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# Isotopic tracing of sediment components assimilated by epibiontic juveniles of *Holothuria scabra* (Holothuroidea)

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*Despite* Holothuria scabra*'s wide distribution and status as one of the best candidates for sustaining the development of tropical sea cucumber aquaculture, very few data are available regarding the organic fraction it assimilates in practice. In this study we report experimental results where* H. scabra*'s diet was supplemented with various $^{15}$N-labelled organic fractions of sediment. We used juveniles weighing between 38 and 88 mg at the beginning of the experiment (ca 2 cm long and 30 days old). Their growth was measured over a four-week period and their $^{15}$N composition recorded. The results showed that* H. scabra *juveniles assimilated all added organic components from both dissolved and particulate fractions of the sediment. Bacteria seem to be an important food source for juveniles, even more so than microphytobenthos (diatoms).*

**Keywords:** holothuroids, *Holothuria scabra*, isotopic tracers, deposit-feeder, sediment

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## INTRODUCTION

Aspidochirote holothuroids are amongst the most important bioturbators of sediments in many marine ecosystems (Massin, 1982). Aspidochirotes ingest the uppermost few millimetres of surface sediment, include organic and inorganic compounds, and reject the non-assimilated fraction in their faeces (Uthicke, 1999). During transit through the digestive tube, organic and inorganic fractions are digested, but only a portion of the ingested molecules is assimilated into the organisms' tissues. It is now broadly accepted that holothuroids assimilate carbon from bacteria and diatoms, in addition to carbon made labile as a result of microbial degradation (Yingst, 1976; Massin, 1982; Lopez & Levinton, 1987). However, this understanding is based on a very small number of direct and indirect observations, either using labelled tracers or deduced from various experiments on sea cucumbers, respectively. Amongst the direct observations, Yingst (1976) ably demonstrated that *Parastichopus parvimensis* assimilates species of diatoms of the genus *Nitzschia* and uptakes labelled carbon ($^{14}$C) from bacteria. Baird and Thistle (1986) demonstrated that exopolymers produced by the estuarine marine bacterium *Pseudomonas atlantica* could be a source of nutrition for the deposit-feeding holothuroid *Isostichopus badionotus*. Several studies exploring sea cucumber assimilation include indirect observations showing that their foregut contains more bacteria and diatoms than either the surface sediment or the hindgut (Taddei, 2006; Plotieau *et al*., 2013a). These studies suggest that holothuroids select bacteria and diatoms in the sediment and/or culture them in the foregut and then digest them. Moreover, some species are able to make patch selectivity: *Stichopus chloronotus* selects sediments with the highest content of microalgae (Uthicke & Karez, 1999). In the same way, Slater and Jeff (2010) demonstrated that *Australostichopus mollis* displayed better growth when higher microphytobenthic activity was recorded. Some Mediterranean holothuroids ingest both coarse and fine sediment, while others select fine to very fine sediment (Mezali and Soualili 2013). Belbachir *et al.* (in press) also demonstrated that Mediterranean holothuroids show selectivity for organic matter: *H. sanctori* is the most selective species, followed by *H. forskali, H. poli* and *H. tubulosa*. Q2 They attribute these differences to the various micro-distribution of species in the different habitats of *Posidonia* meadows.

*Holothuria scabra* occurs in areas of shallow sea in the Indo-Pacific, featuring sandy–muddy bottoms and generally colonized by seagrass beds. *H. scabra* is an important member of these ecosystems (Wolkenhauer *et al*., 2010); it has a diurnal cycle with adults that remain buried in the upper layer of sediment during the day and move out to forage at night (Mercier *et al*., 1999). Although *H. scabra* is widely distributed and is one of the best candidates for the development of a sustainable tropical sea cucumber aquaculture, very little information is available on the fraction of organic sediment it assimilates. In this study we tested various $^{15}$N-labelled sediment components to better understand what fractions of the sediment organic matter are incorporated into its tissues.

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## MATERIALS AND METHODS

Experiments were conducted at the Polyaquaculture Research Unit of the Institut Halieutique et des Sciences Marines (University of Toliara; Madagascar) (www.polyaquaculture.mg) using *H. scabra* juveniles obtained from Madagascar Holothurie S.A. (Eeckhaut *et al.*, 2009). Larvae were raised for 2 wk in hatchery tanks and, after metamorphosis, juveniles of *H. scabra* were kept in the tanks for a further 2 wk. Following this, they were used for the experiments (30 d old, *ca* 2 cm long and from 38 to 88 mg; see Table 1).

In order to study which organic fractions from the sediments are assimilated by *H. scabra*, five treatments and one control were conducted. In each of these treatments and in the control, 32 *H. scabra* individuals (192 individuals in total) were first acclimatized in the aquaria for 1 wk before the beginning of the study and then reared for a further 3 wk in aerated 50 l aquaria containing a 5 cm layer of sediment taken from natural seagrass beds (density of 150 ind $m^{-2}$).

All living individuals were weighed each week (at T0, T1, T2 and T3) over the duration of the study. A weight record was made by immersing individuals freshly collected from the experiment tank in sterile seawater. Weights were measured three times for each individual (replaced in sterile sea water for 10 min between each measurement) with a high precision balance (precision of 1 mg). With the exception of the experiment with *Clostridium* (where a high mortality was recorded) a minimum of 15 individuals from each experimental group were weighed each week. Mortality rates were also recorded.

After the acclimatization period (T0), 1 wk later (T1) and at the end of the treatment 3 wk later (T3), six juveniles from each of the six aquaria were placed separately in a tank containing only seawater for 48 h in order to eliminate gut content and any labelled compounds not integrated in their tissues. They were then oven-dried at 60°C for 48 h, before being crushed with a mortar and pestle to obtain a fine powder. The powder was then acidified with 37% fuming HCl in a bell jar for 48 h in order to remove skeleton carbonates. Isotopic ratios and elemental content measurements were performed with a mass spectrometer (VG Optima, Isoprime, UK) coupled to a C–N–S elemental analyser (Carlo Erba, Italy) for combustion and automated analysis. Relative concentration of nitrogen is expressed as a percentage relative to dry weight ($N\%_{DW}$). Isotopic ratios are presented as δ values (‰), expressed relative to atmospheric $N_2$. Reference materials were IAEA-N1 ($\delta^{15}N = +0.4 \pm 0.2‰$). Experimental precision (based on the standard deviation of replicates of an atropina standard) was 0.4‰. Atom% notation ($^{15}N$ atom%) was also used to calculate the quantity of tracer assimilated by *H. scabra* over time at the end of the experiment. This quantity was calculated according to:

$$^{15}N_{excess} = \%^{15}N_{excess} \times N\%(DW) \times \text{ final biomass}$$

$\%^{15}N_{excess}$ was obtained by subtracting the natural abundance of $^{15}N$ in holothurian tissues (0.376 atom%) from the measured $^{15}N$ abundance.

Because this quantity is dependent of the initial amount of tracer added in the aquarium, we also calculated the integration percentage (i.e. the percentage of the initial quantity of $^{15}N$ added to the experimental aquarium and effectively assimilated in the holothurian tissues).

ANOVA were performed on the growth data in order to compare mean weight, with significant differences determined by Tukey's HSD test ($\alpha$: 0.05) (Statistica 7.0). To detect any effects of treatments over time, an ANCOVA analysis was realised with R 2.15.0. For isotope ($^{15}N$ values), non-parametric Mann–Whitney *U*-tests were performed (*a*: 0.05) (Statistica 7.0).

## The control

The aquarium contained sediment, seawater and 32 individuals. Isotopic analyses were made on the individuals at T0, T1 and T3 to determine $^{15}N$ levels in standard rearing conditions.

## Treatments 1 and 2: assimilation of compounds from $^{15}N$-labelled bacteria of the genera *Vibrio* (closest strain in blast search: JQ665337.1) and *Clostridium* (closest strain in blast search JF836014.1)

These experiments investigated the potential of *H. scabra* to assimilate organic components from *Vibrio* and *Clostridium*. *Vibrio* is a genus commonly observed in seawater, marine sediments and in the digestive tube of *H. scabra* (Plotieau *et al.*, 2013a). *Clostridium* is less common in marine environments and has not been recorded in the list of the 114 phylotypes identified in the digestive tube of *H. scabra* (Plotieau *et al.*, 2013a). Bacteria were cultured in Petri dishes with LB medium (tryptone (10 g $l^{-1}$), yeast extract (5 g $l^{-1}$) and agar (15 g $l^{-1}$), NaCl (30 g $l^{-1}$), with MilliQ water containing $^{15}N$-alanine 98% (Eurisotop, France) (5 mg per petri dish

**Table 1.** Mortality rate, growth rate calculated at the end of the experiments and mean weight (±SD) of *H. scabra* juveniles. T0, T1, T2, T3 = time at the beginning, after 1, 2 and 3 wk of the experiments, respectively. Values in a same column sharing at least one symbol (a, b) did not differ significantly (Tukey's HSD test; a = 0.05). Q5

| | Mortality (%) | Mean weight (g) | | | | Growth rate (mg $j^{-1}$) |
|---|---|---|---|---|---|---|
| | | T0 | T1 | T2 | T3 | |
| Control | 5 | $0.038 \pm 0.041^a$ | $0.030 \pm 0.026$ | $0.020 \pm 0.026$ | $0.022 \pm 0.021^a$ | −0.76 |
| $^{15}N$-labelled *Vibrio* | 15 | $0.064 \pm 0.051^a$ | $0.087 \pm 0.066$ | $0.146 \pm 0.121$ | $0.181 \pm 0.176^b$ | 5.57 |
| $^{15}N$-labelled *Clostridium* | 88 | $0.036 \pm 0.019^a$ | $0.032 \pm 0.022$ | $0.031 \pm 0.012$ | $0.040 \pm 0.019^a$ | 0.19 |
| $^{15}N$-alanine | 3 | $0.043 \pm 0.037^a$ | $0.053 \pm 0.037$ | $0.175 \pm 0.097$ | $0.230 \pm 0.164^b$ | 8.90 |
| $^{15}N$-alanine + antibiotics | 15 | $0.066 \pm 0.038^a$ | $0.084 \pm 0.057$ | $0.075 \pm 0.066$ | $0.097 \pm 0.090^b$ | 1.48 |
| $(^{15}NH_4)_2SO_4$ + antibiotics | 3 | $0.088 \pm 0.048^a$ | $0.108 \pm 0.163$ | $0.107 \pm 0.109$ | $0.112 \pm 0.069^b$ | 1.14 |

containing 9 ml of LB medium). Alanine is an important amino acid present in the bacterial wall (Schleifer & Kandler 1972). In order to check incorporation of $^{15}$N by cultured bacteria (*Vibrio* and *Clostridium*), three samples of each bacterial strain were rinsed three times with 0.22 μm filtered seawater and oven-dried at 60°C for 48 h before measurements with a mass spectrometer (see below).

Gram coloration was achieved in order to select one gram-negative (*Vibrio*) and one gram-positive (*Clostridium*) bacteria from the sample groups of pure cultures (Adamse, 1970). For each bacterial strain, the content of three Petri dishes (3 g w/w) was added once a week to the experimental tanks. Before these additions, living bacteria were rinsed three times with 0.22 μm filtered seawater in order to remove non-integrated $^{15}$N-alanine.

In order to identify the two strains of bacteria in the cultures and to check for any contamination that might occur during the 4 wk duration of the study, three samples of each bacterial culture were fixed in absolute ethanol (100%) at the beginning of the experiment and again after 1, 2 and 3 wk. Bacterial DNA from 5–10 mg of fixed samples was extracted using an Invisorb spin tissues minikit (Invitek) and a 550 bp-long 16S rRNA gene fragment was then amplified by touchdown-PCR using the protocol developed by Plotieau *et al.* (2013a). Generated sequences were submitted to the BLAST database (http://www.ncbi.nlm.nih.gov/BLAST) in order to identify the closest species, found in each case to be *Vibrio* and *Clostridium*, respectively.

## Treatments 3 and 4: assimilation of $^{15}$N-alanine in the presence or absence of antibiotics

These experiments investigated the ability of *H. scabra* to assimilate alanine dissolved in water and the role of bacteria in this assimilation. Accordingly, in experiment 4, $^{15}$N-alanine (12 mg) was added to the aquarium each week for 3 wk (concentration of 0.24 mg $l^{-1}$). In experiment 5, $^{15}$N-alanine (12 mg) and antibiotics (4 g ampicillin and 1 g streptomycin according to Malmcrona-Friberg (1986) and Mary *et al.* (1993)) were added each week over the duration of the study in a separate experimental tank. The hypothesis tested was that the following: if $^{15}$N-alanine was assimilated directly from seawater by *H. scabra*, their tissues would contain more $^{15}$N than juveniles in the control. Conversely, if bacteria took part in the assimilation of $^{15}$N from alanine, the concentration $^{15}$N in *H. scabra*'s tissues reared with $^{15}$N-alanine + antibiotics would prove to be less abundant than the concentration in juveniles reared only with $^{15}$N-alanine.

## Treatment 5: assimilation of organic compounds from autotrophic microorganisms

Ammonium is the preferred nitrogen source for most autotrophic bacteria and other microautotrophs (Von Wirén & Merrick, 2004). $^{15}$N-ammonium sulfate 99% (Eurisotope, France) (300 mg) and antibiotics (4 g ampicillin and 1 g streptomycin) were added to the aquarium each week for the duration of the study. Antibiotics were added to enhance the development of labelled non-bacterial microorganisms. At the end of the experiment, if the $^{15}$N amount in *H. scabra*'s tissues was found to be higher than in the control, this would suggest that *H. scabra* was able to assimilate organic compounds from autotrophic microorganisms.

## RESULTS

At the end of the experiment, the percentage of dead individuals in the control was of 5%. The mortality rates after 4 wk varied significantly according to the treatment applied (Table 1). More than 85% of juveniles died when $^{15}$N-alanine labelled *Clostridium* were added. *Clostridium*-fed individuals did not grow during the study period: their weight only progressed from 36 to 40 mg in 4 wk. Diseased juveniles appeared 1 wk after *Clostridium* introduction, with juveniles presenting spots on their tegument. A few days following this, the juveniles died and their bodies completely deteriorated and liquefied. Juveniles that fed on $^{15}$N-alanine labelled *Vibrio* and on sediments with $^{15}$N-alanine + antibiotics had a mortality rate between 155 and 22%. The lowest mortality rates (less than 5%) were obtained when $^{15}$N-alanine or $^{15}$N-ammonium sulfate + antibiotics were introduced to the aquaria.

The individuals undergoing all treatments grew more than those in the control (Table 1), with recorded growth rates varying between 1.14 and 8.90 mg $j^{-1}$. Juveniles in the control did not grow well, with a recorded growth rate of −0.76 mg $j^{-1}$; as such, the average weight of control individuals at the end of the experiment that was not different than that recorded at the beginning. The average weight of juveniles undergoing the following four treatments differed from that of the control at the end of the treatment: juveniles reared with $^{15}$N-alanine labelled *Vibrio*, juveniles reared with $^{15}$N-alanine, those with $^{15}$N-alanine + antibiotics and those with $(^{15}NH_4)_2SO_4$ + antibiotics (ANOVA plus Tukey; $p < 0.05$) (Table 1). The highest growths were observed for juveniles reared with $^{15}$N-alanine (8.9 mg $j^{-1}$) and those feeding on *Vibrio* (5.57 mg $j^{-1}$); the lowest growths were recorded for individuals fed with *Clostridium* (0.19 mg $j^{-1}$).

There was no difference in $\delta^{15}$N values over time for the control individuals. $\delta^{15}$N values at the end of the all treatments differed significantly from the control, showing a significant incorporation of $^{15}$N in all treatments (Mann–Whitney *U*-tests; $p > 0.05$). Assimilation seems particularly rapid in the ammonium sulphate treatment, as values of $\delta^{15}$N reached more than 2000‰ after 1 wk of experiment (Figure 1).

Treatments conducted with *Clostridium* and *Vibrio* showed a similar pattern of evolution for $\delta^{15}$N, indicating that in both cases there was an assimilation of $^{15}$N from labelled bacteria into holothurian tissues: the $\delta^{15}$N of holothurian tissues passed from 14.2 to 47.0‰ in individuals fed with *Clostridium* and from 15.6 to 115.5‰ in individuals fed with *Vibrio* (Figure 1). Although the $\delta^{15}$N values in the tissues of individuals fed with *Clostridium* increased over time (indicating that they had eaten these bacteria and assimilated their components) the high mortality rate (88%; Table 1) and the low growth (0.19 mg $j^{-1}$; Table 1) suggest that some of these components were toxic for the holothuroids. The values of $\delta^{15}$N of juveniles reared with $^{15}$N alanine labelled *Vibrio* (from ±15.62‰ to ±115.47‰) indicated that there was significant assimilation of *Vibrio* during the study duration (Figure 1). Moreover, growth (5.57 mg $j^{-1}$; Table 1) was

Fig. 1 - B/W online, B/W in print

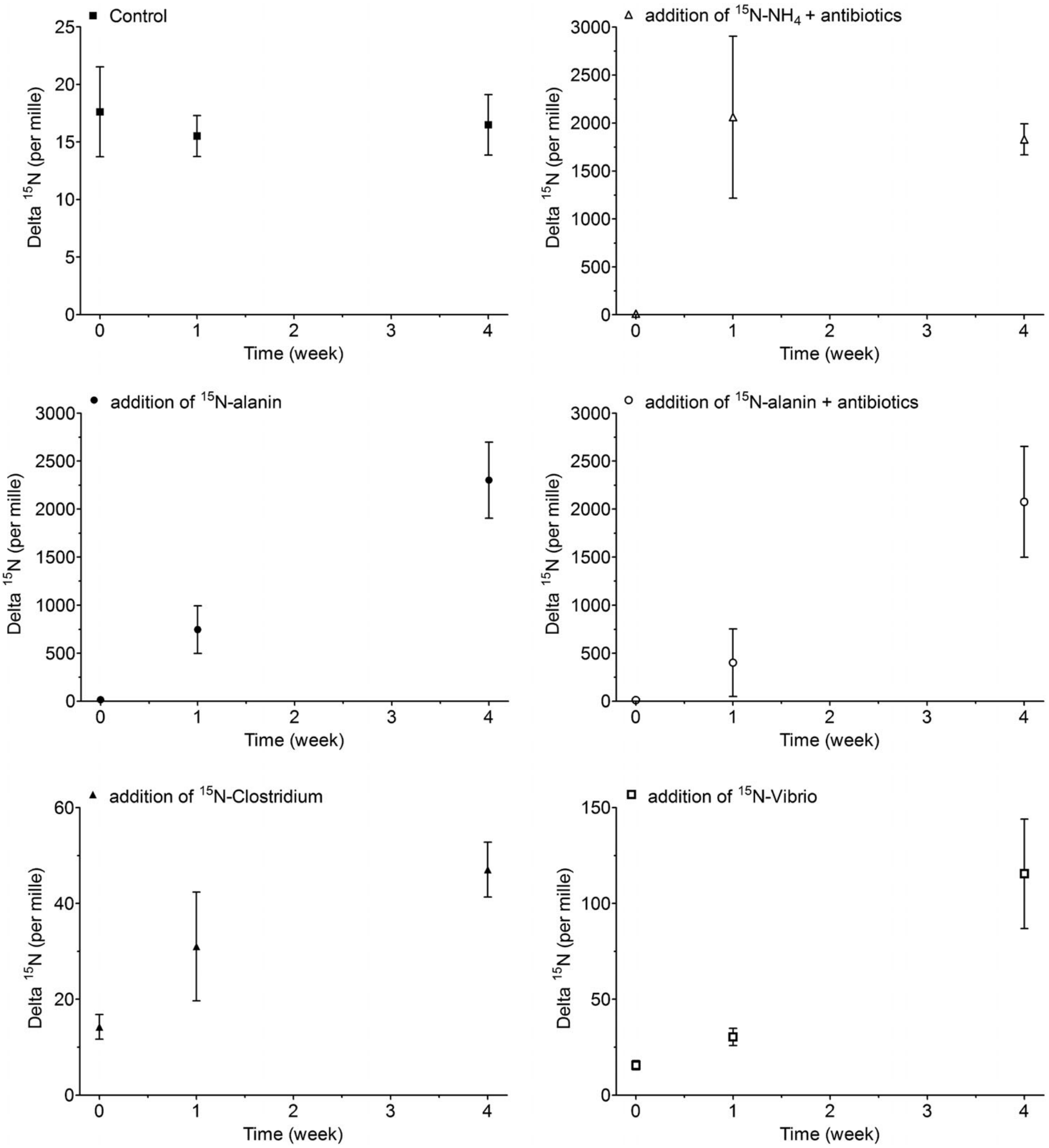

**Figure 1.** Mean $\delta^{15}N$ values (±s.d.) in ‰ measured in *Holothuria scabra* tissues reared in aquaria in presence of different $^{15}N$-tracer treatments.

significantly higher than that of the control individuals and 29 times higher than that of individuals fed with *Clostridium* ($p < 0.05$).

*H. scabra*'s tissues showed a high $^{15}N$ labelling when $^{15}N$-alanine was added, either alone or with antibiotics, to the aquaria (Figure 1), demonstrating that bacteria is part of *H. scabra*'s source of alanine, with the rest being derived directly through uptake from sea water. Over the same period no significant difference ($p > 0.05$) in $\delta^{15}N$ values was observed between juveniles reared with alanine alone and those reared with alanine and antibiotics (Figure 1). $\delta^{15}N$ values in juveniles reared in presence of $^{15}N$-ammonium sulfate and antibiotics were significantly different than values for juveniles in the control (Figure 1).

As we did not add the same quantity of labelled substances in each experimental tank, δ values are not directly indicative of the level of assimilation. For this reason we calculated a normalized tracer assimilation rate where each $^{15}N$ assimilated quantity over the whole experiment time was normalized with the quantity of labelled tracer added during this time (Table 2). We found that only a small percentage of the tracer was incorporated in the holothurian tissues (between 0.0015 and 6.43%). The percentage of assimilated tracer was, however, higher in the alanine treatment than in either the

**Table 2.** Proportion of $^{15}N$ assimilated over time relative to initial $^{15}N$ tracer added quantity according to the experimental treatment.

| Isotopic tracer | Normalized tracer assimilation (%) |
|---|---|
| $^{15}N$-labelled *Vibrio* | 0.35 |
| $^{15}N$-labelled *Clostridium* | 0.0015 |
| $^{15}N$-alanine | 6.43 |
| $^{15}N$-alanine + antibiotics | 2.56 |
| $^{15}N$ ammonium sulfate + antibiotics | 0.69 |

alanine + antibiotic treatment, the ammonium sulfate treatment, the $^{15}$N alanine labelled *Vibrio* treatment or the $^{15}$N alanine labelled *Clostridium* treatment (Table 2).

## DISCUSSION

This study concerned 30 d old juveniles of *Holothuria scabra* (15 d of larval development and 15 d of postmetamorphic development). The use of these juveniles allowed the recording of assimilated tracers in entire individuals, and also created the opportunity to work with a high number of holothuroids (192 individuals) treated with labelled tracers in controlled conditions. The survival rate in the control individuals was similar (in fact slightly higher) to that of individuals reared by Madagascar Holothurie S.A.: the survival rates of juveniles fed over 8 wk by Madagascar Holothurie S.A. varied from 725 to 84% for the same population density used here (150 ind m$^{-2}$) (Lavitra *et al.*, 2009). Lavitra *et al.* (2009) observed that the growth rate of freshly metamorphosed juveniles of *H. scabra* is very slow at the beginning of the post-metamorphic development phase, a result also observed in the course of this study, where the growth rates varied from −0.76 to 8.9 mg d$^{-1}$.

The main finding of this study is that the food sources for *H. scabra* are varied and come from a combination of dissolved nutrients, heterotrophic bacteria and autotrophic microorganisms: all the components tested here were assimilated to some extent, although at different rates and with different effects on the growth of the holothuroids.

The four most abundant amino acids in the integument of *Apostichopus japonicus* are alanine, lysine, glutamic acid and aspartic acid (Gao *et al.*, 2011). The three first amino acids are also present in bacterial walls (Schleifer and Kandler, 1972). In the course of this study we observed that alanine could be rapidly taken up by *H. scabra* in its dissolved form (i.e. treatment with alanine + antibiotic). Free amino acids are an important constituent of dissolved organic matter, particularly in the seagrass sediments where *H. scabra* juveniles metamorphose. This could, therefore, represent an easily accessible food source for young holothuroids. Alanine incorporation may also be mediated by the microbial biomass (i.e. treatment with alanine alone), though more slowly. Bacterial consumption was also emphasised through the use of *Vibrio* and *Clostridium* bacteria, which were evidently assimilated by *H. scabra*. Nevertheless, *Clostridium* probably involved the uptake of harmful toxic substances into holothuroids, leading to a significant mortality rate. Previous studies have already demonstrated the role of bacteria in the diet of the holothuroids *P. parvimensis* (Yingst, 1976) and *I. badionotus* (Baird & Thistle, 1986). The present research team also recently demonstrated that bacterial concentration decreased significantly in substrates subject to holothurian farming (Plotieau *et al.*, 2013b). *Vibrio* are a very common bacteria found in marine sediment (Baross & Liston, 1970; Ward-Rainey *et al.*, 1996) and in the sediment transiting through *H. scabra*'s gut (Plotieau *et al.*, 2013a). *Clostridium* is a genus less common than *Vibrio* in marine sediment (Yakimov *et al.*, 2005) and it was not observed in the 114 phylotypes revealed in *H. scabra*'s gut (Plotieau *et al.*, 2013a). *Vibrio* supplementation of the sediment had a positive effect on the growth of *H. scabra*. Nevertheless, the proportion of tracer assimilated during alanine treatment tended to be higher than that in *Vibrio* treatment. This could partly be explained by the results described in one of our recent studies (Plotieau *et al.*, 2013a) regarding the bacterial composition of the sediment passed through the gut. We found that the sediment bacterial community entering the holothurian gut is very diverse and changes significantly from the foregut to the hindgut: many bacterial strains disappear, but *Vibrio* is the most represented genus in the gut sediment (although this is not the case in the sediments on which *H. scabra* feeds). This result suggests that *Vibrio* are bacteria well adapted to resist the digestion process of *H. scabra*.

Our treatment using ammonium sulphate also showed an incorporation of the isotopic tracer. There is actually no evidence that heterotroph marine animals take ammonium from the seawater as a nitrogen source. Conversely, ammonium is the preferred nitrogen source for most microautotrophs (Von Wirén and Merrick, 2004) and, therefore, the $^{15}$N ammonium sulfate was first integrated into microautotrophs before their eventual incorporation into *H. scabra*'s tissues. As antibiotics were used, most ammonium served the growth of microautotrophs other than bacteria, and (probably mostly for diatoms) as a major contributor in microphytobenthos. Incorporation of the tracer in this experiment showed lower tracer assimilation than during the treatments using alanine (both with and without antibiotics) or *Vibrio*. This suggests that dissolved organic matter and bacteria are very important food sources for *H. scabra* juveniles in comparison to microphytobenthos. Yingst (1976) showed that *Parastichopus parvimensis* assimilates bacteria, diatoms of the genus *Nitzschia* and photoautotroph flagellates *Dunaliella*. She also observed that sea cucumbers do not assimilate organic compounds from the green algae *Ulva* or from the red algae *Gelidium*. The algae she used provided little direct nutritive value to the sea cucumbers, but did feed the bacteria attached to their surface. This is consistent with various studies indicating that plant material does not provide an important source of nutrients for many deposit feeders (Newell 1965; Odum, 1971; Fenchel, 1972).

In conclusion, elements assimilated into the tissues of *H. scabra* juveniles come from a mixture of dissolved nutrients, heterotrophic bacteria and autotrophic microorganisms. Therefore, the diet of this holothuroid is more complex than generally assumed. These components found in the sediment are all assimilated to some extent but at different rates and with different effects on the growth of the holothuroids. As *H. scabra* is a commercial species of high value, supplementation of sediment in aquaculture by bacteria or microautotrophs such as diatoms should be considered and analysed further.

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