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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 (1 mm-mesh) and M: garden netting (5 mm-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.

Keywords: Periphyton; Fouling; Artificial substrate; Marine pond; Aquaculture

24 **1. Introduction**

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

38 Variation of periphyton quantity and quality depends on a range of factors such as (i)
39 submersion time (Azim and Aseada, 2005), (ii) substrate type (Ramesh et al., 1999;
40 Keshavanath et al., 2001; Azim et al., 2002a), and (iii) light intensity and quality (Kirk 1994;
41 Goldsborough et al., 2005). The latter is strongly influenced by the depth of the substrates
42 (Asaeda and Son 2000). Thus, Azim et al. (2001a, 2003b) waited minimum 2 weeks to allow
43 periphyton to develop on the substrates before stocking fishes. Keshavanath et al. (2001)
44 observed that fish production based on periphyton depends on artificial substrate type and
45 preferred to use bamboo rather than PVC pipes or sugarcane bagasse bundles when culturing
46 masheer (*Tor khudree*) fingerlings. Azim et al. (2001b, 2002a, 2004a) and Keshavanath et al.
47 (2001) pooled several sub-samples of periphyton collected at equally spaced depths along
48 vertical substrates to analyse the composition of periphyton. This pooled sample was

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

52 The potential contributions of semi-extensive aquaculture to environmental protection
53 and restoration of coastal areas have been clearly recognised within EU policy. The
54 SEACASE program (Sustainable extensive and semi-extensive coastal aquaculture system in
55 Southern Europe) was started in 2007 to develop sustainable extensive and semi-extensive
56 coastal aquaculture systems in Southern Europe (Conceição et al., 2007). The present
57 SEACASE study is a preliminary assessment of the feasibility to grow periphyton on artificial
58 substrates in temperate marine ponds. The effects of submersion time, substrate type, water
59 depth, and total or partial sampling methods, on the quantity and quality of periphyton
60 collected, were evaluated. The goals of this study were to identify (i) the best periphyton
61 substrate type and (ii) a methodology of periphyton sampling for further studies on
62 periphyton-based marine aquaculture.
63

64 **2. Materials and methods**

65

66 *2. 1. Experimental site and design*

67

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

74 whereas it was 66.3 ± 4.6 cm after 30 submersion days. The mean submerged surface area (±
75 S.E.) was 713 ± 9.3 cm² and equal for each substrate type. Eleven poles or strips (called units)
76 of each substrate type were deployed in the marine pond. The units were put 20 cm apart from
77 the closest other units in 4 parallel rows with 11 units each within a 1.0 m x 2.4 m plot,
78 randomly assigning the different unit types to the available locations. The different strip types
79 were suspended in the water column from iron bars fixed on a horizontal wooden frame
80 standing slightly above the surface on poles driven in the bottom, while the pole units were
81 standing in the sediment, under the iron bar.
82
83 *Total sampling: Influence of substrate type and submersion time*
84
85 On sampling days, four units of each substrate type were randomly collected. All the
86 periphyton on each unit was collected. Sampling was done 15 (23 May 2007; T_{15d}) and 30
87 days after submersion (4-5 June 2007; T_{30d}). Collected units were not placed back. In total, 32
88 units were collected (4 units/type/date x 4 types x 2 dates).
89
90 *Sub-sampling: Influence of substrate type and submersion depth*
91
92 At the end of the experiment (T_{30d}), the remaining 3 units of each substrate type (W, S,
93 m, M) were sampled in a random order. The submerged area of each unit was divided in five
94 15-cm-segments starting from the bottom (Fig. 2a: 1: 0-15 cm, 2: 15-30 cm, 3: 30-45 cm, 4:
95 45-60 cm, 5: 60-75 cm). Each 15-cm sub-sample (i.e. 1 to 5; Fig. 2a) was completely cleaned.
96 The order of the segment cleaning was randomly assigned for each unit. Each sub-sample was
97 next separately stored. In total, 60 samples were collected (3 units/type x 4 types x 5 sub-
98 samples/unit).

99

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

101

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

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

108 These data were compared with the results of the whole unit samples (T) collected on the
109 same day (30d). 28 data were thus used for each set of comparison ((4 units/types x 4 types) +
110 (S-3 or S-5 sampling method 3 units/type * 4 types)).
111 Three units (one W, m and M) were incorrectly treated and could not be included in the data
112 set. It explains why the total degree of freedom was lower than expected (Tables 1 through 3).

113

114 *2.2. Sampling and storage*

115

116 At T_{15d} and T_{30d}, water temperature (°C), salinity, pH were measured with a multi-
117 parameter probe (HI9828 HANNA) at the water top 15 cm of three sites in the pond, at 5:00
118 PM. Mean water temperature, salinity and pH (± SE) were 24.4 ± 0.76 °C, 32.2 ± 0.14 ppt and
119 8.1 ± 0.07 at T_{15d} vs. 26.3 ± 0.93 °C, 32.9 ± 0.14 ppt and 8.2 ± 0.03 at T_{30d}. Mean oxygen
120 concentration (± SE) was at 6.4 ± 0.1 $mg.L^{-1}$ (92.8 ± 2.2 %) T_{15d} and 6.9 ± 0.1 $mg.L^{-1}$ (102.7
121 ± 2.7%) at T_{30d}. The water samples were collected immediately after the probe recording.
122 Means of suspended matter (± SE) and particulate organic matter were 13.7 ± 1.04 $mg.L^{-1}$ and
123 1.9 ± 0.2 $mg.L^{-1}$, respectively, at T_{15d}. The suspended matter was composed of 85.9 ± 0.4 % of

124 inorganic matter. At T_{30d}, mean Chl *a* was 5.3 ± 0.3 μg.L^{-1}. Chlorophyll pigments included
125 15.2 ± 1.2 percent of Phaeophytin *a*.
126
127 *Periphyton*
128
129 The order and the location of collected units were randomly assigned. Each unit was
130 sampled by putting a PVC tube (diameter of 6 cm x 110 cm of length) over it and closing it
131 with a 100 µm-meshed stopper to avoid periphyton loss. The length of the submerged part of
132 the collected substrate was measured in order to calculate the exact substrate area with
133 periphyton (cm²). Each unit was carefully and completely cleaned with fingers and a
134 toothbrush into a plastic flask with a fixed volume of 0.7 µm-filtered sea-water (200 ml for
135 total unit samples and 40 ml for 15-cm samples). All material from the inner part of the net of
136 meshed substrates was removed. Each sample was next sub-sampled using a Motoda box-
137 splitter (Motoda, 1959): 1/8 part was stored in a dark box at - 20°C for Chl*a* analysis, 7/16
138 parts were stored with 4% formalin for taxonomic analysis, and 7/16 parts were used for
139 periphyton weight analyses, putting it directly in pre-weighted box at 60°C.
140
141 *2.3. Sample analyses*
142
143 *Dry weight and Ash free dry weight*
144
145 Periphyton samples were dried at 60°C for 72h, weighed (DW: dry weight), and burned for 4h
146 at 450°C to calculate the ash-free dry weight (AFDW; Byers et al., 1978). DW, AFDW and
147 the weight of ash (ASH) were measured to the nearest 10^{-5} g with an AE240 Mettler Toledo
148 Balance. As filtered sea-water was used to clean units, "salt correction" was applied on

149 periphyton weight. The effects of added filtered seawater (7/16 of 200ml or 40 ml according
150 to the type of sampling) on DW, ASH and AFDW of periphyton were determined considering
151 the salinity of the cleaning water and the corresponding calibration curves (DW ($g.L^{-1}$) = 1.17
152 Salinity (R^2=0.99), ASH ($g.L^{-1}$) = 0.94 Salinity (R^2=0.99), and AFDW ($g.L^{-1}$) = 0.23 Salinity
153 (R^2=0.96). These equations were established using based on DW, AFDW and ASH content of
154 three replicates of 0.7 µm-filtered water in which the salinity was either 0, 10.7, 20.4, 28,
155 28.7, 36.37, 36.42, 38.03, 38.12, 40.17 or 40.38 ppt. Sea-water (28 to 40) was collected in
156 marine ponds. Water in which the salinity ranged from 10 to 20 corresponded to diluted sea
157 water by Milli-Q water (0 ppt).
158 Values were reported to the total sample volume (200 or 40 ml) and to the total length of the
159 unit. DW, ASH and AFDW were thus expressed in $mg.cm^{-2}$.
160
161 *Chlorophyll a and Phaeophytin a*
162
163 Chlorophyll *a* (Chl *a*) and phaeophytin *a* (Phaeo *a*) observed in periphyton were
164 determined with a Turner TD 700 fluorometer after 12 hours of acetone extraction at 4°C in
165 the dark without and with acidification. Nine ml of 100% acetone were added to 1 ml-
166 periphyton as could performed Azim's team (M.C.J. Verdegem, Pers. Com.). Chl *a* and Phaeo
167 *a* data were reported to the total sample volume (200 or 40 ml) and to the total length of the
168 unit. Values were expressed in $\mu g.cm^{-2}$ for periphyton. The ratio of phaeophytin vs. sum of
169 chlorophyll pigments was also calculated as (Phaeo *a*).(Phaeo *a* + Chl *a*)$^{-1}$ and expressed in %
170 (% Phaeo *a*). The autotrophic index (AI) was calculated as: AFDW ($mg.cm^{-2}$)/ Chl *a* ($\mu g.cm^{-2}$
171) * 1000 µg/mg (APHA 1992).
172
173 *2.4. Statistical analyses*

174

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

183

184 **3. Results**

185

186 *3.1. Total sampling: Influence of submersion time and substrate type*

187

188 According to the ANOVA results (Table 1), dry weight, ash free dry weight,
189 chlorophyll *a* and phaeophytin *a* varied significantly among submersion time (TIME; Table
190 1). Means were greater at T_{30d} than at T_{15d}. Mean AFDW and Phaeo *a* were more than twice
191 higher at T_{30d} than at T_{15d} (DW: 6.3 $mg.cm^{-2}$ vs. 2.3 $mg.cm^{-2}$ and Phaeo *a*: 0.2 $\mu g.cm^{-2}$ vs. 0.1
192 $\mu g.cm^{-2}$; Fig. 3a, b).
193 Substrate type (TYPE) significantly affected the amount of periphyton collected in
194 terms of DW, AFDW, Chl *a* and Phaeo *a* (Table 1). Tukey HSD tests revealed that means of
195 DW, AFDW, Chl *a* and Phaeo *a* were larger on mosquito screen (m) that on the other
196 substrate types (M, S or W; Fig. 3c, d). Mean periphyton DW and total chlorophyll pigment
197 varied between 3.4 and 6.4 $mg.cm^{-2}$ (Fig. 3c) and between 0.5 and 1 $\mu g.cm^{-2}$, respectively,
198 among substrate types (Fig. 3d).

199 The interaction of both factors (TYPE x TIME) was a significant variation source of %
200 Phaeo *a* (Table 1). Relatively more Phaeo *a* was present on mosquito screen at T_{30d} (m-T_{30d}:
201 27.7%) than at T_{15d} (m-T_{15d}: 16.7%). In contrast, mean % Phaeo *a* did not significantly differ
202 over time on wooden poles (W), fiber-glass (S) and garden netting (M). Means (± SE) were
203 respectively 30.2 ± 0.9 %; 28.2 ± 1.1 % and 18.7 ± 1.6 %. At T_{15d}, a higher % Phaeo *a* was
204 observed on smooth substrates (W, S) than on meshed substrates (m, M). At T_{30d}, the %
205 Phaeo *a* observed on wooden poles (W) was higher than on garden netting (M).
206 The autotrophic index was significantly different for the factors TYPE and TIME, and
207 showed a significant interaction (Table 1). The mean AI observed on wooden poles (W) was
208 more than 6 times lower at T_{15d} (1554 ± 410) than at T_{30d} (9449 ± 1479). In contrast, the mean
209 AI observed on the other substrates (S, m, M) did not vary over time.
210
211 *3.2. Sub-sampling: Influence of substrate type and submersion depth*
212
213 Chl *a*, % Phaeo *a* and the AI were significantly different among substrate type (Table
214 2). HSD tests showed that at T_{30d}, the Chl *a* mean was greater on meshed substrates and
215 fiberglass than on wooden poles (m, M, S: 0.6 ± 0.12 $\mu g.cm^{-2}$ > W: 0.26 ± 0.08 $\mu g.cm^{-2}$). At
216 T_{30d}, % Phaeo *a* varied such as W > S, M ≥ M, m. The AI mean was almost three times higher
217 on wooden poles (W: 2815 ± 816) than on the other substrates (S, M, m: 939 ± 299).
218 Periphyton DW, Chl *a* and Phaeo *a* significantly changed with depth (DEPTH; Table
219 2). More periphyton was collected at 60-75 cm depth than at 0-15 cm depth (Fig. 4).
220 Respectively 2 and 12 times more DW and total chlorophyll *a* was collected in the bottom 15
221 cm than at the top 15 cm. Mean differences between sampling depths of DW and Phaeo *a*
222 were not statistically significant whereas means seemed to increase between 15 and 60 cm
223 depth. In contrast, Chl *a* increased gradually with depth (Fig. 4b). The mean AI (± SE) was

224 more than three times larger in the top 15 cm (3406 ± 1002) than between 15 and 60 cm (900
225 ± 260). The % Phaeo *a* did not vary with depth whatever the type substrate (Table 2) and was
226 25.7 ± 2.05%.

227

228 *3.3. Total vs. sub-sampling: comparison of both sampling methods*

229

230 *Five sub-sampling*

231

232 The mean DW, AFDW, Chl *a* and Phaeo *a* differed significantly between sampling methods
233 (SAMPLING (S-5 vs. T); Table 3). Means were higher with the total sampling (T) than the S-
234 5 sub-sampling method whatever the substrate type (Fig. 5a, b). It was particularly right for
235 periphyton quantity rather than quality. DW and AFDW determined through S-5 sampling
236 were 2 and 8 times, respectively lower than T means (Fig. 5a) whereas the mean of
237 chlorophyll pigment obtained with S-5 sampling corresponded to 82.6% of means obtained
238 with total sampling T (Fig. 5b).

239

240 *Three sub-sampling*

241

242 Sampling was a significant source of variation for DW and AFDW (SAMPLING (S-3
243 vs. T); Table 3). More DW and AFDW were measured with total sampling (T) than with the
244 S-3 sub-sampling method (Fig. 5a). As S-5 means, S-3 means of DW and AFDW were 2 and
245 8 times, respectively, lower than the T means (Fig. 5a). In contrast, Chl *a* and Phaeo *a* means
246 did not significantly differ between S-3 and T (Fig. 5b).

247

248 **4. Discussion**

249

250 *4.1. Marine periphyton and its variation sources*

251

252 *Marine periphyton*

253 A thin mat of matter was observed on all the immersed surface of the different types of
254 substrate after 15 days of submersion. The inorganic fraction of periphyton (ASH) could
255 originate from trapping of suspended inorganic particles. The latter would be favoured during
256 resuspension caused by wind driven turbulence or people working around units during
257 sampling. The organic matter (AFDW) fraction originated from the accumulation of detritus,
258 bacteria, fungi, flora and fauna on substrates. The presence of photosynthetic pigments (Chl *a*
259 and Phaeo *a*) could indicate flora colonization of artificial substrates. The presence of
260 phaeophytin *a* indicated that the flora observed was partly degraded (15 to 30 %). The mean
261 autotrophic index ranged between 250 (60-75 cm section of mosquito screen at T30d) and
262 9450 (wooden poles at T_{30d}). These high values indicate that the periphyton contained mainly
263 heterotrophic organisms and dead organic matter, as specified by Huchette et al. (2000) for an
264 AI above 200. *In situ* observations showed that periphyton was also composed of detritus and
265 small-sized organisms as harpacticoid copepods (Richard et al., unpublished data).

266

267 *Submersion time*

268

269 A significant increase of periphyton DW, AFDW and photosynthetic pigments was
270 shown on all substrate types. According to periphyton colonization models (Hoagland et al.,
271 1982; Steinman, 1996), AFDW and Chl *a* levels increase exponentially until a biomass peak.
272 Organisms at the base of the biofilm become light and nutrient limited, eventually die and
273 detach from the substrate (Hansson et al., 1992; Asaeda et al., 2000; Keshavanath et al.,

274 2001a; Azim and Aseada, 2005). In this investigation, periphyton was still in its accretion
275 phase on day 30 on all substrate types. As noted Eding et al. (2006), biofilm establishment
276 seems to be slower in marine than in freshwater. It would be better to wait a minimum of 4
277 weeks rather than 2 as Azim et al. (2001a, 2003b) did in freshwater, before introducing fish in
278 marine periphyton-based ponds.

279

280 *Substrate type*

281

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

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

299 The substrate type could also influence the nature of the heterotrophic associated community.
300 Richard et al. (2007) observed that mesh substrates, as aquaculture pens, offered appropriate
301 structures for infauna, as *Corophium* sp. whereas newly submerged smooth substrate,
302 favoured epifauna recruitment. In this study, some polychaete tubes were observed on
303 mosquito screen, but not on smooth substrates. The results of this study indicate that more and
304 qualitatively better periphyton grew on mosquito screen than on the other substrates.
305
306 *Submersion depth*
307
308 The light intensity and its spectral composition change with depth, influencing the
309 quality and type of flora (Boston and Hill, 1991; Hansson, 1992; Kirk, 1994), as periphyton
310 (Goldsborough et al., 2005). In contrast to the observations of Azim et al. (2002a), periphyton
311 DW and chlorophyll pigments increased with depth in this investigation. A decrease of the 10
312 cm-water level at T_{30d} could explain why less periphyton was collected on the 0-15 cm part of
313 substrates than on the deeper parts. Nevertheless, the lower chlorophyll pigment concentration
314 observed on the 15-60 cm part of substrates compared to the deeper part (60-75 cm) could be
315 due to a photo-inhibition processes, as Hansson (1992) suggested when periphyton Chl *a* was
316 negatively correlated with light. Unfortunately, light incidence was not measured during this
317 study.
318 Maximal periphyton biomass could be observed where the combination of light and nutrient
319 are optimal (Hansson et al., 2002). In this way, periphyton observed on the deeper part of
320 substrates could have the advantage over the one observed on the surface part by benefiting
321 from nutrient released at the water-sediment interface. Moreover it could benefit from
322 trapping suspended sediment and microphytobenthos present at the bottom of the pond.
323

324 *4.2. Comparisons*

325

326 *Sampling method*

327

328 The sum of 3 samples taken between 0-15 cm, 30-45 cm and 60-75 cm (S-3 method)
329 led to comparable Chl *a* and Phaeo *a* means with the ones obtained with total sampling (T).
330 That was not the case with S-5 method. Nevertheless, the DW and AFDW of the periphyton
331 collected with both sub-sampling methods (S-5 and S-3) were significantly lower than with
332 total sampling. The sub-sampling, especially the one of mesh substrates, necessitated extra
333 handling for cutting before periphyton collection. Each handling event results in losses,
334 making both the S-5 and S-3 methods less accurate than whole unit sampling. Total sampling
335 was easier and more periphyton was collected. In further studies, the total sampling method
336 will be preferred to sub-sampling one.
337 In this investigation, the use of 200 ml of filtered salt-water for unit cleaning induced
338 over-estimation of periphyton weight. The DW, the ASH and the AFDW added when
339 cleaning 750 cm²-periphyton substrate with 200 ml of 0.7 µm seawater of 32.55 ppt were
340 respectively 10.1, 8.1 and 2 $mg.cm^{-2}$. These values are very important compared to the real
341 periphyton weight (Table 4), especially for DW and ASH. Without the salt correction, ASH
342 would be more than 6 times greater than the real values (with correction) at T_{15d} and 3 times at
343 T_{30d}. Analysis of three blanks of cleaning water should be envisaged at each sampling date in
344 subsequent studies. To avoid the salt correction, the use of milliQ water could be envisaged in
345 case where the determination of periphyton weight would be the only analysis to carry out on
346 the sampled unit. The periphyton fauna and flora could be analysed from other units cleaned
347 with filtered seawater to avoid osmotic shock of the living cells.

348

349 *Periphyton in other aquatic systems*

350

351 Absolute values which described the quantity and the quality of periphyton developed
352 on our substrates deployed in marine water were different with the one observed mainly in
353 freshwater by others authors (Table 4). The mean DW observed on our substrates reached 8.8
354 $mg.cm^{-2}$ on mosquito screen m at T_{30d} (Table 4). This is relatively high since 10 studies out of
355 13 found a DW < $5mg.cm^{-2}$. Maximal mean organic periphyton (AFDW) observed in this
356 study (4.5 $mg.cm^{-2}$) was greater than means observed by others authors which generally did
357 not exceed 1 $mg.cm^{-2}$ with the exception of Azim et al., 2002b (Table 4). In contrast to this
358 investigation, in most of the cited studies, periphyton substrates are simply removed from the
359 water causing probably a lot of loosely attached to be lost and could explain lower mean of
360 AFDW. The use of a tube with a stopper for substrate sampling is recommended to avoid
361 underestimation of periphyton development.
362 High autotrophic index of this investigation (Table 4) was induced by greater AFDW but also
363 by very low chlorophyll *a* concentration observed on substrates (0.4 to 0.6 $\mu g.cm^{-2}$; Fig. 3b).
364 Numerous studies observed Chl *a* levels above 10-15 $\mu g.cm^{-2}$ (Azim et al., 2001b,c, 2002a
365 2003a; Keshavanath et al., 2001; Table 4). Low periphyton concentration could originate
366 partly from the use of inert substrate (Huchette et al. 2000; Azim et al. 2003b; Liboriussen
367 and Jeppesen, 2006; This study: Table 4) rather than nutrient-leaching substrate (Azim et al.,
368 2001b, 2002a, 2002b). Nevertheless, others factor could influence the primary productivity,
369 such as temperature, light and nutrient availability (Liboriussen and Jeppesen, 2005; Vermaat
370 et al., 2005). The high densities of periphyton recorded by Azim et al. (2001b, 2002a, 2002b)
371 and Keshavanath et al. (2001) were observed in tropical ponds in Bangladesh and India with
372 more light and higher temperatures than in temperate ponds in France, in the Netherlands
373 (Azim et al., 2003b) or Denmark (Liboriussen and Jeppesen, 2006). It is the same in water

374 column where mean Chl *a* was above 200 µg.L^{-1} in Bengali fresh ponds (Azim et al. 2002b),
375 whereas it was 5 µg.L^{-1} in our temperate marine pond. Productivity in freshwater is generally
376 higher than in marine water. However, the ponds were fertilized with urea, manure, food in
377 most studies listed in Table 4 whereas our pond was not fertilized. Azim et al. (2001c, 2003a)
378 showed that periphyton biomass increased with increasing fertilization rate up to a maximum.
379 Thus, in future studies, as part of EU policy of environmental protection and restoration of
380 coastal areas, fertilized effluents of intensive farms could be used to maximise periphyton
381 production and the associated production of herbivorous fishes.
382
383 The present investigation showed that (i) periphyton biomass in a marine pond
384 increased between day 15 and day 30, (ii) more periphyton was collected on mosquito screen
385 than on wooden poles, fiberglass strips or garden netting, (iii) periphyton biomass increased
386 with water depth submersion, (iv) sub-sampling methods underestimated periphyton
387 development compared to whole unit sampling, and (v) a correction of periphyton biomass
388 must be carried out for the dissolved inorganic salts present in marine or brackish systems
389 using blank weight of cleaning salt filtered water. The use of a tube with stopper for substrate
390 sampling will reduce periphyton sampling losses. Finally, the autotrophic fraction in the
391 periphyton communities was very low compared to periphyton developed on biodegradable
392 substrates used in fish cultures in fertilized tropical ponds. Thus, pond fertilization and use of
393 biodegraded substrates (i.e. long-time submerged wood) should be envisaged in further
394 studies on periphyton-based marine aquaculture in temperate regions.
395
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397

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409

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533

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_{15d}, T_{30d}) 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*)$^{-1}$) and autotrophic index (AI: AFDW.Chl a^{-1}) 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
		log DW			**log (AFDW + 1)**			**AI**		
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		
		Chl *a*			**Phaeo *a***			**% Phaeo *a***		
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*)$^{-1}$) and autotrophic index (AI: AFDW.Chl a^{-1}) 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	
			√ DW				log (AFDW + 1)				log 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 Chl *a*				log Phaeo *a*				% 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	MS	F	P	MS	F	P	MS	F	P	MS	F	P
		log DW			**log AFDW**			**Chl *a***			**Phaeo *a***		
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^{-2})	Periphyton quality (range of mean Chl a, Phaeo µg.cm^{-2})
Boston and Hill 1991	American streams	(16 to 22°C)	**NF**	Ceramic tiles an 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 m²)	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 m²)	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 5m²)	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	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 m²)	April to September (26.4 to 31.7°C)	**CF:** * + Rice brain and mustard oil cake	Bamboo, Jutestick, Kanchi	presence	DW : 0.5 to 4.5	Chl *a*: 5 to 18
Azim et al., 2002b	Polyculture of carps in Bengali fresh ponds (75 m²)	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 m²)	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^3) in The Netherlandans	August to October (22.5°C)	**CF:** NaNO3 + 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	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 m²)	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 (25m²)	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^{-2}	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 (± Standard Error) periphyton dry weight (a, c) and chlorophyll pigment (b, d) observed on substrates according to a, b) submersion time (TIME: T_{15d}, T_{30d}) 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 (± 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 (± 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

Figures

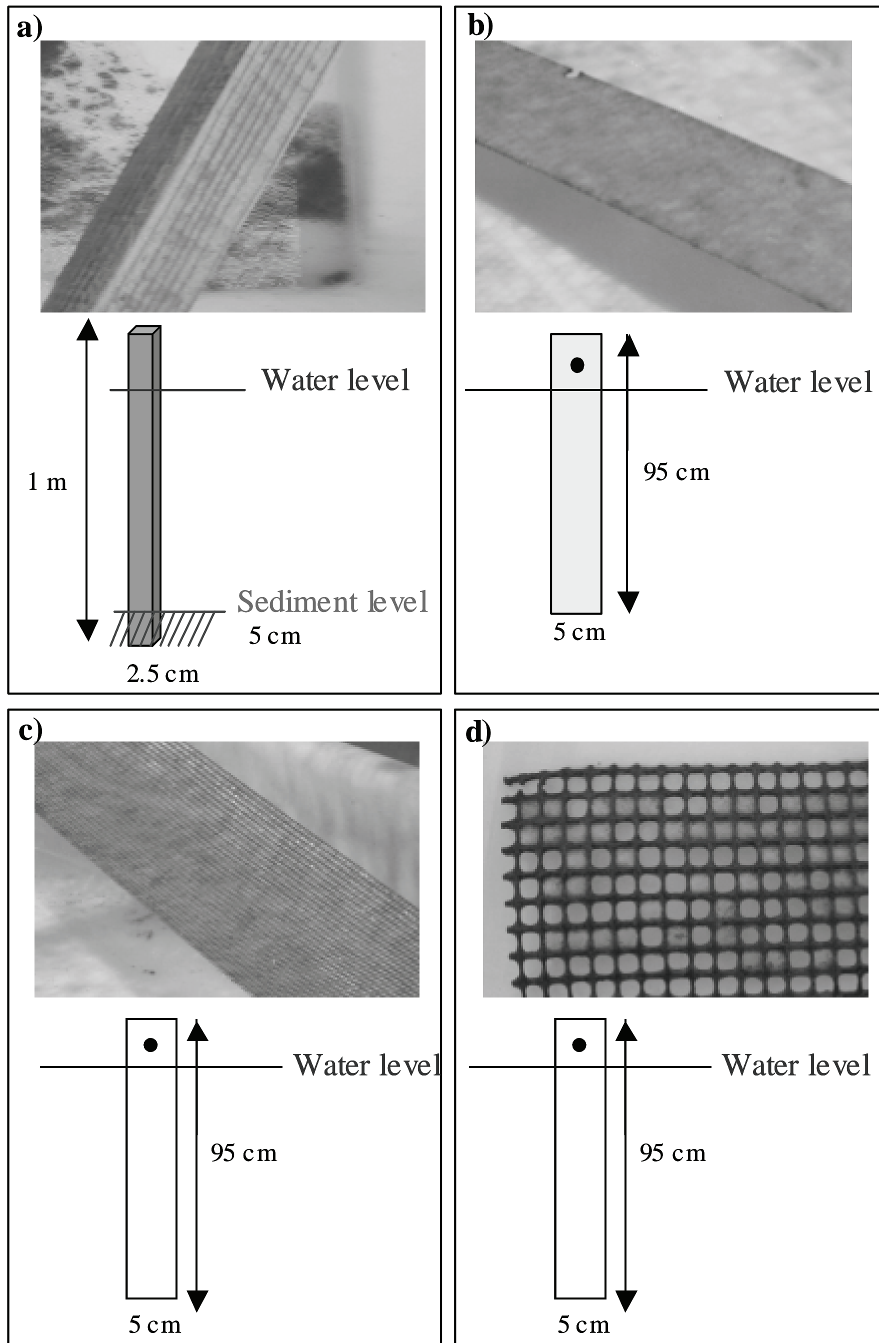
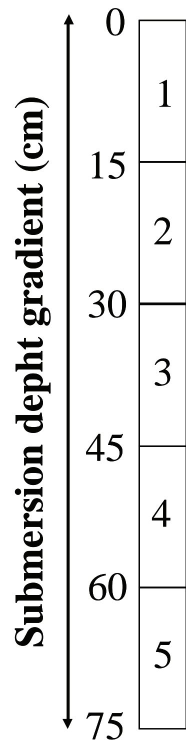


Fig. 1. Richard et al.



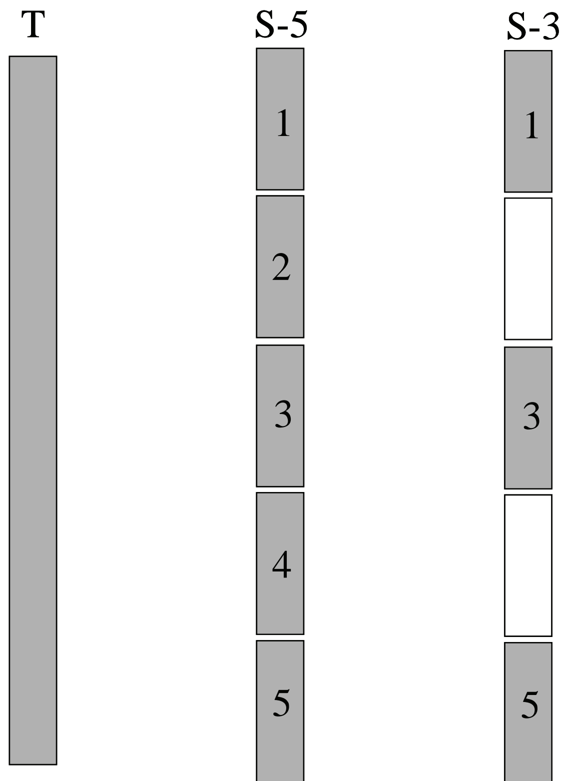
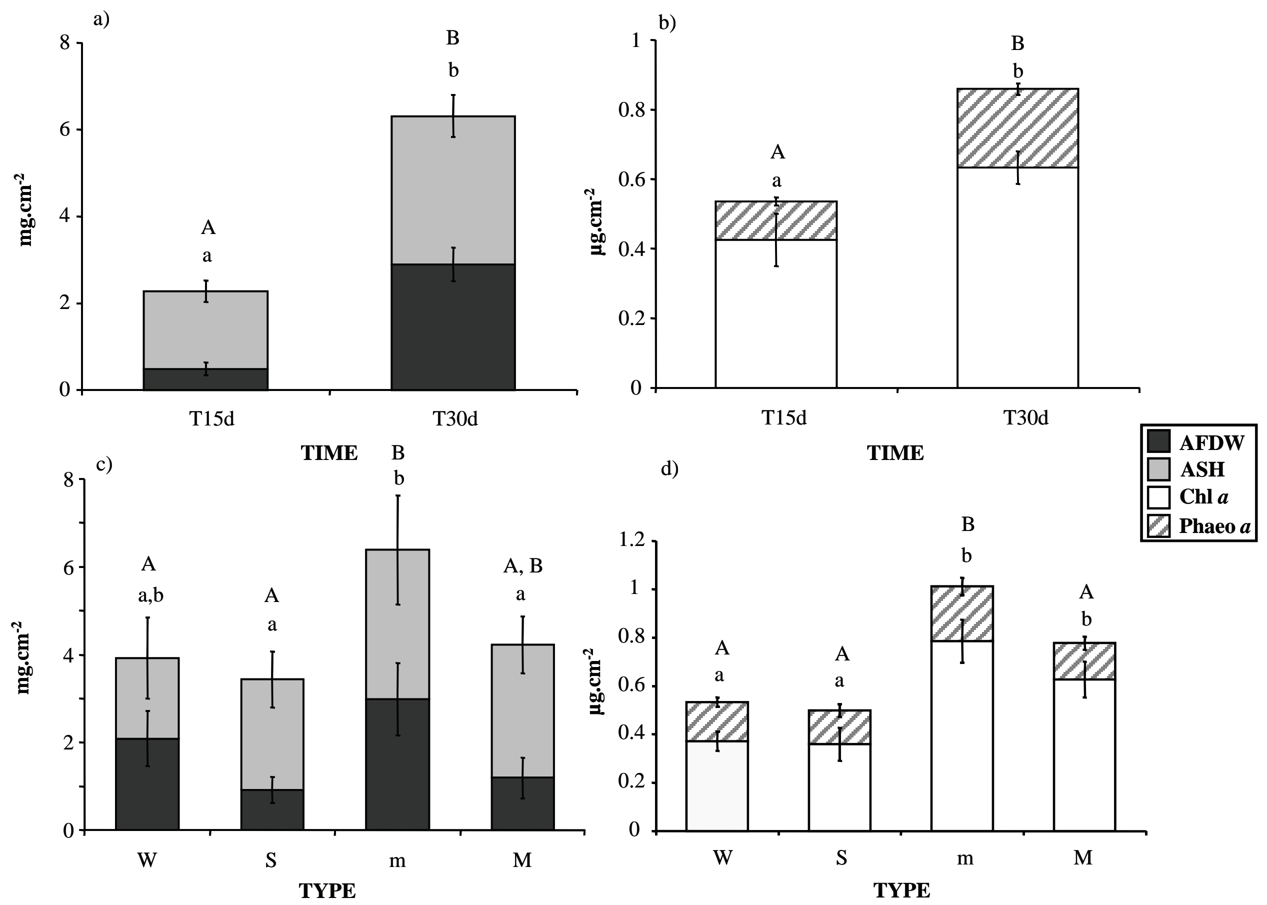


Fig.2. Richard et al.



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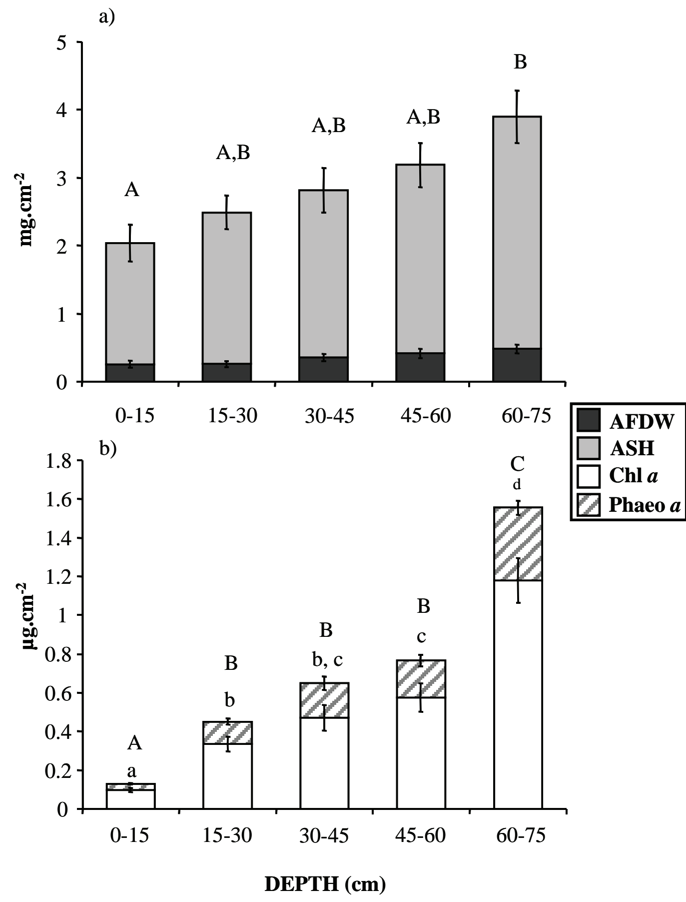

Fig. 4. Richard et al.

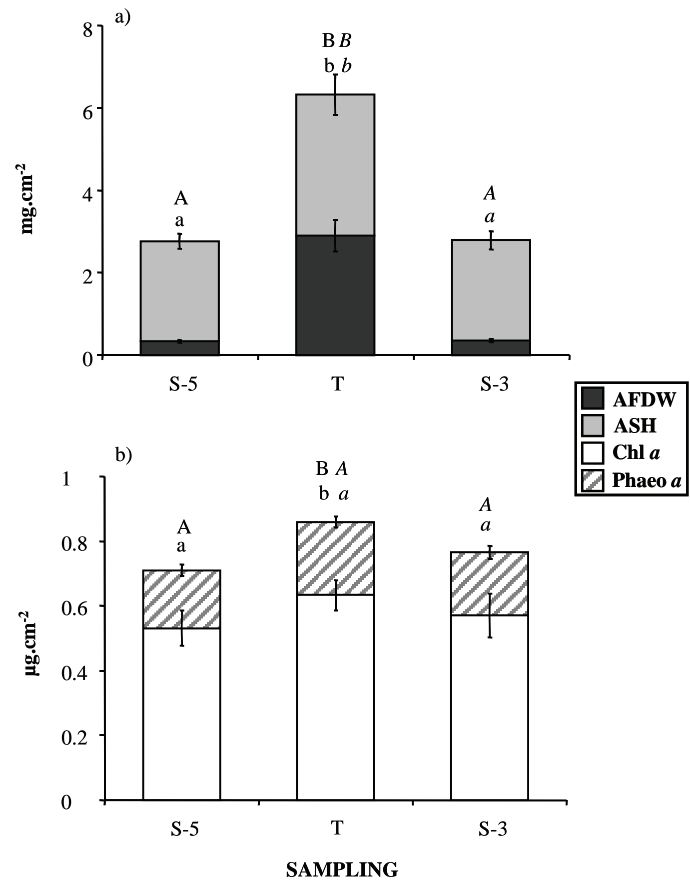

Fig. 5. Richard et al.