

## Early colonization on Artificial Seagrass Units and on *Posidonia oceanica* (L.) Delile leaves

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**ABSTRACT.** Many epiphytes grow on *Posidonia oceanica* (L.) Delile leaves but early stages of that colonization are not well known. To study this early colonization without destroying the plant, Artificial Seagrass Units (ASUs) were utilised. The first nine days of colonization by macroscopic eukaryotic organisms on natural *P. oceanica* leaves and on ASUs were studied. The capability of those ASUs to mimic *P. oceanica* in the long term was also evaluated. Indeed, early colonists of a substrate can influence the settling of later ones by “priority effects”. Thus if the pioneer community is the same on both substrates, they will more likely be the same after a longer exposure time. On both substrates, colonization began by the settling of crustose-calcareous algae and foraminiferans. The number of organisms increased more quickly on ASUs than on natural leaves but Shannon-Wiener diversity index was higher for *P. oceanica* leaves. The low colonization rate on natural leaves may have been due to different microclimatic conditions on the two substrates and to a less developed biofilm than on ASUs. The high diversity observed on natural leaves was mainly related to the presence of bryozoan ancestrulae, which were absent on ASUs. Different microhabitats on each substrate (different algae morphotypes) can explain this difference. Thus, at such an early colonization stage, pioneer communities were different on the two substrates, suggesting that later communities would be different too. However, ASUs could be used in environmental perturbation studies instead of natural leaves, thanks to their high colonization rate.

**KEY WORDS:** seagrass, artificial substrata, epiphytes, colonization, substrate preferences.

## INTRODUCTION

In the Mediterranean coastal zone, the seagrass *Posidonia oceanica* (L.) Delile forms dense meadows. The leaves of this seagrass are long (up to 130 cm) and have the longest life span among seagrasses (up to 56 weeks) (THELIN & BOUDOURESQUE, 1983; GOBERT et al., 2005). Thus, they can support an important epiphytic community that is composed of many organisms such as bacteria, diatoms, dinoflagellates, red and brown macroalgae, protists, sponges, bryozoans, hydrozoans and annelids (KERNEIS, 1960; MAZZELLA et al., 1981; CASOLA et al., 1987; MAZZELLA et al., 1989; JACQUEMART & DEMOULIN, 2006; MABROUK et al., 2011; LEPOINT et al., 2014). In total they can represent up to 40 % of the leaf biomass (GOBERT et al., 1995) and their density increases with leaf age (NOVAK, 1984). *P. oceanica* leaves grow from

a basal meristem. Youngest parts of the plant are situated at the basis of the leaves and in the centre of the shoots. They grow from the centre to the external part of the shoot, tightly packed with older leaves. Epiphytes are thus more abundant on the outer leaves of the shoots than on the inner ones and on the leaf tips than on the bases (VAN DER BEN, 1971; CASOLA et al., 1987). On the young parts of the plant, the epiphyte cover is mainly constituted of bacteria and diatoms that form a biofilm (NOVAK, 1984). Biofilms are important in marine environments because they condition the settling of organisms on immersed substrates (CRISP & RYLAND, 1960; KEOUGH & RAIMONDI, 1995; DE TROCH et al., 2005). On older parts of *P. oceanica*, macroepiphytes become dominant, even if they can be secondarily colonized by bacteria or other micro-organisms (NOVAK, 1984).

Epiphytes also contribute to the food webs associated with the *P. oceanica* ecosystems (MAZZELLA et al., 1992; LEPOINT et al., 2000; MICHEL, 2011). Their biomass, biodiversity, abundance and/or coverage are used in pollution studies because they react more quickly to environmental perturbations than does the plant itself, thanks to their rapid turnover and their high nutrient uptake rates (MORRI, 1991; PERGENT-MARTINI et al., 2005; LEPOINT et al., 2007; GOBERT et al., 2009).

The daily evolution of early epiphyte settling on *P. oceanica* leaves is not known, although it can influence subsequent epiphytic assemblages. Indeed, according to the theory of CONNELL & SLATYER (1977), the first colonists of a substrate influence later colonists by facilitating or inhibiting their settling and persistence. First colonizers are able to influence the recruitment of other organisms by creating some specific conditions in the environment or by limiting the possible interspecific interactions with newly-arriving species (BOLOGNA & HECK, 1999; IRVING et al., 2007). These phenomena are called “priority effects” (BELYEA & LANCASTER, 1999). The identification of early colonizers is thus essential to understanding the dynamics of the epiphytic community living on *P. oceanica* leaves.

Artificial Seagrass Units (ASUs) are interesting in the study of the epiphyte colonization of

seagrass leaves because they can be set up anywhere, regardless of conditions (BARBER et al., 1979; LEE et al., 2001) and they allow the control of parameters such as leaf number, shoot density, leaf length and width, as well as the surface available for colonization (BOLOGNA & HECK, 1999; TRAUTMAN & BOROWITZKA, 1999). Moreover, they represent a non-destructive method, which is particularly important when working with protected species such as *P. oceanica* (DONNARUMMA et al., 2014).

The aims of this study were to identify the first sessile macroscopic eukaryotic organisms settling on the basal parts of *P. oceanica* leaves and ASUs, day by day during nine days, and then to determine if those ASUs are able to mimic natural leaves, in order to use them instead of the natural plant to study the colonization of *P. oceanica* by epiphytes or to detect environmental perturbations. Indeed, if the pioneer community composition and abundances are the same on ASUs as on *P. oceanica*, subsequent colonists will more likely be the same on both substrates too (CONNELL & SLATYER, 1977; IRVING et al., 2007; MAGGI et al., 2011).

## MATERIALS AND METHODS

This study was realised from March 5<sup>th</sup> to March 14<sup>th</sup> 2005, at a depth of 10 m, in front

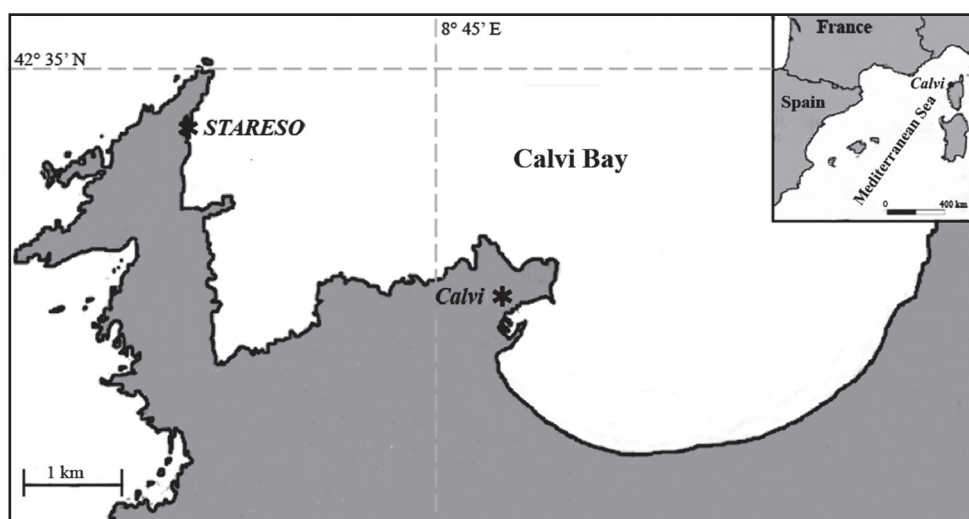


Fig. 1 – Sampling site. The sampling site is situated in front of the research station STARESO, Corsica, France, in the *P. oceanica* meadow, at a depth of 10 m.

of the STARESO research station (Fig.1, Calvi Bay, Corsica, France, 42°35' N, 8°43' E). This site is characterized by a continuous *P. oceanica* meadow where the shoot density varies from 400 to 700 shoots.m<sup>-2</sup> at a depth of 10 m (SOULLARD et al., 1994).

Every Artificial Seagrass Unit (ASU, Fig. 2) was made of a black PVC ribbon (width = 1 cm, length = 50 cm, thickness = 0.5 mm) with a float (Eppendorf tube) at the free end to maintain those ASUs in the same position as natural leaves. They were fixed on a ballasted PVC tube and the whole structure was put inside the meadow. At the same time (day 0 of the experiment), ten *P. oceanica* shoots were marked with the punching hole method (ZIEMAN & WETZEL, 1980) to assure that both substrates had been exposed to the same pool of colonists during the same period. Indeed, as *P. oceanica* leaves grow from a meristem situated in the centre of the shoot, it was assumed that the zone of the youngest marked leaf (GIRAUD, 1979) situated under the hole experienced the same exposure time as the ASUs. As the youngest parts of *P. oceanica* leaves are wrapped in the sheaths of old leaves, it was impossible to define more precisely this newly exposed zone.

Every day during nine days (after day 0), one ASU and one marked natural shoot were picked up by scuba diving. The youngest punched leaf of each natural shoot was selected (GIRAUD, 1979) and the inner and outer surfaces of the freshly exposed zone were observed (0 to 4.2 cm<sup>2</sup>) with a magnification of fifty times, to determine the density of sessile macroscopic eukaryotic organisms. To compare with natural leaves, only the basal parts (0-5 cm) of ASUs were observed.

Organisms were determined at phylum level. To compare the contribution of each phylum on both substrates, relative abundances (percentage of the total number of organisms in each sample) were calculated. Sample diversity was also estimated using Shannon-Wiener diversity index, calculated with a DIVERSE analysis in PRIMER 6 (CLARKE & WARWICK, 2001).

A preliminary study was carried out on the samples collected after four days of exposure using scanning electron microscopy (SEM), in order to illustrate some epiphytes and the development of the biofilm on every substrate. A portion of 0.5 cm<sup>2</sup> was selected at the bases of natural and artificial leaves and fixed in 4% seawater glutaraldehyde for a few days, at 4°C.

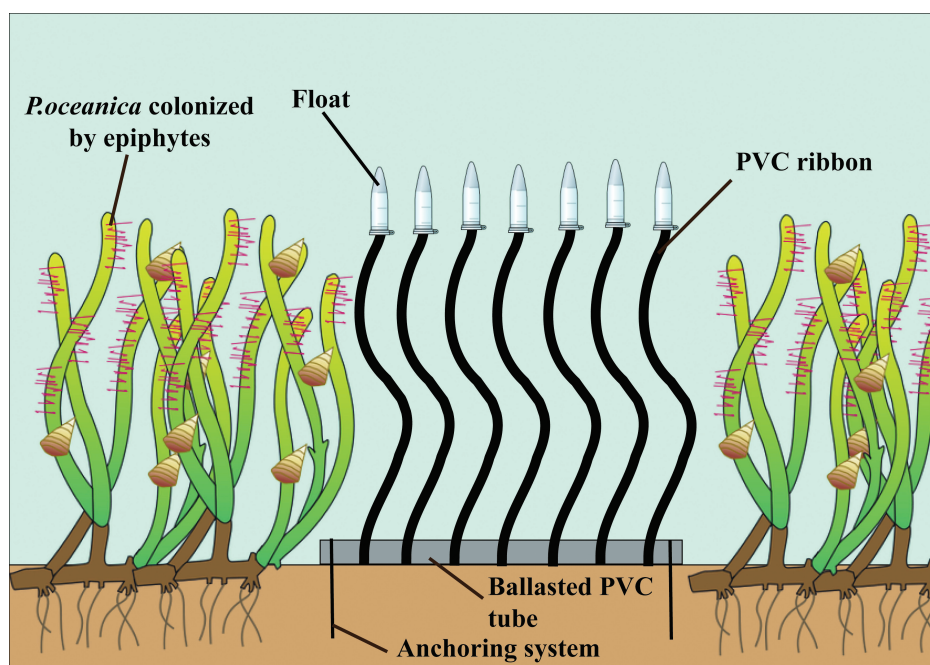


Fig. 2 – Experimental device for the Artificial Seagrass Units (symbols used with the courtesy of the Integration and Application Network, University of Maryland Center for Environmental Science ([ian.umces.edu/symbols/](http://ian.umces.edu/symbols/))).

TABLE 1

Shannon-Wiener diversity index ( $H'$ ) calculated for each sampling day from phylum abundances on ASUs and *P. oceanica* natural leaves (N.D.: no data).

| Duration of the colonization (days) | 1 | 2    | 3    | 4    | 5    | 6    | 7    | 8    | 9    |
|-------------------------------------|---|------|------|------|------|------|------|------|------|
| ASUs                                | / | 0.26 | 0.17 | 0.29 | 0.12 | 0.56 | 0.60 | N.D. | 0.68 |
| Natural leaves                      | / | 0.00 | 0.00 | 0.64 | 0.64 | N.D. | 0.67 | N.D. | 0.92 |

They were then rinsed in seawater filtered on a 0.22  $\mu\text{m}$  filter, post-fixed in 1% seawater  $\text{OsO}_4$ , rinsed again in seawater and critical point dried. They were mounted on aluminium stubs and coated with a thin layer of gold-palladium for the SEM observation. It is worth mentioning that the use of distilled water in the preparation of SEM samples of both studied substrates seemed to wash out most of the organisms. It should therefore be avoided.

RESULTS

On both substrates (Fig. 3), the first sessile macroscopic eukaryotic organisms settled after 2 days. The number of colonists increased with time but this increase was more important for

ASUs. Indeed, the colonist density was only 29 organisms per square centimetre ( $\text{org.cm}^{-2}$ ) on *P. oceanica* leaves after nine days while it reached 355  $\text{org.cm}^{-2}$  on ASUs. Except for the third sampling day, the total abundance was always 3 to 12 times higher on ASUs than on natural leaves.

Shannon-Wiener diversity index ( $H'$ , Table 1) increased throughout the experiment for natural leaves while this increase was only clear after 5 days of exposure for ASUs. From the fourth sampling day,  $H'$  was always higher for the basal parts of natural leaves than for those of ASUs.

After only 4 days (Table 1, Fig 4), sessile communities colonizing natural leaves were more diverse than those settling on ASUs. This fact is also clear when looking at phyla relative abundances. On both substrates, the observed phyla were macroalgae, ciliates, foraminiferans and bryozoans, but their relative abundances (Fig. 4) were different.

On natural leaves (Fig. 4A), the community showed a dominance of algae (20 % after 5 days to 100 % after 3 days) and foraminiferans (15 % after 7 days to 100 % after 2 days). After 7 days, bryozoans became important too (5-6 %).

On ASUs, the dominant phylum, before 6 days of exposure, was algae (Fig. 4B, between 85 % after 4 days and 97 % after 5 days). From the 6th sampling day to the end of the experiment, there was a switch between algae (39 % after 9 days) and foraminiferans (61 % after 9 days).

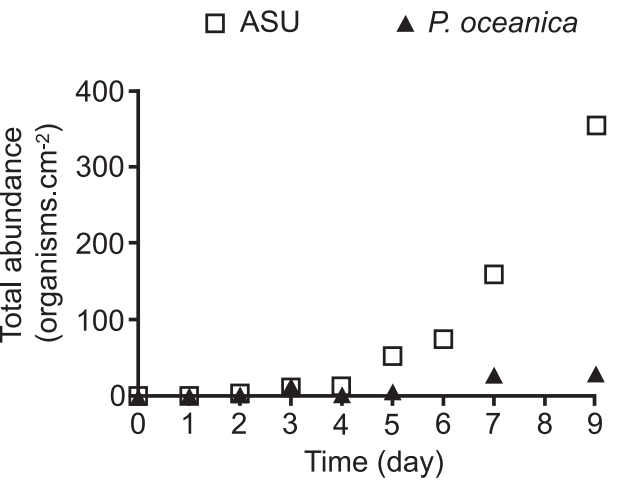


Fig. 3 – Evolution of epiphyte density ( $\text{organisms.cm}^{-2}$ ) over time on the bases of natural leaves (triangles) and ASUs (squares).



Whenever other phyla were observed, they represented less than 2 % of the total abundance on both substrates.

### DISCUSSION

Epiphyte biomass on *P. oceanica* leaves increases with leaf age and follows a sigmoid distribution (CEBRIÁN et al., 1999). The stabilization of the curve is reached when there is a balance between epiphyte growth and losses, due, for example, to grazing pressure (VAN MONTFRANS et al., 1984; BORUM, 1987; ALCOVERRO et al., 1997). In this study, the obtained curves (Fig. 3) are in the exponential part of the sigmoid curve because of the shortness of the experiment (BORUM 1987). Indeed, the exposure time here is 9 days while CEBRIÁN et al. (1999) have shown that epiphyte biomass on *P. oceanica* leaves reaches the upper part of the sigmoid distribution after 270 days.

The curve for ASUs showed a highly increasing density of organisms towards the end of the experiment, suggesting that colonization was in an active phase. This rapid increase in colonist density may be due to a “snowball effect”. Indeed, it was proven that colonization modifies the leaf surface and favours the recruitment of new colonizers (NOVAK, 1984; BOLOGNA & HECK, 1999; IRVING et al., 2007). So, in the early phase of colonization, if more organisms settle on a substrate, the colonization by new organisms is favoured.

In comparison with ASUs, the increase in epiphyte density on *P. oceanica* leaves was low, although the Shannon-Wiener diversity index was higher. These observations are in accordance with those of other authors comparing ASUs with aquatic macrophytes (CATTANEO & KALFF, 1978; NOVAK, 1984; EDGAR, 1991). The difference between both types of substrates can be explained by the existence of a longer lag phase before active epiphyte colonization for *P. oceanica* than for ASUs. This lag phase is the period needed by a substrate to be ready to be colonized. It depends on bacterial communities living on the leaves and on the maturity of the biofilm, which grows importantly during the early stages of colonization (KEVERN et al., 1966; NOVAK, 1984; KEOUGH & RAIMONDI, 1995, 1996; DAHMS et al., 2004). SEM observations of samples of each substrate, after 4 days of exposure, confirmed that a more complex biofilm was present on ASUs than on natural leaves (Fig. 5A–B).

Another reason for a lower colonization rate of natural leaves in comparison with ASUs is the production of secondary metabolites, such as phenolic compounds, by the plant. Those chemicals can influence the biofilm and the epiphytic cover that settle on seagrasses by acting as an antifouling agent or by selecting species adapted to resist those compounds (HARRISON & CHAN, 1980; NOVAK, 1984; JENSEN et al., 1998). For *P. oceanica*, the production of phenolic compounds presents a seasonal pattern, depends on environmental stress (competition, sewage...) and is higher in intermediate leaves than in adult

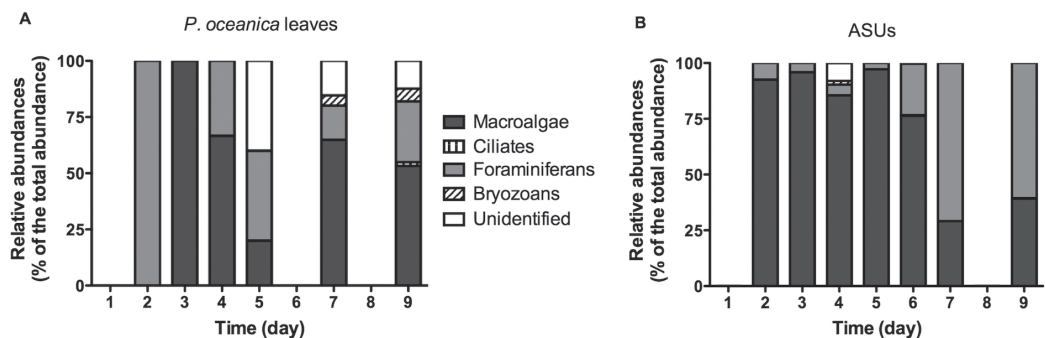


Fig. 4 – Evolution of relative abundances of the observed phyla (% of the total abundance) over time for natural leaves (A) and ASUs (B). Unidentified: phyla unidentified at the used magnification level.

ones (CUNY et al., 1995; AGOSTINI et al., 1998; DUMAY et al., 2004). As this study focused on the bases of intermediate *P. oceanica* leaves, the high production of phenolic compounds in those leaves could have influenced the epiphytic cover, either directly or by modifying the biofilm composition.

The difference in microclimatic conditions on the surface of the two substrates could be responsible for the observed differences too. Natural leaves are slightly curved, so the water circulation and the light conditions on their surfaces are probably different to those existing on the completely flat ASUs. The dominant bryozoan on *P. oceanica* leaves, an obligate epiphyte of this plant, *Electra posidoniae*, is

almost entirely restricted to the plant internal curved face (MATRICARDI et al., 1991). So, the scarcity of bryozoans on ASUs and particularly of *E. posidoniae*, also observed by GAMBI et al. (2011) and DONNARUMMA et al. (2014), is probably due to the flatness of ASUs as well as to the absence of the chemicals cited above.

Different microclimatic conditions can also be created on a substrate by the existence of different microhabitat structures that can influence the colonization by micro- or macro-organisms (NOVAK, 1984; GARTNER et al., 2013). According to CASOLA et al. (1987), after the biofilm development, the epiphytic community succession on *P. oceanica* leaves is characterized by, at first crustose-calcareous

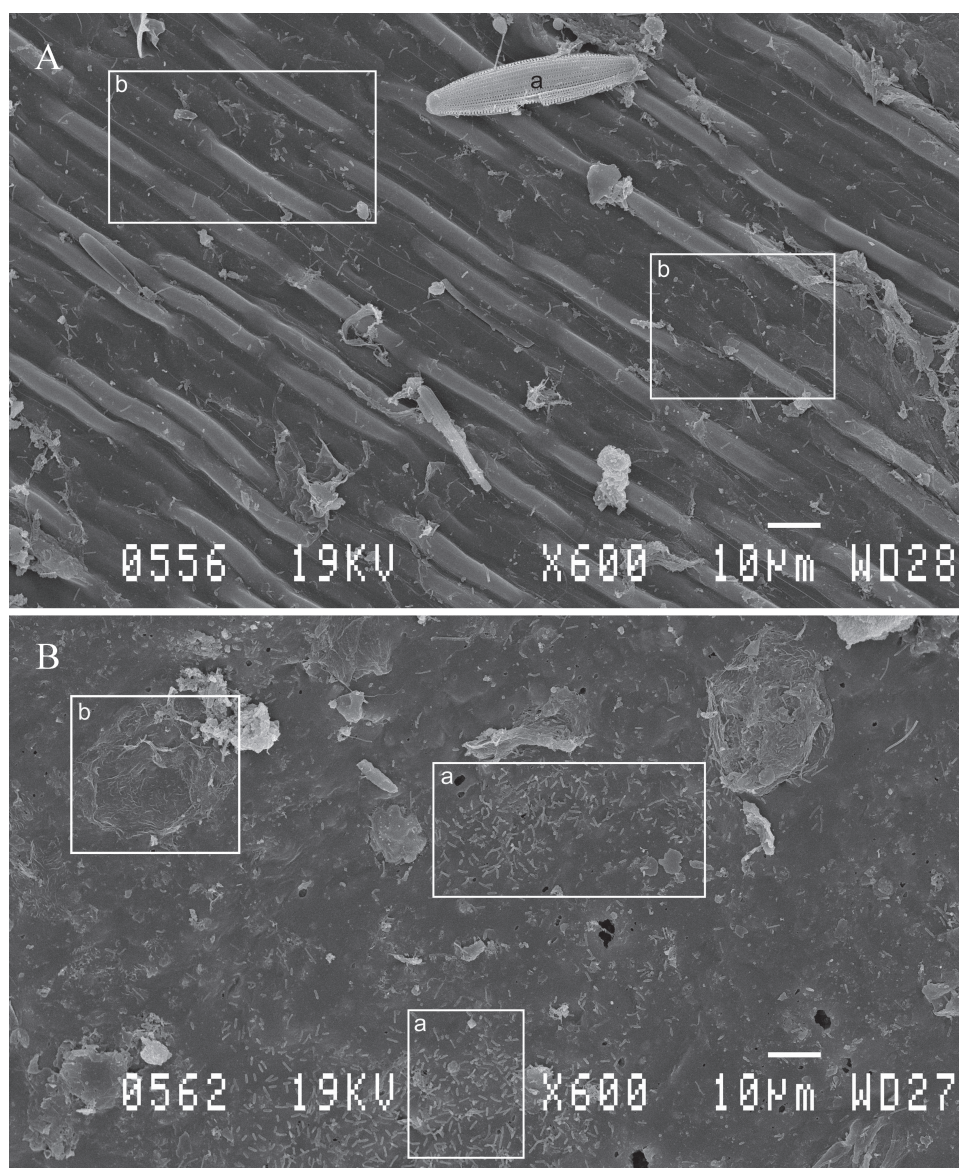


Fig. 5 – SEM pictures (magnification: 600x) of the basal portions of the *P. oceanica* leaf (A) and the ASU (B), showing the difference in microhabitat structure between the two substrates after 4 days of exposure. A. a = diatom, b = bacterial biofilm in formation. B. a = developed bacterial biofilm, b = biofilm agglutinated by mucus.



forms (foraminiferans, bryozoans, crustose-calcareous algae), then crustose-noncalcareous forms and finally erect forms. So the community goes from a simple habitat structure (i. e. mono-layered biofilm) to a more complex one (i. e. multi-layered structure). Here, the morphology of the algal community was different between the two substrates (Fig. 6). On natural leaves, the classical succession was observed. Crustose-noncalcareous algae became important after 7 days of exposure and some filamentous and erect algae were observed too. On ASUs, crustose-calcareous algae were dominant throughout the exposure period. Thus, as the microhabitat structures and the microclimatic conditions were different on both substrates, the observed communities were different too.

The dominance of crustose-calcareous algae and the scarcity of sessile fauna on artificial *Posidonia* leaves have also been observed by MICHEL (2011) and DONNARUMMA et al. (2014), after respectively 76 days and three months of *in situ* exposure. This suggests that the community structure that is observed here on ASUs is already representative of the later communities. That also underlines the role of microhabitat structure and microclimatic conditions in the establishment of colonists on immersed substrates.

Leaf movements can also influence algal epiphyte biomass (LAVERY et al., 2007). Here, every ASU was formed of a single PVC blade ending with a float. Thus, each ASU probably moved more in the water column than would a natural leaf, especially an intermediate one,

which is tightly packed with the other leaves of the seagrass shoot. However, the high density of epiphytes observed on ASUs is in contradiction with results of LAVERY et al. (2007), suggesting that movement was not a limiting factor in our experimental design.

However, the experimental protocol can explain the low colonization rate observed on natural leaves in comparison with ASUs. The very young parts of *P. oceanica* studied here were situated in the centre of the tuft formed by the leaves. In that zone, leaves are tightly arranged side by side so the leaf surface is less accessible for epiphyte propagules or larvae than on ASUs that are not organised in tufts. This low epiphytic cover of the youngest and basal parts of *P. oceanica* shoots has already been observed by CASOLA et al. (1987). It explains only the difference in colonization rate between the two substrates but not the difference in community structures. Indeed, the classical succession on *P. oceanica* leaves was identified on natural leaves after only nine days of exposure while the community structure on ASUs was different (see above).

## CONCLUSIONS

At the end of this study, we can hypothesize that the community present on ASUs after a few months inside the meadow would be different to the one on natural leaves, because the first colonization stages are not the same. Despite the absence of replication and the shortness of this study (nine days of exposure), first steps of

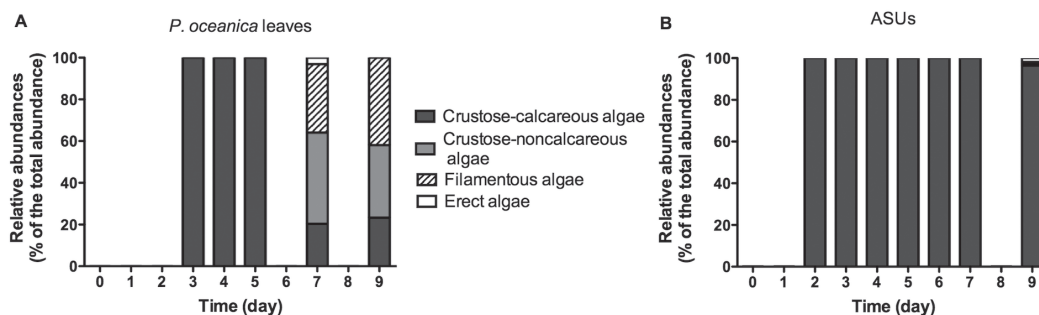


Fig. 6 – Evolution of relative abundances (% of the total abundance of algae) of the different morphological groups of algae observed on the bases of natural leaves (A) and ASUs (B) over time.

the classical epiphyte succession on *P. oceanica* leaves (CASOLA et al., 1987) were observed, while the community on ASUs was different. Moreover, dominant (crustose calcareous algae) and absent (bryozoans) groups observed on ASUs in this study were the same as those observed by other authors after a longer exposure time: MICHEL (2011), 76 days, same ASUs as here; DONNARUMMA et al. (2014), 3 months, different ASUs), suggesting that our results and conclusions are reliable. However, the same study should be performed with more replicates in order to strengthen these conclusions. It would also be interesting to follow the settling of colonists on a longer time scale and to use SEM to study quantitatively the composition and evolution of the biofilm.

ASUs could, however, be useful for the study of environmental perturbations without the use of *P. oceanica*, thanks to the rapidity of their colonization. In that case, further experiments should first characterize the communities present on ASUs in pristine and perturbed locations.

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