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2 ***HYDROBIA ULVAE*: A DEPOSIT-FEEDER FOR CLEANING**

3 **LIVING HARD-SHELLED FORAMINIFERA**

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ABSTRACT

This study proposes a new method for fast and inexpensive extraction of a large number of living foraminifera for laboratory cultures. The method is a significant improvement over current extraction methods, which are highly time-consuming. Several treatments were designed to test the method. Sediment bearing foraminifera from Brouage Mudflat (Atlantic coast of France) was washed through a 50- μm sieve and distributed in glass Petri dishes with 20, 40 and 80 specimens of *Hydrobia ulvae*, a common gastropod from European intertidal mudflats. As a control experiment, one dish was treated similarly but maintained without *Hydrobia*. After two days, most of the sediment in the *Hydrobia* treatments was compacted into small cylindrical gastropod feces and the tests of living benthic foraminifera (*Ammonia tepida* and *Haynesina germanica*) were clean and easily visible. Additional experiments showed that the foraminifera were not ingested by *Hydrobia ulvae*, and could be picked quickly and easily.

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INTRODUCTION

Laboratory studies using living foraminifera for biological and ecological investigation have been used for more than a half-century and provide important, complementary data to field-base studies (e.g., Myers, 1935; Le Calvez, 1938; Jepps, 1942; Arnold, 1954). They have been increasingly used for ecological and environmental studies (e.g., Bradshaw, 1961; Bender and Hemleben, 1988; Bijma and others, 1990; Stouff and others, 1999; Khare and Nigam, 2000; Heinz and others, 2002). These studies require separation of live individuals from the sediment without harming them and efficient techniques to differentiate live and dead individuals. Vital staining, such as the use of fluorescent probes, associated with direct observation of cytoplasm and reticulopodial network allow distinguishing live individuals from dead ones (Murray and Bowser, 2000, Bernhard, 2000). However, before using these methods, it is first necessary to isolate individuals from the sediment. In samples containing a high proportion of mud, the tests are hard to discern, even more so because they are often hidden in small-particle agglutinated cysts, making observation quite difficult. The sediment must be sieved to concentrate the foraminifera before observation (e.g., Bowser and others, 1992; Linke and others, 1995; Moodley and others, 2000), but even after sieving, the tests are still scattered among the coarser sediment particles and incased in their cysts. The foraminifera may be separated from the sediment by using their negative geotaxis, which makes them crawl up the walls of their dishes or microscope slides put in the dishes (Arnold, 1974; Anderson and others, 1991; Bernhard, 2000). However, not all foraminiferal species exhibit such behavior (Bernhard, 2000), and infaunal species must be cleaned and picked out from the sediment with a brush or pipette. This is highly time consuming and may harm living individuals (Anderson and others, 1991; Carey, 1993).

1 This study proposes a new harmless biological technique for concentrating living hard-
2 shelled foraminifera, such as *Haynesina germanica* and *Ammonia tepida*, from muddy
3 sediment using the feeding behavior of a small deposit-feeding gastropod, *Hydrobia ulvae*.

4 5 MATERIAL AND METHODS

6 The area selected for collecting foraminifera and *Hydrobia ulvae* was the upper part of
7 the intertidal Brouage mudflat on the French Atlantic coast at latitude 45° 54' N and longitude
8 1° 7' W (Fig. 1). This area was selected owing to the high density of living foraminifera (110
9 individuals per cm³, Arminot du Chatelêt, per. comm.) and because *Hydrobia ulvae*, a
10 common gastropod from European intertidal mudflats, is the most abundant species among
11 the macrofauna (Haubois and others, 2004). This snail is a deposit-feeder that inhabits muddy
12 sand- and mudflats (Hayward and others, 1998). It ingests sediment and egests inorganic
13 particles compacted into small cylinders (feces).

14 Fig. 1 here

15 The sediment sample was collected at low tide by scraping off the first centimeter of
16 sediment in an area where microphytobenthos was abundant (brown film on surface
17 sediment). Seawater was collected in the same area. If the mud snails and foraminifera cannot
18 be sampled at the same time, then it is necessary to maintain a ready stock of mud snails in the
19 laboratory. This is quite easy since they can be kept living for several weeks if placed in
20 sediment in a cold room (5°C). In the laboratory, 1 g of sediment was sieved with seawater
21 through a nylon mesh of 50-µm openings to eliminate clay- and finer silt-size particles. To
22 eliminate additional fine grains, the remaining material was gently stirred in filtered (0.2 µm)
23 seawater and then allowed to stand for several seconds to let the foraminifera settle to the
24 bottom. Supernatant seawater with fine suspended particles was then eliminated, and seawater
25 re-added. This operation was repeated until the supernatant water was clear. Finally, the

1 processed sediment and foraminifera were distributed in a glass Petri dish (16 cm diameter).
2 Owing to the fragility of foraminiferal tests, it was impossible to stir the sediment strongly.
3 Consequently, the tests remained incased in their fine-particle agglutinated cysts and flocs of
4 fine sediments remained together with rare coarser grain.

5 To test the efficiency of the treatment with *Hydrobia*, triplicate Petri dishes were
6 prepared, by addition of 40 and 80 specimens of *Hydrobia ulvae*, respectively. They were
7 placed in a constant-temperature room (18°C) and kept at a light/dark cycle of 12h/12h for 2
8 days. The dishes were observed every day under a dissecting microscope. After 2 days, the
9 number of hard-shelled foraminifera was counted in all dishes and the species were identified.

10 In addition, experiments were carried out to address the question of whether or not the
11 snails are ingesting any foraminifera. Twenty *Hydrobia* were placed for 48 hours in triplicate
12 Petri dishes, together with sediment collected in an area rich in living foraminifera and sieved
13 like above. At the same time, six other Petri dishes were prepared with the same sediment but
14 without gastropods. Three of these dishes without *Hydrobia* were used to count the number of
15 foraminifera at the beginning of the experiment, the three others were used as controls. At the
16 end of the experiment, 48 hours later, the number of foraminifera was counted in the six
17 remaining dishes (3 with *Hydrobia* and 3 controls without). Moreover, 50 *Hydrobia* were
18 collected in an area rich in living foraminifera and sediment was collected at the same place in
19 order to evaluate the density of living foraminifera. The shells of the *Hydrobia* collected in the
20 field as well as those of the experiments (triplicate Petri dishes with 20 *Hydrobia* after 48 h)
21 were broken and their living material was extracted. Because the guts of *Hydrobia* are very
22 small and difficult to open without potentially breaking foraminiferal tests (if present), we
23 used a process previously devised by one of us (Debenay, pers. comm.) for studying the gut
24 contents of other gastropods: The living material of the gastropods was immersed for 4 days
25 into a sodium hypochlorite solution with available chlorine of ~ 3% in order to remove

1 organics. Control experiments, which consisted in the immersion of foraminifera with
2 cytoplasm (potentially living) in the same solution, had shown that the tests, even agglutinated
3 ones, were very well preserved after 4 days in the solution (Debenay, pers. comm.).

4 To complete this experiment, six foraminifera were left with 2 *H. ulvae* for 24 hours in
5 a Petri dish, and then observed to determine if they were still alive (based on pseudopodial
6 activity).

7 RESULTS

8 EFFECT OF *HYDROBIA ULVAE* GRAZING ON THE SEDIMENT

9 During all the experiments with 40 and 80 gastropods, *H. ulvae* fed on the bottom of the
10 dishes and on the food aggregates surrounding living foraminifera. This activity led to the
11 sorting of the sediment and foraminiferal tests into three components: (1) feces of *H. ulvae*
12 made up of small cylinders of compacted sediment; (2) clean foraminiferal tests; (3) a few
13 remaining mineral sediment particles.

14 During the first day, feces production began, but the tests were still covered with a layer of
15 fine sediment and/or food and were not easy to distinguish. After two days, snails had cleaned
16 all the foraminiferal tests, which had become very easy to locate and pick (5 seconds per
17 specimen). It was easy to count them (Table 1). No improvement of the cleaning could be
18 observed for experiments lasting more than two days. An additional benefit became evident.
19 The foraminifera were unable to reconstruct their agglutinated cysts after cleaning because all
20 the fine particles of sediment were aggregated. It appeared that the foraminifera had been
21 cleaned but not ingested by the mud snail. In the dishes without *H. ulvae*, the tests kept their
22 cysts of organic and mineral particles and could hardly be distinguished (Fig. 2). Furthermore,
23 the compaction of the sediment into feces was more efficient with 80 gastropods than with 40.

24 **Table 1 here**

25 **Fig. 2 here**

EFFECT OF *HYDROBIA ULVAE* ON THE FORAMINIFERA

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2 The experiments were carried out with the same amount of sediment (1 g) in all the
3 Petri dishes. After two days, the number of foraminifera (12 ± 2 *Ammonia tepida* and 4 ± 2
4 *Haynesina germanica*, proportions comparable to those found in the natural tidal flat at
5 Brouage; Armynot du Chatelêt, per. comm.) was not significantly different between
6 treatments with or without 20 *Hydrobia ulvae* (Student t test: $p > 0.05$) and not significantly
7 different from numbers at the start of the treatments (Table 2). The fact that the number of
8 individuals, including juveniles, was not lower in the dishes with *Hydrobia* suggests that the
9 gastropods did not ingest any foraminifera. This inference is corroborated by the absence of
10 foraminifera in the guts of the 60 *Hydrobia* from the dishes.

Table 2 here

11
12 The stomach contents of 50 gastropods collected from Brouage tidal flat contained in
13 total only one small test (*Ammonia*) although foraminifera were abundant and available
14 (~ 1200 living foraminifera in 50 cm^3 - 89% *Ammonia tepida*, 8% *Haynesina germanica*, 3%
15 other species). We assume that this lone foraminifera was attached on the shell or snared in
16 the aperture of a snail but was not actually part of the gut contents. These observations
17 suggest that *Hydrobia* does not ingest foraminifera in the natural environment.

18 In a further test, six foraminifera were placed together with two *H. ulvae* and observed
19 at the start and after 24 hours. The foraminifera maintained pseudopodial activity and
20 appeared unharmed by the gastropods (Fig. 3), even though the gastropods had scraped them
21 clean of the agglutinated cysts that incased the tests.

Fig. 3 here

22
23 Several foraminifera extracted with this method were used for a bacterial grazing
24 experiment. They fed normally on bacteria, showing that their health was not perceptibly

1 affected by the cleaning process. They recovered, returned to their normal behaviors, and,
2 when placed in sediment, they reconstruct their agglutinated cyst.

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DISCUSSION

5 Several of methods for extraction and culture of foraminifera have been published
6 (review in Anderson and others, 1991) but all are highly time-consuming. In this study, we
7 describe a new method for extracting quickly and inexpensively a large number of living
8 benthic shelled foraminifera for culture in the laboratory. This method requires only two hours
9 of actual work to extract 1000 tests (30 minutes for preparation of Petri dishes with sediment
10 containing foraminifera and *H. ulvae* and 1 h 30 min for picking 1000 tests after the sediment
11 treatment by *H. ulvae*). The rest of the work is carried out by *H. ulvae*. In comparison, the
12 picking of 1000 tests from untreated sediment required 20 hours. The grazing activity of
13 *Hydrobia ulvae* results in the formation of small cylindrical feces of compacted sediment and
14 cleaning of agglutinated cysts from tests of living benthic foraminifera. Most of the organic
15 and inorganic particles, even those in the cysts around foraminiferal tests, are grazed by the
16 mud snails and digested or packed into feces. Small isolated particles are no longer available
17 to the foraminifera for construction of its cyst. The cleaning is harmless to the foraminifera
18 since the gastropod does not have any feeding activity towards the foraminifera. This behavior
19 is different from other gastropods, such as *Olivella*, that may selectively ingest living
20 foraminifera, as reported by Hickman and Lipps (1983). However, *Olivella* is much bigger
21 than *Hydrobia* (2 cm instead of 5 mm). Moreover, unpublished studies have been carried out
22 in the île d'Yeu laboratory on *Littorina littorea*, *Littorina saxatilis*, *Gibbula umbilicalis* and
23 *Monodonta lineata*, temperate gastropods much bigger than *Hydrobia*. These studies have
24 shown that very few foraminifera are ingested accidentally by these gastropods when they
25 feed on algae, but that there is no selective ingestion.

1 After testing the abundance of cleaned tests and the incubation times on a sediment
2 sample of 1 g, we concluded that the process is completed after 2 days with 40 *H. ulvae*.
3 About the same number of tests was cleaned in the same amount of time by either 40 or 80 *H.*
4 *ulvae*. The only benefit of using 80 snails rather than 40 was production of feces that were
5 more compacted, which facilitated the picking of living foraminifera. After this treatment,
6 recovery of living specimens with a fine brush was much easier and faster. Moreover, even
7 smaller specimens were clearly discernable, which is not the case when they are hidden in
8 cysts of sediment particles. For the same treatment time (2 days), it is possible to increase the
9 quantity of sediment treated by increasing the abundance of *H. ulvae* in bigger Petri dishes.
10 The active feeding of *H. ulvae* in Petri dishes suggest that this method could be applied to
11 muddy samples in which the mud snail does not occur, such as fine sediments from deeper
12 subtidal habitats or those of the shelf or slope. We suggest too that this method might be
13 adaptable in other coastal and brackish environments where *H. ulvae* does not live, by using
14 other small deposit feeders, such as *Hydrobia salsa* and *Hydrobia totteni* in USA, *Hydrobia*
15 *knysnaensis* in South Africa, and *Hydrobia buccinoides* in Australia. However, these
16 applications require testing.

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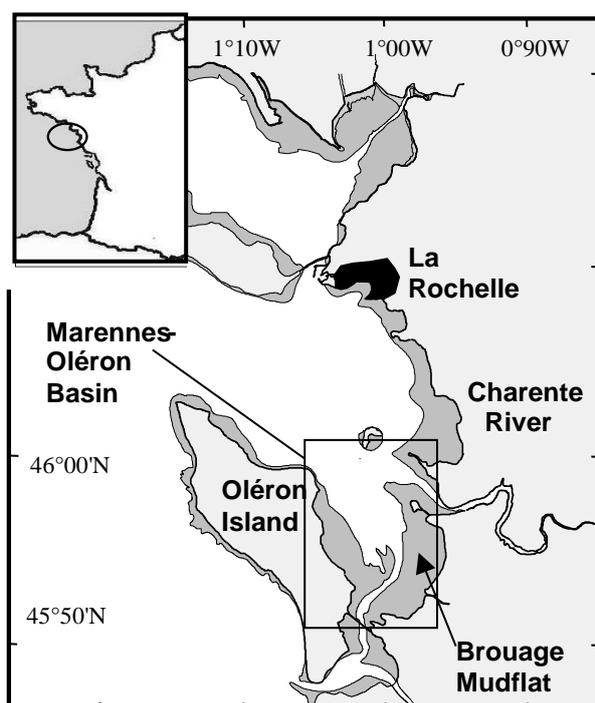
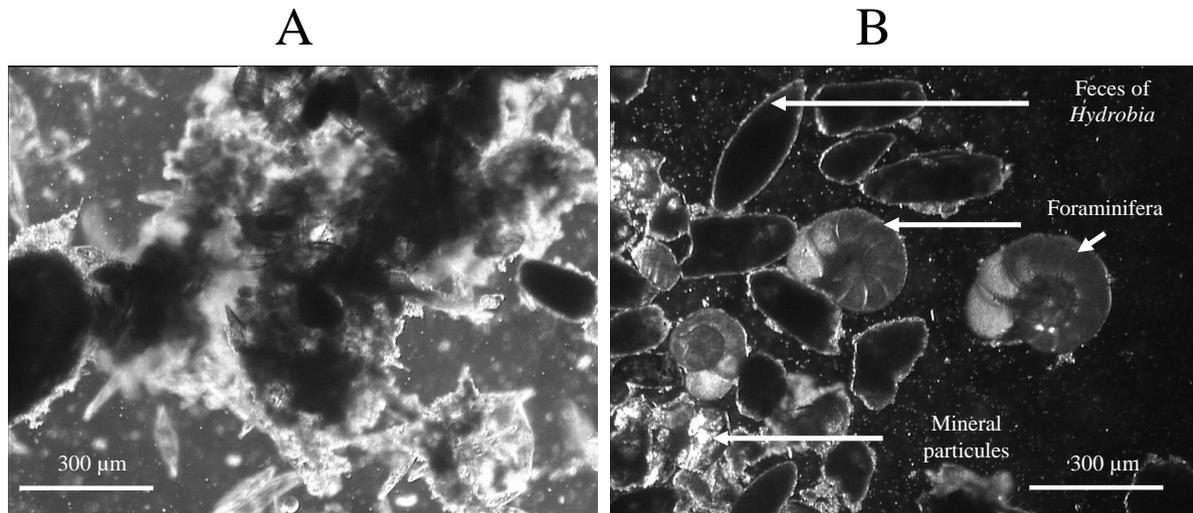


FIGURE 1. Location of the sampling site: Brouage mudflat. White: subtidal area, Dark gray: tidal area.

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10 **FIGURE 2.** Sieved sediment from Brouage mudflat (A) before a treatment with *Hydrobia*
11 *ulvae* (B) after 2 days with *Hydrobia ulvae* (x 200).

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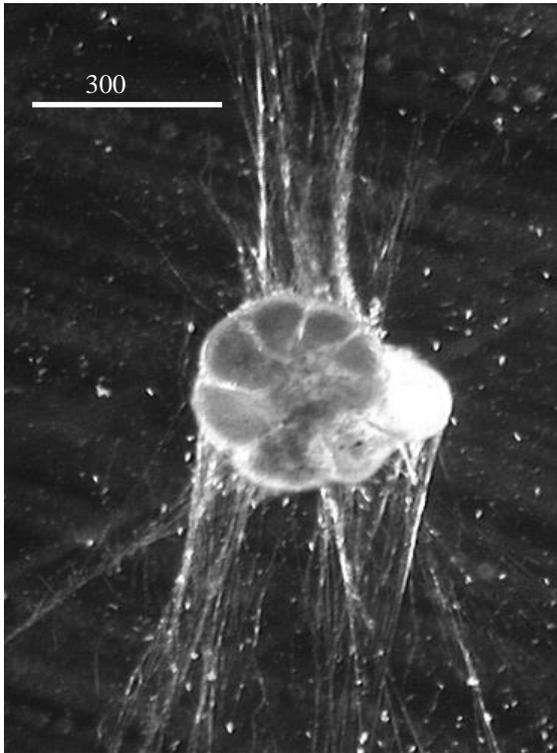


FIGURE 3. Pseudopodia emission of *Ammonia tepida* (x 200).

1 **TABLE 1.** Abundance of foraminifera (Fora g⁻¹dw) (dw: dry weight) in the sediment after
2 treatment with different densities of *Hydrobia* (*Hyd*).
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	Abundance (Fora g ⁻¹ dw)	Abundance (Fora g ⁻¹ dw)
	40 <i>Hyd</i>	80 <i>Hyd</i>
<i>Ammonia tepida</i>	121	129
<i>Haynesina germanica</i>	626	655
Sum	747	784

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1 **TABLE 2.** Abundance of foraminifera (Fora g⁻¹dw) (dw: dry weight) with standard deviation
 2 (SD) in the sediment before (T0) and after 48 h treatment without and with *Hydrobia* (*Hyd*).

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	Abundance (Fora g ⁻¹ dw)		Abundance (Fora g ⁻¹ dw)		Abundance (Fora g ⁻¹ dw)	
	SD	SD	SD	SD	SD	SD
	T0	T0	without <i>Hyd</i>	20 <i>Hyd</i>		
<i>Ammonia tepida</i>	13	3	12	2	13	3
<i>Haynesina germanica</i>	3	1	4	2	4	2
Sum	16	4	17	4	17	5

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