sup>) observed on collected substrates. df: degrees of freedom, SS: sum square, MS: mean square, F: Fischer, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001

Variation source	df	MS	F	P	MS	F	P	MS	F	P	
			$\sqrt{\text{DW}}$			$\log (\text{AFDW} + 1)$			$\log \text{AI}$		
TYPE	3	0.02	0.21	0.8904	0.02	1.35	0.2786	3.21	6.38	0.0021 **	
DEPTH	4	0.36	3.48	0.0204 *	0.05	2.64	0.0556	3.72	7.40	0.0004 ***	
TYPE x DEPTH	12	0.05	0.53	0.8774	0.01	0.79	0.6563	0.50	0.99	0.4823	
Error	27	0.10			0.02			0.50			
			$\log \text{Chl } a$			$\log \text{Phaeo } a$			$\% \text{ Phaeo } a$		
TYPE	3	1.87	21.56	<0.0001 ***	0.39	2.07	0.1255	375.89	20.87	<0.0001 ***	
DEPTH	4	8.02	92.26	<0.0001 ***	7.76	40.86	<0.0001 ***	36.88	2.05	0.1128	
TYPE x DEPTH	12	0.11	1.31	0.2658	0.25	1.32	0.2587	30.57	1.70	0.1176	
Error	30	0.09			0.19			18.01			

Table 3

Results of ANOVAs testing the effect of periphyton sampling method (SAMPLING: T: total vs. S-5: addition of all five sub-samples and vs. S-3: addition of three sub-samples 0: 0-15 cm, 3: 30-45, 5: 60-75 cm), substrate type (TYPE: W: wooden poles, S: fiber-glass strips, m: mosquito screen, M: garden netting) and their interactions on Periphyton dry weight (DW), ash free dry weight (AFDW), Chlorophyll *a* (Chl *a*) and phaeophytin (Phaeo *a*) observed on collected substrates. df: degrees of freedom, MS: mean square, F: Fischer, \* P < 0.05, \*\* P < 0.01, \*\*\* P < 0.001

Variation source	df	log DW			log AFDW			Chl <i>a</i>			Phaeo <i>a</i>		
		MS	F	P	MS	F	P	MS	F	P	MS	F	P
SAMPLING (S-5 vs. T)	1	3.838	74.62	<0.0001 ***	24.383	130.80	<0.0001 ***	0.085	9.30	0.0069 **	0.017	6.46	0.0205 *
TYPE	3	0.118	2.30	0.1124	0.626	3.36	0.0419 *	0.156	17.09	<0.0001 ***	0.008	3.09	0.0532
SAMPLING x TYPE	3	0.109	2.11	0.1342	0.454	2.44	0.0980	0.028	3.08	0.0537	0.007	2.67	0.0786
Error	18	0.051			0.186			0.009			0.003		
SAMPLING (S-3 vs. T)	1	3.833	65.13	<0.0001 ***	23.978	119.66	<0.0001 ***	0.038	3.03	0.0986	0.008	2.94	0.1034
TYPE	3	0.129	2.19	0.1249	0.680	3.39	0.0406 *	0.194	15.61	<0.0001 ***	0.009	3.35	0.0421 *
SAMPLING x TYPE	3	0.105	1.79	0.1858	0.368	1.84	0.1763	0.029	2.34	0.1074	0.007	2.53	0.0895
Error	18	0.059			0.200			0.012			0.003		

Table 4

Ranges of mean variables characterizing quantity and quality of periphyton developed on different submerged substrates in natural and exploited aquatic systems observed by different authors over the world

References	Location	Season (date and range of temperature)	Fertilization	Substrate type	Presence (+) and absence (-) of fish	Periphyton quantity (range of mean DW, AFDW mg.cm <sup>-2</sup> )	Periphyton quality (range of mean Chl a, Phaeo µg.cm <sup>-2</sup> )
Boston and Hill 1991	American streams	(16 to 22°C)	NF	Ceramic tiles and natural rocks	natural presence	AFDW: 0.25 to 2.1	nd
Huchette et al., 2000	Tilapia reared on floating cages in a Bengali fresh farm	March to May (29.5°C)	No fertilization (NF)	Plastic bottle	presence and absence	AFDW: +: 0.5 to 0.9 - : 0.75 to 0.9	Chl a: +: 1 to 1.5, -: 1.2 to 2.8 AI: 300 to 600
Azim et al., 2001b	Polyculture of carps in Bengali fresh ponds (75 m <sup>2</sup> )	Sept to December (23 to 33.7°C)	Continuous fertilization (CF): *	Bamboo	presence	DW: 0.7 to 2.5 AFDW: 0.6 to 0.8	Chl a: 6.5 to 14.8 Phaeo a: 1.7 to 6.6 AI: 50 to 90
Azim et al. 2001c	Bengali fresh ponds (75 m <sup>2</sup> )	July to September (27.8 to 33.1)	CF: 4 rates of *	Bamboo	absence	DW: 0.5 to 5 AFDW: 0.5 to 3.3	Chl a: 1 to 16 Phaeo a: 0.1 to 1 AI: 70 to 300
Keshavanath et al., 2001	Masher fingerlings rearing in indian fresh water tanks (2.5m <sup>2</sup> )	Trial 1: May to June (31.6°C) Trial 2: Dec. to March (26.5°C)	CF: Poultry manure + re-fertilization fortnightly	Bamboo, PVC, sugarcane bagasse	Trial 1: absence Trial 2: absence and presence	Trial 1: DW: 0.5 to 1.9 AFDW: 0.4 to 1.2 Trial 2: DW: 0.2 to 0.9 AFDW: 0.1 to 0.6 DW: 0.5 to 4.5	Trial 1: Chl a + Phaeo a: 2.7 to 12.7 Trial 2: Chl a + Phaeo a: 0.6 to 25.7 AI: 50 to 330
Azim et al., 2002a	Polyculture of carps in Bengali fresh ponds (75 m <sup>2</sup> )	April to September (26.4 to 31.7°C)	CF: * + Rice bran and mustard oil cake	Bamboo, Jutestick, Kanchi	presence	DW: 2 to 10 AFDW: 2 to 6	Chl a: 5 to 18
Azim et al., 2002b	Polyculture of carps in Bengali fresh ponds (75 m <sup>2</sup> )	August to November (27.1 to 32.7 °C)	CF: *	Bamboo	presence	DW: 2 to 10 AFDW: 2 to 6	Chl a: 10 to 45 Phaeo a: 1 to 30 AI: 100 to 350
Azim et al., 2003a	Bengali fresh ponds (75 m <sup>2</sup> )	Trial 1: May to July Trial 2: Aug to September	CF: Trial 1: * Trial 2: 3 levels of *	Trial 1: Bamboo, Kanchi, Hizol Trial 2: Bamboo	absence	DW: Trial 1: 2 to 5 Trial 2: 0.9 to 2.6	Chl a: Trial 1: 2.8 to 12 Trial 2: 1.4 to 11.4
Azim et al., 2003b	Tilapia rearing in fresh tanks (1 m <sup>3</sup> ) in The Netherlands	August to October (22.5°C)	CF: NaNO <sub>3</sub> + single supersphosphate (SSP) each week	Glass slides	presence and absence	DW: +: 0.2 to 0.4 -: 0.3 to 0.6 AFDW: +: 0.05 to 0.25 -: 0.15 to 0.35 DW: 1.75 to 3.75	Chl a: +: 0.5 to 2.5, -: 1 to 5 AI: 70 to 150
Azim et al., 2004a	Polyculture of carps in Bengali fresh ponds (75 m <sup>2</sup> )	December to April (17 to 28°C)	CF: *	Bamboo	presence	DW: 1.75 to 3.75	nd
Azim et al., 2004b	Polyculture of carps in Bengali fresh station and farm ponds	June to November (21 to 33°C)	CF: * + rice bran and oil cake	Bamboo	presence	DW: 0.8 to 7	nd
Keshavanath et al., 2004	Tilapia rearing in Indian fresh water tanks (25m <sup>2</sup> )	May to August (25 to 31)	Punctual fertilization at start: Poultry manure	Bamboo	presence	DW: 0.1 to 0.35 AFDW: 0.1 to 0.3	Chl a + Phaeo a: 1 to 4
Liboriussen and Jeppesen 2006	Danish lakes	May to September (16 to 21°C)	NF	Strips of Tape	natural presence	DW: 1 to 2	Chl a: 1.5 to 4
Khatoon et al., 2007	Malaysian brackish water shrimp pond	(30.1 to 33.3°C)	CF: TSP at start, daily shrimp pellets	Bamboo, PVC pipes, plastic sheet, fibrous scrubber, ceramic tile	Presence of shrimp	nd, 56 to 168 polychaete tube.cm <sup>-2</sup>	Chl a: 0.01 to 0.1
Richard et al. (This study)	French marine pond	May to June (24.4 to 26.3°C)	NF	Four substrates (M, m, S, W)	absence	DW: 1.5 to 8.8 AFDW: 0.2 to 4.5	Chl a: 0.4 to 0.6 Phaeo a: 0.1 to 0.2 AI: 250 to 9450

\*: Fortnightly cow manure, urea, Triple super phosphate TSP, nd: no data

### Figures captions

Fig. 1: Pictures and schemes of the four types of periphyton substrate deployed in the marine pond: a) wooden poles (W), b) fiber-glass strips (S), b) mosquito screen (m) and d) garden netting (M)

Fig. 2: a) Scheme of sub-sampling of the submerged substrate surface carried out along the submersion depth gradient, b) scheme of three methods of sampling (Total, 5 sub-samples, 3 sub-samples)

Fig. 3: Mean ( $\pm$  Standard Error) periphyton dry weight (a, c) and chlorophyll pigment (b, d) observed on substrates according to a, b) submersion time (TIME: T<sub>15d</sub>, T<sub>30d</sub>) and c, d) substrate type (TYPE: W: wooden poles, S: fiber-glass strips, m: mosquito screen, M: garden netting). Different letters indicate statistically difference among variation source. Lower cases are linked to means represented by the bars of the bottom (AFDW, Chl *a*). Capital letters are associated with DW and Phaeo *a* means

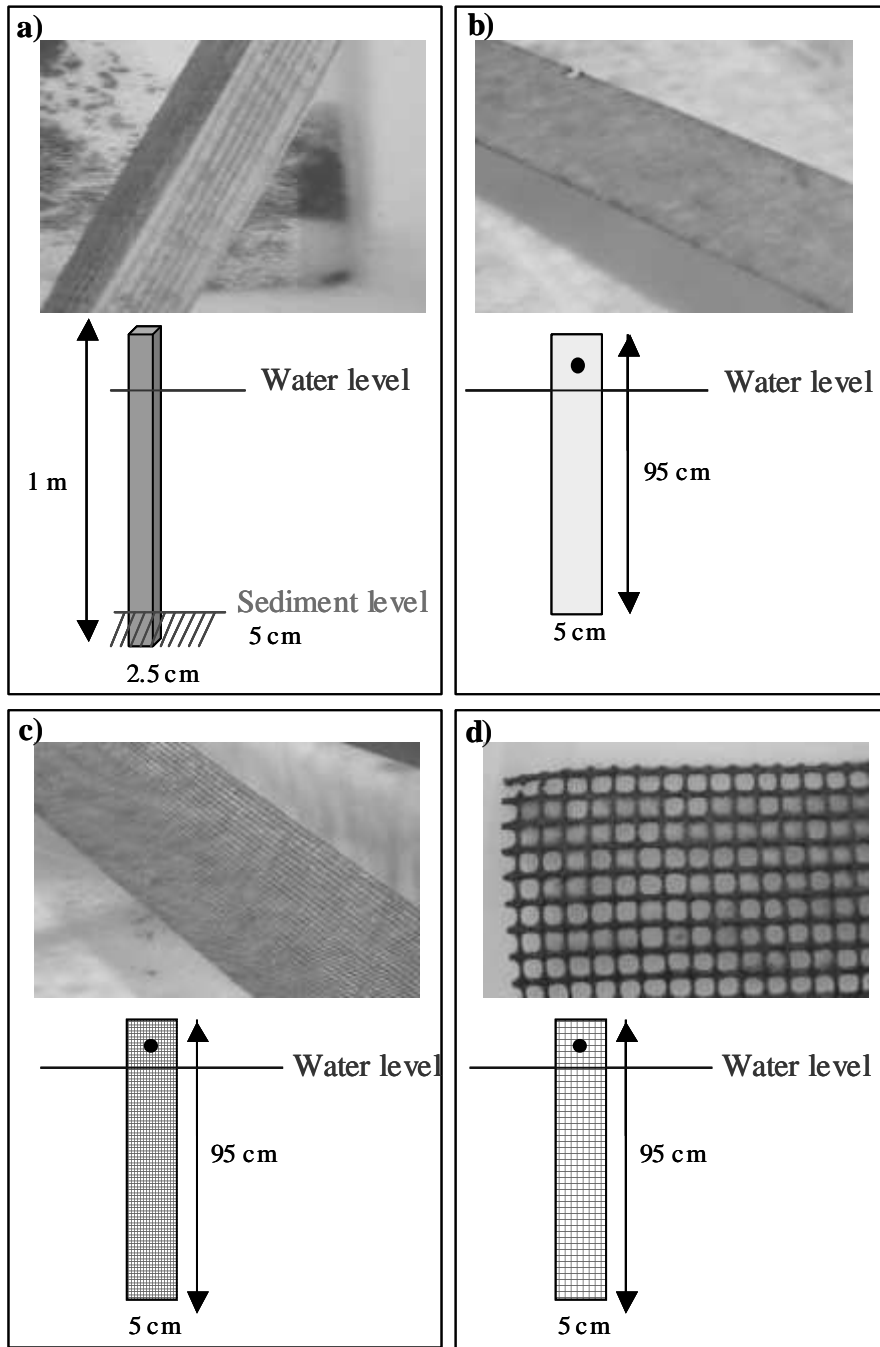
Fig. 4: Mean ( $\pm$  Standard Error) periphyton dry weight (a) and chlorophyll pigment (b) observed on substrates according to the submersion depth (DEPTH; 1: 0-15 cm; 2: 15-30 cm; 3: 30-45 cm, 4: 45-60 cm, 5: 60-75 cm). Different letters indicate statistically difference among depth. Lower cases are linked to means represented by the bars of the bottom (AFDW, Chl *a*). Capital letters are associated with DW and Phaeo *a* means

Fig. 5: Mean ( $\pm$  Standard Error) periphyton dry weight (a) and chlorophyll pigment (b) observed on substrates according to the sampling method (SAMPLING: S-5: addition of five sub-samples; T: total sample; S-3: addition of three sub-samples 0: 0-15 cm, 3: 30-45, 5: 60-75 cm). Different letters indicate statistically difference among sampling method. Normal letters are used for the T vs. S-5 comparison, whereas italic letters are used for the T vs. S-3

comparison. Lower cases are linked to means represented by the bars of the bottom (AFDW, Chl *a*). Capital letters are associated with DW and Phaeo *a* means

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Figures

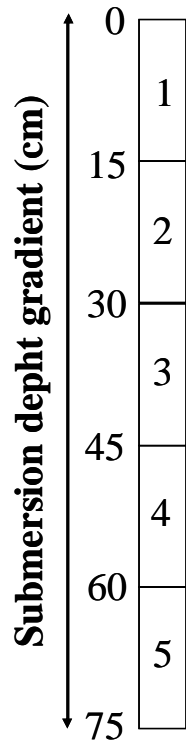


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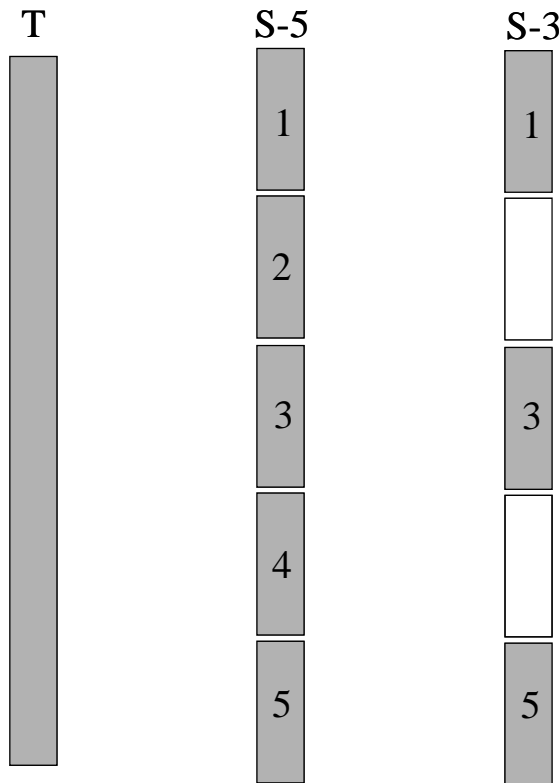
Fig. 1. Richard et al.

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a) Sub-sampling



b) Total sample 5 sub-samples 3 sub-samples



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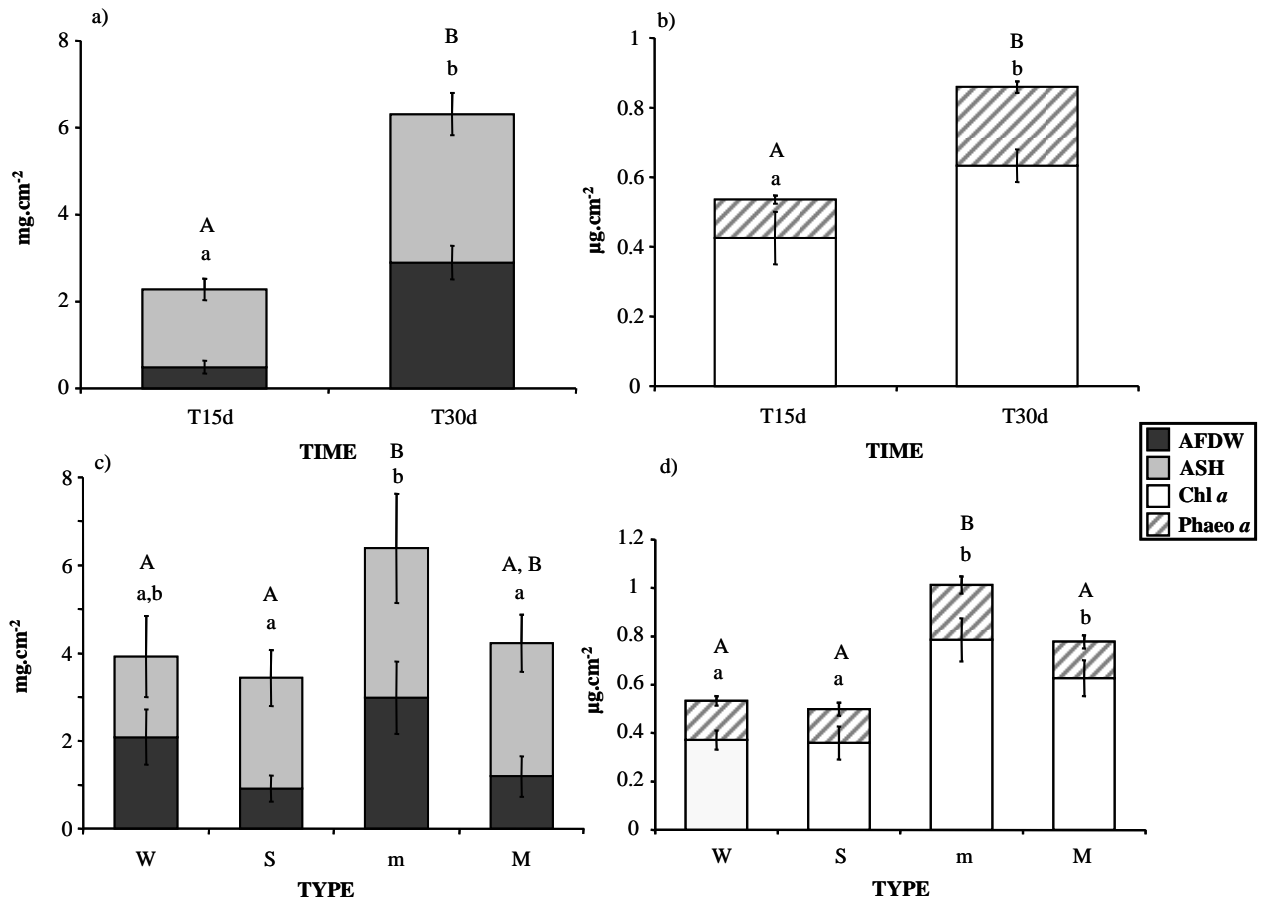
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Fig.2. Richard et al.



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Fig. 3. Richard et al.

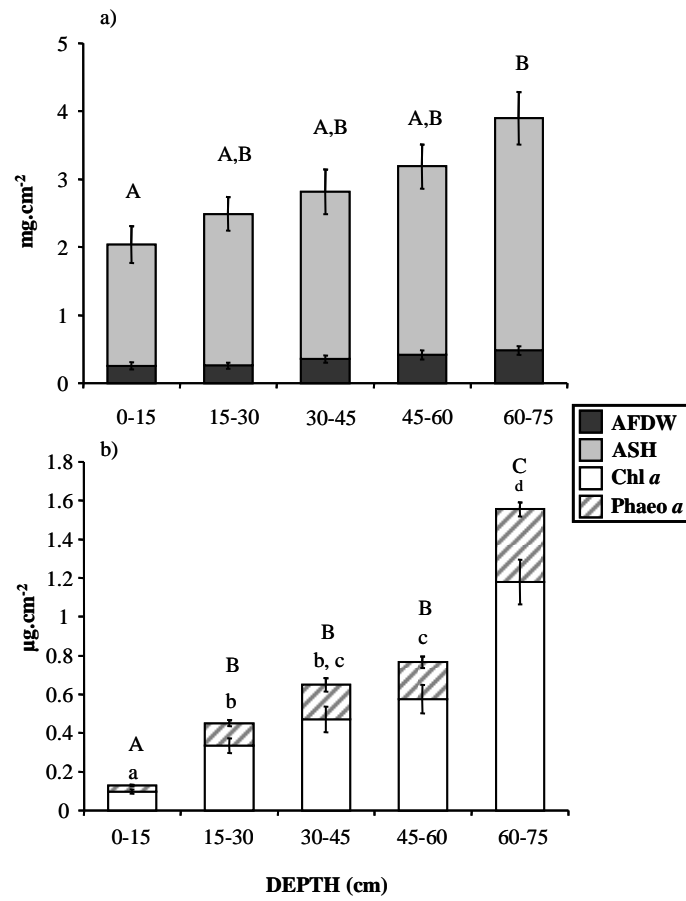
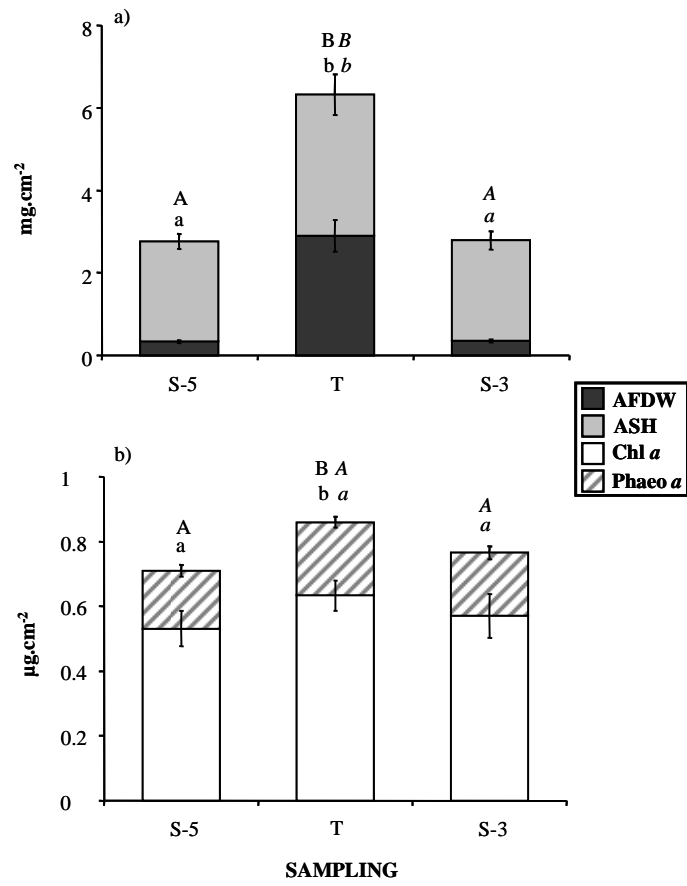


Fig. 4. Richard et al.

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Fig. 5. Richard et al.

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