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Methods for removing epiphytes from seagrasses: SEM observations on treated leaves

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Abstract

Several techniques commonly used to remove epiphytes from seagrasses were tested on the same *Posidonia oceanica* (L.) Delile material: shaking, scraping, sonicating and soaking in acid. The effects of each treatment on the epiphyte removal rate and on the seagrass itself were assessed by scanning electron microscopy. The advantages and disadvantages of each technique are discussed. It appears that the combination of an acid treatment with moderate scraping provides the best results as nearly all epiphytes are removed without seriously damaging the leaf substratum.

Keywords: Cleaning techniques; Epiphytes; Seagrasses; SEM

1. Introduction

Within the various coastal biocenoses, seagrass meadows must certainly be considered as systems of major importance, owing to their impact on the biogeochemical cycles or on the hydrodynamic and sedimentary processes in infralittoral/sublittoral areas. During the last two decades, many large research projects have been devoted to different aspects of seagrass biology, running from molecular physiology to global ecosystem studies (see e.g. Den Hartog, 1970; McRoy and Helfferich, 1977; Larkum et al., 1989; Phillips and McRoy, 1990). For such ecological approaches, and especially for those dealing with stock and flux evaluations, it is necessary to distinguish between the different compartments of the ecosystems, and thus to consider separately seagrasses and both plant and animal epiphytic communities.

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Several techniques have been proposed to remove epiphytes from seagrass leaves. Some of them use chemical treatment such as soaking in diluted acids (Mazzella and Ott, 1984; Ott, 1990; Nieuwenhuize et al., 1994), others resort to mechanical methods such as the use of water flow (Hickman, 1971) or lyophilization following quick freezing (Penhale, 1977), or simply scraping with a blade (Libes, 1986). Other techniques have been tested on freshwater macrophytes, such as the use of enzymes and EDTA (Delbecque, 1985), or the 'superficial film method' (Sládecková, 1962). However, few authors have verified whether the plant material was still intact after removal of the epiphyton, whether the cleaning was thorough, or whether the physiological response of treated material was close to the response in natural conditions. The latter problem cannot easily be solved, as biochemical pathways (e.g. photosynthesis, respiration, or nutrient uptake) are not necessarily affected in the same way by cleaning techniques. Moreover, the examination of potential morphological injuries requires fixed material, which therefore cannot be used for physiological measurements.

This paper reports the use of scanning electron microscope observations to analyze the efficiency of the different techniques used for removing epiphytes, and the relative damage they cause to the leaves of the most important Mediterranean seagrass species, *Posidonia oceanica* (L.) Delile.

2. Materials and methods

The samples of *Posidonia oceanica* were collected in the Gulf of Calvi, Corsica (France). The gulf has an area of about 22 km^2, and 48% of this area comprises a *Posidonia* meadow starting just below the sea surface and reaching a depth of 38 m (Bay, 1984). Seagrass density decreases from about 480 individual shoots per m^2 at 10 m depth to about 170 shoots per m^2 at 30 m depth (Soullard et al., 1994). Phenological and ecological aspects of this *Posidonia* bed have been extensively described by Bay (1984), Dauby (1989), Frankignoulle and Bouquegneau (1990), Dauby et al. (1995) and Gobert et al. (1995).

Seagrass shoots were collected at 15 m depth in November. At that period the oldest leaves reach their maximum size; most of them are decaying or have been grazed, but a certain number are still complete and fully covered by epiphytes. About 20 well-developed shoots were uprooted by scuba divers and placed in plastic bags to avoid mechanical removal of epiphytes during the transfer to the laboratory. Intermediate leaves (according to Giraud, 1979) with no necrosed tips were then chosen and the distal half (most covered by epiphytes) was cut into pieces about 3 cm long. Twelve batches of three *Posidonia* pieces each (from three different shoots) were made up and allocated to twelve different treatments, as shown in Table 1.

The *Posidonia* pieces were immediately fixed in 4% seawater glutaraldehyde at room temperature for 5 days, then gently rinsed in seawater and post-fixed in 1% SW OsO_4 for 4 h. After a last rinsing in distilled water, the material was progressively dehydrated up to 70° ethanol, wherein it was stored for 6 days. The final dehydration was performed in dimethoxipropane (15 min) and was followed by three rinsings in 100° ethanol before inclusion in Peldri® resin. The latter was washed out by freeze-drying for 20 h (−55°C, 100 mT). The material was mounted on a thin aluminium stub by gluing with Tempfix® resin, and

Table 1
The twelve treatments tested for removing epiphytes from *Posidonia* blades

1. Proof-sample; no treatment
2. Stirring in distilled water for 0.25 h
3. Soaking in 0.2 N hydrochloric acid for 30 min at room temperature + rinsing
4. As Treatment 3 but with 10% acetic acid
5. As Treatment 3 but with 2 N orthophosphoric acid
6. Soaking in 0.2 N NaOH for 15 min at room temperature + rinsing
7. Soaking in 10% household bleach for 15 min + rinsing
8. Scraping with a razor blade
9. Sonication at 50 KHz in seawater for 15 s
10. As Treatment 9 but for 30–40 s
11. Soaking for 5 min in 0.2 N hydrochloric acid + blade scraping + rinsing
12. Sonication in 0.1 N phosphoric acid for 5 s + rinsing

metallized with gold–palladium (15 nm, 30 mA). Observations were performed with a JEOL JSM-840A scanning electron microscope.

The whole surface of the three *Posidonia* pieces (i.e. about 9 cm^2) in each batch was carefully scanned at medium magnification ($\times$ 100) in order to display possible differences between leaves or to show individual inhomogeneities. This examination revealed that pieces taken individually displayed a similar structure (leaf substratum state and epiphyte community) throughout their surface. However, some differences were noted in the epiphyte community when comparing the three pieces in the same batch; this is likely to be due to differences in the epiphyton settlement upon the living leaves. High-resolution observations ($\times$2000 to $\times$4000) displayed local variations which are attributable to discontinuities in the epiphytic covering, especially for large taxa such as worms or bryozoa. The photomicrographs shown in this paper were, of course, 'selected' from a great many; they nevertheless represent 'mean observed conditions' rather than restricted situations.

3. Results

3.1. Untreated samples (Treatment 1)

The proof-samples macro- or microscopically display most of the common *Posidonia* leaf substratum epiphytic phyla as described, e.g. by Peres and Picard (1964) or Van der Ben (1971): cyanobacteria, diatoms, fungi, small branched or flat (*Myrionema*) brown algae, encrusting calcareous red algae (*Melobesia* spp.), foraminifers, hydroids, bryozoa (*Electra posidoniae* Gautier), serpulid worms or tunicates. Among these different taxa, diatoms indisputably form the major part of the epiphytic felt (Fig. 1), followed by melobesias.

The covering (in terms of colonized surface) ranges from about 20% to more than 60%, depending on the sample observed. The biomass of epiphytes averages, for that period of the year (autumn), 0.1 g dry weight per g dry weight of *Posidonia* (Gobert et al., 1995).

Fig. 1. Untreated sample. View showing the importance of bacteria and diatoms in the epiphytic felt. Plate width: 50 μm.

3.2. *Rinsed samples (Treatment 2)*

Stirring the seagrass blades in distilled water for several minutes was thought to remove loosely attached epiphytes by the combined effect of mechanical disturbance and osmotic shock. Photomicrographs (Fig. 2) show that a large fraction of the diatom community has disappeared from the *Posidonia* leaf surface, but that some diatoms species—probably those which were better attached—remain. Light-microscope observations show that most of the attached algae have blown up, but still remain gripped by their stem. Neither the encrusting epiphytes nor the *Posidonia* leaf itself appear to be significantly affected by this treatment.

3.3. *Samples treated with acids (Treatments 3–5)*

As many epiphytes contain a significant fraction of inorganic carbonates (melobesias, bryozoa, tubicolous polychaetes), acidification should help in removing them from the leaf substratum. SEM observations show that the effect of all the acids tested (HCl, H_3PO_4, CH_3COOH) was satisfactory in detaching encrusting algae, but that some calcareous types (serpulids, bryozoa) withstood the treatment (Fig. 3). With the times and concentrations used in this study, no degradation of the leaf matrix was noticed. On the other hand, very small organisms such as diatoms appeared to resist the acid treatments and were only poorly removed.

Fig. 2. Sample stirred in distilled water. Note the persistence of some diatoms and encrusting epiphytes. Plate width: 200 μm.

3.4. Samples treated with bases (Treatments 6 and 7)

It might be thought that dilute alkali, such as sodium hydroxide or hypochloride, would help in removing lightly attached epiphytes as they perform a digestion of the organic matter. Photomicrographs of the samples treated with NaOH (Fig. 4) show that, with the concentration used (0.2 N), the effect on the epiphyton was almost indiscernible. However, the walls of the external cell layer of the *Posidonia* leaves appeared to have been altered, i.e. scratched. Samples treated with bleach did not provide any useful picture as all burst into pieces during the dehydration procedure prior to inclusion, indicating a strong alteration of the leaf structure.

3.5. Mechanical treatments (Treatments 8–10)

The most commonly used technique (but maybe the most time-consuming) is certainly scraping with a thin sharp tool: razor blade, metal or plastic scalpel, glass plate, etc. Of course this technique is mainly suitable for ribbon-shaped seagrasses such as *Posidonia oceanica*. Photomicrographs show that the 'removal rate' is very good (Fig. 5), as large epiphytes (even calcified ones) as well as inconspicuous forms were removed by scraping (a clean new razor blade was used). Nevertheless, some spots of epiphytes remained here and there, probably in the places where the leaf was not perfectly flat. The whole of the leaf epidermis was more or less seriously affected by this treatment, with signs of laceration

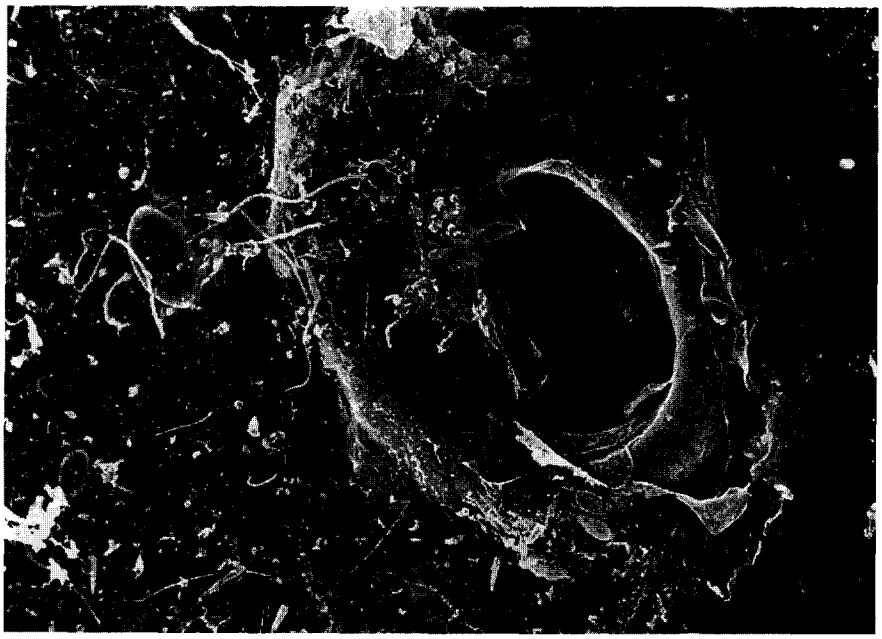

Fig. 3. Sample treated with dilute HCl. Some calcareous types (here the serpulid worm *Spirorbis*) are not completely dissolved. Plate width: 350 μm.

tracks, torn cells and damaged leaf edges. Samples with the least injuries also had fewest epiphytes removed. Thus it appears that the pressure exerted on the scraping tool is directly related to the quality of cleaning but inversely related to the degree of preservation of the frond.

Ultrasonication is commonly used by biologists to clean equipment or to disintegrate organic bodies. We tried to immerse seagrass material in a US-tank filled with filtered seawater in order to see if ultrasound could help in detaching epiphytes. It appears that even with short sonication times (15 s) most of the small epiphytes were either destroyed (diatom frustules burst, Fig. 6) or washed away; larger epiphytes (melobesias, bryozoa) began to be disintegrated, but still remained widely attached to the leaf substratum. With longer treatments, the latter organisms also disappeared, but the *Posidonia* leaves themselves started to fissure and showed various other injuries (Fig. 7).

3.6. Mixed treatments (Treatments 11 and 12)

Two treatments were tested (acid + scraping, acid + sonication) using a combination of dilute acid and a mechanical process. Scraping with a blade after soaking in HCl gave very good results, as almost the whole epiphyton (from cyanobacteria to the largest animals) disappeared from the leaf surface. Moreover, as the calcareous encrusting epiphytes were 'pre-digested', the pressure on the blade needed to remove them is reduced, and thus the *Posidonia* leaf stayed almost undamaged (Fig. 8).

Fig. 4. Sample treated with NaOH. View showing the persistence of the epiphytic felt. Plate width: 200 μm.

Fig. 5. Blade-scraped sample. Note the relative cleanness and the laceration tracks. Plate width: 250 μm.

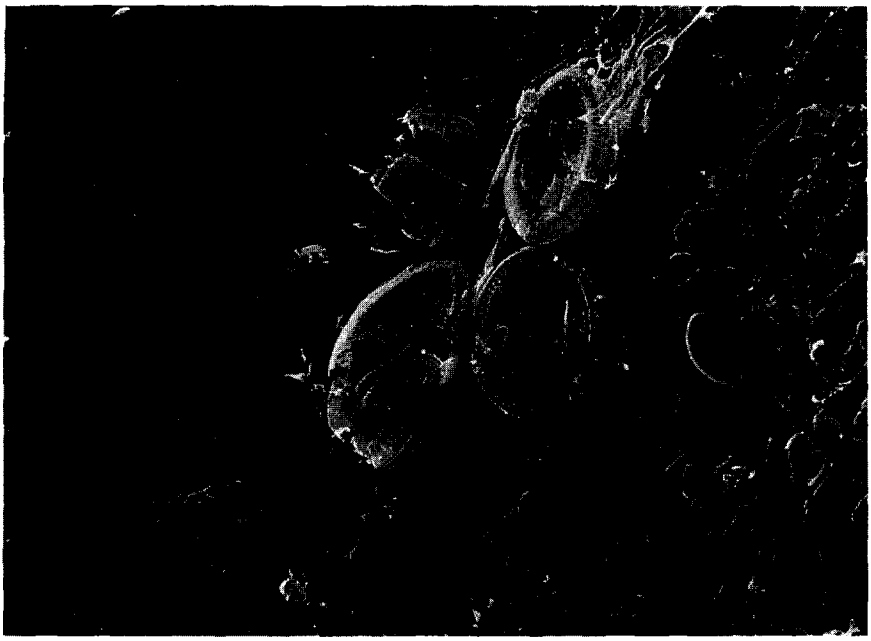

Fig. 6. Sample lightly treated by ultrasonication. View showing burst diatom frustules. Plate width: 180 μm.

Fig. 7. Sample heavily treated by ultrasonication. Fissures in the leaf are clearly visible. Plate width: 1800 μm.

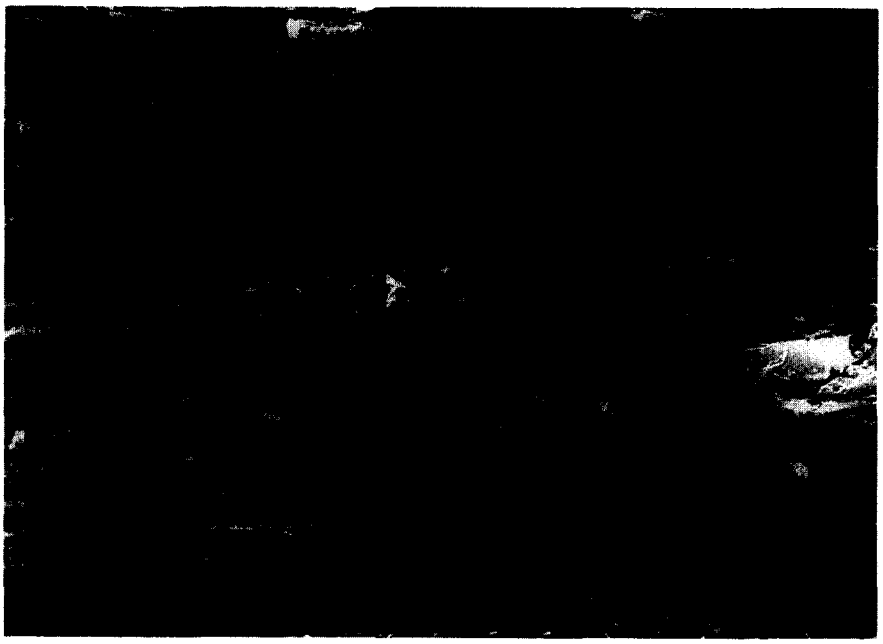

Fig. 8. Sample treated with acid and then scraped. Almost all the epiphytes have been removed and the leaf surface appears almost undamaged. Plate width: 175 μm.

The results of the acid + ultrasound treatment were much less promising as no noticeable difference was observed compared with the separate treatments. Moreover, in time acids are likely to corrode the stainless steel coating of the ultrasound tank.

4. Discussion and conclusion

Among the techniques applied to try to remove epiphytes, the best is indisputably slight acidification followed by blade scraping (Treatment 11), which ensures almost complete cleaning of the leaf substratum with very little damage. This technique was also recommended by Mazzella and Ott (1984), who preferred acetic to hydrochloric acid as it was more efficient at dissolving any carbonates in the organic matrix. These authors recorded an epiphyte removal rate greater than 90%. The treatment has only two disadvantages: it takes a relatively long time, and it cannot be used when the total weight of epiphytes needs to be known, as carbonates can represent more than 40% of their dry weight (Bay, 1984).

Scraping untreated material has routinely been used in various studies (Libes, 1986; Pergent et al., 1994; Gobert et al., 1995). It partially solves the weight problem, but can provide overestimations of the mass of epiphytes as seagrass material may also be carried away. This treatment is in any case unsuitable for morphological studies because of the wounds inflicted to the leaves. The use of 'soft' blades (such as plastic) should minimize injuries (G. Pergent, personal communication, 1993), but probably results in incomplete

Table 2
Advantages and disadvantages of the different techniques used to remove epiphytes from seagrasses

Technique	Removes micro-epiphytes	Removes calcareous epiphytes	Removes erect epiphytes	Allows epiphytes to be weighed	Allows seagrass to be weighed	Preserves seagrass undamaged
Shaking	+	−	−	−	−	+
Acids	−	+	−	−	−	±
Bases	−	−	−	−	−	−
Scraping	+	+	+	±	±	−
Sonication	+	+	−	−	±	−
Acid + scraping	+	+	+	±	+	+
Freezing[a]	+	+	?	?	?	+

[a]According to Penhale, 1977.

cleaning. However, this technique, together with the former one (and maybe also with lyophilization), is the only one that removes large encrusting epiphytes such as serpulids or bryozoa.

The lyophilization technique as proposed by Penhale (1977) has not been tested in the framework of this study. Penhale estimated that 70–90% of the epiphytes were removed (after having scraped seagrasses—*Zostera*—with a flat spatula, however) without damaging the leaves. We have already worked on seagrass material which has been stored for several months in a deep-freeze; when brought to room temperature, most of the epiphytes simply flaked off the leaves.

Ultrasonication appears only to be suitable for removing lightly attached micro-epiphytes such as diatoms. A good removal rate of the whole epiphyton can only be obtained with prolonged treatments, but these severely alter the leaf substratum. Good separation of diatoms can more easily and efficiently be made simply by shaking the sample vigorously in water.

The use of acid solutions alone is acceptable to remove calcified epiphytes such as melobesias. As other large epiphytes resist the treatment, a stronger acid concentration or a protracted immersion time may be more appropriate, but there is an obvious risk of attacking the seagrass organic matter itself. Nieuwenhuize et al. (1994) recently showed that acid treatments caused leaching and digestion of *Zostera* leaf material (positively correlated with the concentration of the acid; either acetic, hydrochloric or phosphoric), resulting in the loss of certain organic compounds. Nevertheless, the acid treatment could prove sufficient for some studies as it removes the taxa that form the bulk of epiphyte biomass at some periods of the year. Such a treatment followed by (not combined with) ultrasonication may provide results as good as those obtained by acid + scraping.

As was mentioned in the Introduction, the physiological response of treated material can be seriously altered by the separation technique. The importance of such a modification is likely to depend upon the pathway considered and upon the treatment inflicted, but unfortunately this cannot be determined on preserved seagrass material such as we analysed. It can, however, reasonably be thought that treatments inducing morphological injuries (hard

scraping, sustained sonication) or leakage of biological constituents (concentrated acids) are affecting the seagrass physiology more or less seriously . On the other hand, techniques which preserve the integrity of the substratum (e.g. diluted acid + light scraping) are quite likely not seriously to disturb the main physiological mechanisms. However, most of the assessments of physiological rates of seagrass communities which are of interest to researchers (such as primary production or nutrient uptake) are now usually performed in situ, with (isotopic) tracers (Phillips and McRoy, 1990). These techniques do not require the removal of epiphytes prior to measurements being taken, as the separation is done afterwards.

Finally, the choice of a particular technique of epiphyte removal depends upon the research objectives. All treatments have their own advantages and disadvantages (Table 2), and it is important that care is taken in selecting the technique appropriate for the seagrass species in question (Short, 1990) and the aims of the investigation.

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