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Abstract

Sorting of soft-bottom macrobenthos entangled with *Posidonia oceanica* fibers is time-consuming and tedious because of the weak color contrast. This paper describes a staining-destaining technique that produces good contrast between soft-bottom macrobenthos and *Posidonia oceanica* fibers. The method has been tested on Corsican samples in oligotrophic areas characterized by small-size soft-bottom macrofauna. Our technique saves sorting time (-24%) and is simple to implement.

Keywords: Staining, Destaining, Macrobenthos, Sorting, *Posidonia oceanica* fibers, Soft-bottom.

Introduction

Study of soft-bottom macrobenthos is one of the common methods used in the assessment of coastal water quality in many parts of the world (e.g.: Labrune *et al.*, 2007; Dauvin *et al.*, 2007; Mangialajo *et al.*, 2007; Massé, 2000). The implementation of biotic indices based on this assemblage requires sample processing. In the Mediterranean Sea, the sorting is the most time-consuming step, especially when *Posidonia oceanica* (L.) Delile 1813 fibers, coming from adjacent meadows, are present in abundance.

Around Corsica, the oligotrophic seawater conditions are characterized by small-size soft-bottom macrobenthos (unpublished data). Moreover, because the *P. oceanica* meadow here is well developed, dead leaves are often exported and degraded outside the bed (e.g.: Mateo & Romero, 1997), and it is very common to find these seagrass fibers mixed in with sampled sediment.

Generally, a flotation technique is used to separate the sediment and the lighter fraction including the dead macrofauna. In our case, this low-density fraction also includes the *P. oceanica* fibers. To facilitate sorting, a staining technique is usually used, but all organic matter, including plant fibers, is colored. Therefore, it would be advantageous to find an agent with low staining power on *P. oceanica* fibers.

The main objective of this study was to adapt the staining method to facilitate the sorting of the floating fraction composed of soft-bottom macrobenthos and *P.*

oceanica fibers. Moreover, as the natural pigmentation of soft-bottom animals is necessary for identification, the possibility of destaining the fauna after the sorting step was also investigated.

Material and Methods

Sediment samples were collected along the Corsican coast at different sites by scuba diving with an Ekman grab (0.09 m²) and treated following the ISO Standard 16665 (ISO, 2005).

Each sample was separated by the flotation technique and only the lighter fraction was treated by staining.

Four common stains were tested: rose bengal, methylene blue, lugol, and eosin [2% aqueous eosin and 2% laboratory eosin B as suggested by Williams (1974)].

Stains were individually tested to achieve optimum contrast between soft-bottom macrobenthos and *P. oceanica* fibers. When the best stain with the maximum contrast observed was determined, the volume of this stain (2 ml, 4 ml, or 8 ml per 25 g of mixture) was refined. When the best stain and volume were found, different staining times (5, 10, 15, and 30 min) were tested to determine which time resulted in the greatest contrast between animals and *P. oceanica* fibers.

The time saved using the best stain was determined by comparing the sorting time between two conditions (uncolored macrobenthos in a black background basin (classical method) *vs.* colored macrobenthos in a white

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background basin (adapted method)). Timings of two sorters, one a beginner and one an expert, were compared.

Two destaining solutions were tested: 95% ethanol (Williams, 1974) and an alkaline solution (pH = 9) (Thiel, 1966). Three combinations of destaining solution were tested: ethanol alone, alkaline solution alone, and an ethanol-alkaline solution mixed bath (1:1). The optimum conditions were determined and then repeated several times to verify performance.

Results

Only eosin solutions were effective at improving the contrast between soft-bottom macrobenthos and *P. oceanica* fibers. In fact, the two variants of eosin tested both have a good affinity for macrobenthos and were not permanently fixed on *P. oceanica* fibers (Table 1). Subsequently, only 2% aqueous eosin, which is cheaper and easier to obtain, was used for the next steps of this study.

A volume of 4 ml of eosin per 25 g of mixture gave the best distinction between animals and plant fibers.

A staining time of 15 min resulted in good contrast after the cleaning and discharging steps (Table 2).

Using this process, both sorters were significantly faster when sorting a colored soft-bottom macrobenthos-*P. oceanica* fibers mix: the beginner saved 22% of time and the expert saved 26% when they used the adapted method *vs.* the classical method (p ≤ 0.05, Table 3).

When the staining was done immediately before sorting, the destaining consisted of one bath with 95% ethanol. When staining was completed some hours before use, the destaining consisted of two baths using alkaline solution (Table 4). In the latter case, each bath was 30 min in duration: the first one with only alkaline solution, the second with the alkaline solution and 95% ethanol in a 1:1 mix. Finally, in all cases, organisms were stored in 95% ethanol.

An outline of the method is shown in Table 5.

Conclusions

This paper describes an adapted technique to facilitate the sorting of macrobenthos entangled with *P. oceanica* fibers. The optimum results were obtained by staining the flotation part of a sample with 2% eosin solution in a proportion of 4 ml of solution per 25 g of mixture for a coloration time of 15 min followed by a cleaning-discharging step. This technique allows a mean time-saving in the separation process of 24%. The destaining method (using 95% ethanol solution or an alkaline solution) helps to preserve the natural pigmentation of organisms after a maximum time of 1 h.

Table 1: Staining of soft-bottom macrobenthos-plant fibers.

Tested stain	Coloration effect
Rose bengal	All fibers (animals and plant fibers)
Methylene blue	All fibers (animals and plant fibers)
Lugol	None
2% Aqueous eosin	Animal only
2% Eosin B	Animal only

Table 2: Staining time (min) of eosin and observed effects on soft-bottom macrobenthos and on *P. oceanica* fibers.

Time (min)	Effects on soft-bottom macrobenthos	Effects on *P. oceanica* fibers
5	Few organisms, weakly colored	No color
10	Weakly colored	Very weakly colored
15	Well colored	Weakly colored, rapidly disgorged
30	Well colored	Colored and destained after 2 h

Table 3: Total number (n) of basins sorted, time (min) to sort 25g of sample (uncolored = uncolored macrobenthos sorted on black background (classical method); colored = colored macrobenthos sorted on white background (adapted method)) and time saving (%).

	Total (n)	Uncolored time (min)	Colored time (min)	Time saving (%)	p-value
Beginner	66	40min5s ± 18s (n = 38)	31min51s ± 17s (n = 28)	22	p ≤ 0.05
Expert	71	25min22s ± 10s (n = 42)	18min46s ± 9s (n = 29)	26	p ≤ 0.05

Table 4: Method used and observed effect of tested destaining agent.

Destaining agent	Use	Effect
95% Ethanol	Directly after staining	Good artificial destain
Alkaline solution	A long time after staining	After two baths: good destain

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Table 5: Outline of proposed method.

	Step
1.	Clean the sample with seawater and separate the heavy from floating fractions by the flotation technique (repeat, if necessary)
2.	Weigh the floating fraction to determine the quantity of eosin to be used
3.	Color the sample with an eosin-seawater mix (proportion: 4 ml of eosin per 25 g of mixture) for exactly 15 min
4.	Clean the floating fraction with seawater
5.	Place the sieve with the floating fraction in a box with seawater for destaining
6.	Just before sorting, rinse the floating fraction again, then proceed to sorting
7.	Keep the extracted soft-bottom macrobenthos in 95% ethanol

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