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* Manuscript



# Bacterivory by benthic organisms in sediment: quantification using $^{15}N$ enriched bacteria

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## Abstract

The fate of benthic bacterial biomass in benthic food webs is a topic of major importance but poorly described. This paper describes an alternative method for evaluation of bacterial grazing rate by meiofauna and macrofauna using bacteria pre-enriched with stable isotopes. Natural bacteria from the sediment of an intertidal mudflat were cultured in a liquid medium enriched with $^{15}NH_4Cl$. Cultured bacteria contained 2.9 % of $^{15}N$ and were enriched sufficiently to be used as tracers during grazing experiments. Cultured-bacteria presented a biovolume (0.21 $\mu m^3$) and a percentage of actively respiring bacteria (10 %) similar to those found in natural communities. The number of Operational Taxon Units found in cultures fluctuated between 56 and 75 % of that found in natural sediment. Despite this change in community composition, the bacterial consortium used for grazing experiments exhibited characteristics of size, activity and diversity more representative of the natural community than usually noticed in many other grazing studies. The bacterial ingestion rates of three different grazers were in the range of literature values resulting from other methods: 1149 ngC $ind^{-1}$ $h^{-1}$ for the mud snail *Hydrobia ulvae*, 0.027 ngC $ind^{-1}$ $h^{-1}$ for the nematode community, and 0.067 ngC $ind^{-1}$ $h^{-1}$ for the foraminifera *Ammonia tepida*. The alternative method described in this paper overcomes some past limitations and it presents interesting advantages such as short time incubation and *in situ* potential utilisation.



## KeyWords

Bacteria, grazing, sediment, stable isotope, tracer


## Introduction

Development of improved methods for measuring bacterial abundance and production have radically changed the perception of the role of bacteria in pelagic marine ecosystems.

Bacteria are known to play a major role in organic matter degradation and regeneration of nutrients. Moreover the "microbial loop" model (e.g. Azam et al. 1983) considers bacteria as a "link" more than a "sink", increasing the ratio of primary production available for higher trophic levels. Therefore, bacteria appear to play a major role in pelagic foods web models (e.g. Vézina and Savenkoff 1999).

Bacterial abundance in marine soft sediments is relatively constant, around $10^9$ cells.ml$^{-1}$ porewater (Schmidt et al. 1998), being a thousand times more abundant than in pelagic systems. Moreover, high rates of production have been measured in aquatic sediments (e.g. van Duyl and Kop 1990). These findings have driven a debate on the fate of bacteria in benthic food webs. Due to technical limitations, studies dealing with benthic bacterivory are not as developed as pelagic ones (Kemp 1990).

Pelagic bacteria are mainly grazed by protozoa (e.g. Sherr and Sherr 1994) and a similar pattern was expected in benthic systems (van Duyl and Kop 1990; Bak et al. 1991; Hondeveld et al. 1994). Nevertheless, numerous authors consider the bacterial grazing by benthic ciliates and flagellates as insignificant (Alongi 1986; Kemp 1988; Epstein and Shiaris 1992; Epstein 1997). Depending on the studies, meiofauna grazing is considered either as (i) high enough to structure microbial communities (Montagna 1984b), (ii) using 3 % of bacterial production (van Oevelen et al. 2006a), or (iii) negligible (Epstein and Shiaris 1992). Data on macrofaunal grazing rates are not less variable than on meiofaunal ones. In a synthesis, Kemp (1990) asserted that bacteria density is not high enough to play a major role in macrobenthos diet. These contrasting conclusions probably reflect the use of different methods. In conclusion, it appears that drawing a general view of the role played by microfauna, meiofauna and macrofauna in bacterial grazing is presently difficult (Kemp 1990).

Most of the benthic studies on trophic process employ tracers. Labels can be added directly to sediment. In such a situation, bacteria incorporate labels and are simultaneously grazed by

predators (Montagna 1995; van Oevelen et al. 2006a; van Oevelen et al. 2006b). This technique minimizes disturbance of the spatial distribution and metabolism of grazers and bacteria (Carman et al. 1989). Nevertheless, only a small part of the bacterial assemblages takes up detectable quantities of labels (Carman 1990b). Moreover, the main drawback to this method is that a large part (up to 83 % in Montagna & Bauer (1988)) of the total labels uptake may be attributable to processes other than grazing. Grazers may become labelled by absorption and adsorption of dissolved organic matter (DOM) (Montagna 1984a) or by uptake of labels by non-prey microorganisms associated with grazers (e. g. epicuticular or gut microorganisms) (Carman 1990a).

To reduce this bias, microbial prey can be prelabelled with fluorescent products, or isotopes either stable or radioactive. Fluorescent Labelled Bacteria (FLB) with monodispersed FLB or whole-sediment staining methods are used mainly to assess grazing activity of small predators like flagellates and ciliates (Novitsky 1990; Epstein and Shiaris 1992; Hondeveld et al. 1992; Starink et al. 1994; Hamels et al. 2001). Meiofauna studies using FLB are seldom because FLB detection is difficult and time consuming in large sized grazers. Consequently, only a small number of specimens can be examined preventing detection of inter individual variations in grazing rate. Therefore, grazers like nematodes are able to discharge various digestive enzyme to realise extracorporeal hydrolyse of food (Riemann and Helmke 2002). In such a case, pre-digested FLB ingested are impossible to detect in grazers. Nevertheless bacterivory levels by foraminifera (Langezaal et al. 2005) and nematodes (Epstein 1997) were assessed using FLB.

Bacterivory assessment by the way of adding radioactive or stable isotope to sediment was performed on meiofauna and macrofauna (e. g. Montagna 1984b; Sundback et al. 1996; van Oevelen et al. 2006a; van Oevelen et al. 2006b).

Bacterivory assessment using prelabelled bacteria was performed with radioactive isotopes (Rieper 1978; Carman and Thistle 1985). To our knowledge, the use of stable isotopes on prelabelled bacteria has never been performed until present. Compared to radioactive isotopes, bacteria enriched with stable isotopes are more convenient to use, since they can be used *in situ* without negative environmental effects and legal restrictions. This method will help investigators who are limited by radioactive material prohibition.

The aim of this paper is to describe a method using $^{15}N$ stable isotope to prelabel bacteria in the view to assess bacterivory of large size benthic organisms (meiofauna and macrofauna). Experiments were performed mainly to assess the validity of this method, taking in consideration size, diversity, and activity of the prelabeled bacteria in order to be close to natural population parameters. The method was applied to 3 grazers from an intertidal mudflat in order to appreciate its potential generalization: one mollusc *Hydrobia ulvae*, a nematode community and the foraminifera *Ammonia tepida*.

## Material and methods

*Bacterial culture*

Superficial sediment (1 cm) was collected from the Brouage intertidal mudflat located in the eastern part of Marennes-Oléron Bay (45°55N, 1°06W) on the Atlantic Coast of France. One ml of this sediment was added to 20 ml of bacterial liquid culture medium and kept in the dark at 13°C during 24 hours. The liquid bacterial culture medium was composed of: peptone 3 $g.l^{-1}$ (BioRad), yeast extract 1 $g.l^{-1}$ (BioRad), $^{15}NH_4Cl$ 1 $g.l^{-1}$ (99 % $^{15}N$-enriched $NH_4Cl$ CortecNet); sodium glycerophosphate 0.025 $g.l^{-1}$ and sequestren Fe 6 $g.l^{-1}$. It was completed with 0.2 µm filtered distilled water (500 ml) and 0.2 µm filtered sea water (500 ml) at pH 7.4. The first culture was subcultured during 24 hours under the same incubation conditions in the view to reach approximately $2 \times 10^9$ $cells.ml^{-1}$. Bacteria were rinsed (i.e. separated from

culture medium) by the means of 3 centrifugations (3500 g, 10 min, 20°C) in 0.2 µm filtered sea water, then frozen in liquid nitrogen and kept frozen (-80°C) until grazing experiments.

The bacteria $\delta^{15}N$ was measured on an Eurovector Elemental Analyser coupled with an Isotope Mass Ratio Spectrometer (Isoprime, Micromass). Nitrogen isotope composition is expressed in the delta notation ($\delta^{15}N$) relative to air $N_2$: $\delta^{15}N = [(^{15}N/^{14}N)_{sample} / (^{15}N/^{14}N)_{reference})-1] \times 1000$. Rinsing efficiencies were tested using bacteria cultured in the medium previously described with non-enriched $NH_4Cl$. These bacteria were killed by formalin (2 %), placed in the $^{15}N$-enriched culture medium previously described, harvested by the means of 3 centrifugations (3500 g, 10 min, 20°C) before isotope ratio measurement.

*Cultured bacteria size*

Size of bacteria from original sediment and cultures were measured. For sediment samples, particle-associated bacteria were detached by pyrophosphate (0.01M) and sonication. Bacteria were stained using 4,6-diamidino-2-phenylindole dihydrochloride (DAPI) (2500 $\mu g.l^{-1}$) and filtered onto 0.2 µm Nucleopore black filters (Porter and Feig 1980).

Length (L) and width (2r) of each bacteria was determined by a computer-assisted image analysis (AxioVision Release 4.3) with an epifluorescence microscope (AxioSkop 2 mot plus – Zeiss) equipped with a charge-coupled device camera (AxioCam MRc5 – Zeiss). Bacterial biovolumes ($V$) were calculated for cultured bacteria (N = 1981) and natural bacteria (N = 1806) as follows: $V = \pi r^2.(L-2/3\ r)$ (Fuhrman 1981).

*Cultured bacteria activity*

Frozen aliquots of cultured bacteria were thawed and immediately incubated with 5-cyano-2,3-ditoyl tetrazolium chloride (CTC) (final concentration of 5 mM). After 2, 3 and 5 hours of incubation, experiments were stopped with formalin (2%) and stored at 4°C. Bacterial samples were processed as described above for the DAPI staining in order to simultaneously count total cells (UV excitation) and active cells (green excitation) on same slide.

*Cultured bacteria diversity*

The bacterial diversity of original sediment and culture aliquots was assessed. The DNA was extracted using an Ultraclean Soil DNA Kit (MO BIO, Oxyme) for sediment samples and a QIAamp DNA Mini Kit (Qiagen) for cultures. Bacterial 16S rRNA gene fragments of about 520 bp (the V6-V8 regions of 16r DNA (Gelsomino et al. 1999)) were amplified by PCR using primers 968fGC (5'-AACGCGAAGAACCTTAC-3'[with GC clamp 5']) and 1401r (5'-CGGTGTGTACAAGGCCC-3').

PCR products (300 ng) were loaded onto polyacrilamide gel (8% w/v, 7M urea) in TAE 1 X buffer. Electrophoresis was processed under a constant voltage of 68 V, during 17 h, with a thermal gradient from 66 to 69.7°C increasing at the rate of 0.2°C $h^{-1}$ (Dcode™System: Biorad). The gel was stained with 0.5 $\mu g.ml^{-1}$ Gelstar (BMA) in 1.25× TAE buffer during 30 min and checked through a UV transilluminator system (Versa Doc (Bio-Rad)) equipped with a camera. Temperature Gradient Gel Electrophoresis (TGGE) banding patterns were automatically calculated by the Bionumerix software (Applied Biomaths, Koutrai, Belgium) using the Dice coefficient (DC), without band weighing by both the complete linkage and unweighted pair group method with arithmetic mean (UPGMA) algorithms (threshold of 1%).

*Grazing experiments*

The first centimetre of sediment was collected from a square meter patch during ebb tide from the Brouage intertidal mudflat (France) on March 13, 2006. It was sieved on 500 µm, 200 µm and 50 µm in order to concentrate respectively *H. ulvae*, *A. tepida* and nematodes. Choice of these organisms was driven by their high natural abundance in the study area. Each type of grazer was placed in individual microcosms. Seventeen handpicked specimens of *H. ulvae* were placed in polypropylene Petri dishes (diameter 9 cm). For the foraminifera and nematode experiments, 1 ml of the fraction remaining on the 200 µm and on the 50 µm mesh sieves respectively were placed in 100 ml Pyrex beakers. Each experiment was carried out in

triplicate, along with triplicate controls. Control samples were frozen (-80°C) during 12 hours in order to kill grazers before thawing.

Sediment that passed through the 50 µm mesh was mixed with $^{15}N$ enriched bacteria. Abundance of sediment and cultured bacteria were counted using the methods previously described. This slurry containing $1.05 \times 10^9$ bacteria $ml^{-1}$ and $^{15}N$ enriched bacteria were twice as abundant as natural ones. Seventeen ml of this slurry were placed in *H. ulvae* microcosms and 4 ml were placed in nematode and foraminifera microcosms. Grazing incubations were run in the dark at 20°C. Incubations were stopped by freezing the microcosms at -80°C.

*H. ulvae* were separated by hand from their shell and all specimens of each microcosm were pooled and homogenised using a Potter-Eveljhem. Nematodes were extracted from sediment using ludox (Heip et al. 1985). Approximately 700 nematodes were randomly handpicked from each sample. Foraminifera were stained with rose Bengal in order to identify living specimens. As Rose Bengal is an organic compound, it could affect isotopic composition but control experiments were also stained in order to take this bias into account. For each sample, 150 specimens of *A. tepida* were picked individually and cleaned of any adhering particles.

$\delta^{15}N$ of grazers was determined using the technique described above. Incorporation of $^{15}N$ is defined as excess (above background) $^{15}N$ and is expressed in terms of specific uptake ($I$) (gN $ind^{-1}$). $I$ was calculated as the product of excess $^{15}N$ ($E$) and biomass of N per grazer. $E$ is the difference between the fraction $^{15}N$ in the background ($F_{background}$) and in the sample ($F_{sample}$): $E = F_{sample} - F_{background}$, where $F = {}^{15}N / ({}^{15}N + {}^{14}N) = R / (R + 2)$ and R = the nitrogen isotope ratio. For the $F_{background}$, we used control values measured with killed grazers (frozen). R was derived from the measured $\delta^{15}N$ values as: $R = ((\delta^{15}N/1000)+1) \times R_{airN2}$ with $R_{airN2} = 7.35293 \times 10^{-3}$ (Mariotti 1982). The uptake of bacteria (gC $ind^{-1}$ $h^{-1}$) was calculated as

182 Uptake = ($I$ × (% $C_{enriched\ bacteria}$ / % $N_{enriched\ bacteria}$)) / ($F_{enriched\ bacteria}$ × incubation time). This
183 uptake was multiplied by the ratio between the abundance of total and enriched bacteria,
184 determined from DAPI counts. Uptake ($gC_{bacteria}/h/gC_{grazer}$) were obtained by dividing uptake
185 of bacteria (gC/ind/h) by grazer mean weight (gC / ind).

186 ## Results

187 *Characteristics of enriched bacteria*

188 Bacteria cultured in a liquid medium with a 18 mM $^{15}NH_4Cl$ subsequently centrifuged to
189 remove unincorporated label were found to contain 2.88 ± 0.03 % $^{15}N$. Bacteria killed by
190 formaldehyde before being placed in the same culture medium and centrifuged, contained
191 0.028 % $^{15}N$. Cultured bacteria were enriched enough to allow their detection in the three
192 studied grazers (Tab. 1). Bacterial abundance was not affected by the liquid nitrogen freezing
193 process.

194 Cell volume of cultured bacteria (0.21 $\mu m^3$ ± 0.26) was not significantly (bilateral
195 unpairwise student test; $p = 0.07$) different from cell volume of natural bacteria (0.23 $\mu m^3$ ±
196 0.62).

197 The ratio between active and non active bacteria increased significantly with time in
198 cultured bacteria (Analyse of variance, $p < 0.01$) and evolved from 9.9 to 12.9 % during the
199 first five hours after thawing.

200 The number of Operational Taxon Units (OTU) found in cultures fluctuated between 56
201 and 75 % of that found in natural sediment (Fig. 2). The resulting dendrogram of TGGE
202 patterns for cultured and natural sediment samples displayed two clusters. These clusters, of
203 similar community composition, were defined by 49 % pattern similarity. Subculturing does
204 not seem to affect community composition to a great extent (75 % of similarity) and did not
205 change bacterial diversity. Freezing process induced a decline of 25 % in the diversity of
206 bacteria and slightly affected the bacterial community composition (84 % of similarity).

*Bacterial ingestion rates*

After grazing experiments with pre-labelled bacteria, frozen grazers (control) were systematically less $^{15}N$ enriched than living ones for the three grazers types under study (Tab. 1). $^{15}N$ concentration increased linearly in grazers according to incubation time (Fig. 3). This linearity pointed that ingestion rates were constant during incubation period studied: 2 hours for *H. ulvae* and 5 hours for the nematode community and *A. tepida*. Raw data used for ingestion rates calculations are presented in Tab. 1. The mud snail *H. ulvae* grazed 1149 (± 0.285) ngC $ind^{-1}$ $h^{-1}$, each nematode in the community grazed 0.027 (± 0.005) ngC $ind^{-1}$ $h^{-1}$ and the foraminifera *A. tepida* grazed 0.067 (± 0.013) ngC $ind^{-1}$ $h^{-1}$.

## Discussion

*Discussion on methodology*

- Success of enrichment

Rinsing efficiency was tested by placing non enriched killed bacteria in enriched medium and by separating them from this medium by centrifugations. Those bacteria were poorly enriched in $^{15}N$, showing that the bacterial rinsing centrifugation process was efficient. Thus, $^{15}N$ enrichment of bacteria was due to a bacterial assimilation and not to culture medium remaining between bacterial cells. This high bacterial rinsing efficiency is essential, since some grazers are able to consume directly DOM (Montagna and Bauer 1988) from the culture medium.

There is one disadvantage in using $^{15}N$ instead of $^{13}C$ enriched bacteria. As grazers contain more C than N, more biomass is required for isotopic measurements. However, the use of $^{15}N$ avoids the decalcification step required by $^{13}C$ and bias associated with this decalcification (Jacob et al. 2005). Moreover, grazing experiences are based on the assumption that isotopic composition of bacteria remains constant during the incubation period. The isotopic

231 composition of $^{13}C$ enriched bacteria will vary quickly due principally to respiration loss and
232 to a lower degree to production of DOM (Ogawa et al. 2001; Kawasaki and Benner 2006).
233 The use of $^{15}N$ permits to limit this respiration loss bias so isotopic composition of bacteria
234 remains more stable during incubation.

235 ♦ Size of cultured bacteria

236 Discrimination of prey by grazers on the basis of size can influence the estimate of total
237 bacterivory. Bacterial selection according to size has been well documented in planktonic
238 protozoa (Pérez-Uz 1996; Hahn and Höfle 1999). Most protists graze preferentially on
239 medium-sized bacterial cells, grazing being less efficient with smaller and larger cells (review
240 in Hahn and Höfle 2001). The soil nematode, *Caenorhabditis elegans* feeds on bacteria
241 suspended in liquid and smaller bacteria are better food sources than larger ones for this
242 species (Avery and Shtonda 2003). Since, in our study, cultured and natural bacteria presented
243 a similar average size, it can be inferred that there is only a small bias if any due to cell size
244 selection by grazers.

245 ♦ Activity of cultured bacteria

246 Few data are available on CTC activities of natural benthic bacteria for comparison with
247 our results. In superficial sediments from intertidal mudflats, 4 to 20% of benthic bacteria
248 were found to be active (van Duyl et al. 1999). Proctor and Souza (2001) found 9 to 10 %
249 active cells in river sediments and 25 % in intertidal sediments in the Gulf of Mexico.
250 Halgund *et al.* (2002) detected 46 % active bacteria in lake sediments.

251 Enriched bacteria with activity levels different from those of the natural community may
252 induce a bias if grazers select bacteria according to prey activity. Nematodes can discriminate
253 bacteria exhibiting different physiological or nutritional states (Grewal and Wright 1992).
254 *Pellioditis marina* do not assimilate heat-killed bacteria even though it feeds on live cells at

255 high rates (Moens 1999). In contrast, *Diplolaimelloides meyli* is more attracted by killed than
256 by live bacteria (Moens et al. 1999a). The foraminifera *Ammonia beccarii* collects dead and
257 living stained bacteria without discrimination (Langezaal et al. 2005).

258 Many existing methods to quantify bacterivory use bacterial communities with activity
259 levels different from those of the natural communities. For instance, labels directly added to
260 the sediment are only incorporated by the active fraction of the bacterial community: 100 %
261 of labelled bacteria are active. In contrast, prelabelled bacteria such as standard FLB are
262 generally dead (heat-killed): 0 % of labelled bacteria are active. With the method described in
263 our study, 10 % of labelled bacteria are active after thawing of frozen cultures (Fig. 1). This
264 activity is included in range found in natural environments (van Duyl et al. 1999; Proctor and
265 Souza 2001; Haglund et al. 2002). With the present method, grazers have the opportunity to
266 pick up active or inactive bacteria according to their preference like in the natural
267 environment.

268 ♦ Diversity in the cultured bacteria

269 Subculturing of bacteria produces a final culture free of sediment. Freezing allows storage
270 of aliquots that may be enriched under standardised conditions at any time. The freezing step
271 induces small variations in the diversity of the bacterial community that must be nevertheless
272 kept in mind when using this method.

273 Grazers may be highly selective of prey species. To our knowledge, selection of bacteria
274 has never been observed for macrofauna but demonstrated for nematodes (Moens et al.
275 1999a) and foraminifera (Lee et al. 1966; Bernhard and Bowser 1992; Langezaal et al. 2005).

276 Nematodes used to be considered as generalist feeders, but they were recently shown to be
277 selective feeders exhibiting various preferences for algal and microbial prey. Their
278 reproduction rates differ according to the ingested strain of bacteria (Venette and Ferris 1998;
279 Blanc et al. 2006). Moens *et al*. (1999a) show that monhysterid nematodes are able to select

bacterial strain. Selection can be due to the bacterial size: filamentous bacteria escape uptake by nematodes with small buccal cavities (Blanc et al. 2006). Moens *et al*. (1999a) consider that the chemotaxic responses of nematodes to their bacterial prey may be due more to chemical cues produced by the bacteria than to bacterial cell-wall structure that determine their palatability. Nematodes are also able to significantly modify the composition of a bacterial community by their species-specific bacterial food preferences (De Mesel et al. 2004).

The foraminifera *A. beccarii* distinguishes food and non-food particles during collection (Langezaal et al. 2005). Two allogromiidae species (Foraminifera) have been shown to be non selective grazers, actively harvesting bacterial biofilm from 3 different inocula (Bernhard and Bowser 1992). Lee *et al.* (1966) found that most species of bacteria do not serve as food for foraminifera whereas selected species of bacteria are consumed in large quantity.

Each bacterial species presents characteristics such as cell surface, nutritional quality or chemical cues which may influence bacterial grazer behaviour. These differences have not been evaluated between cultured versus natural bacteria in the present study. However, estimation of total community composition and diversity gives us an approximate idea of these differences. This molecular approach has the advantage to target dominant community members. The cultured community presents 49 % of similarity with the natural bacterial community. Although cultivation of natural bacteria induces a shift in community composition (Fig. 2), this bacterial consortium seems more representative of the natural community than that of many other grazing studies. The majority of experiments that use FLB are done with monospecific bacteria or with a really limited number of bacterial species. Even if natural and cultured community are not strictly identical, the probability for grazers to find and ingest their preferred bacterial species is higher in the supplied bacterial consortium than with monospecific bacteria.

♦ Characteristics of grazing experiment

All various methods developed and applied to measure bacterivory in natural communities possess methodological shortcomings that make interpretation of the resulting data problematic. The method presented in this study, using $^{15}N$ pre-enriched bacteria also presents bias. Sieving the sediment changes the bacterial availability for predators, bacteria being not attached to particle as in natural situation. The best way to minimize this artefact is to add the label directly to sediment in order to label bacteria while they are being grazed. This method is problematic as a high fraction of label found in grazers is due to processes other than grazing as underlined before. This requires control of incubations with a prokaryote activity inhibitor and the effectiveness of this inhibitor has to be tested for each grazer. The pre-enriched bacteria technique does not require the use of such inhibitors and only necessitate one control to determine adsorption of enriched bacteria on grazers.

Nematodes (Gerlach 1978), foraminifera from the genera *Ammonia* (Chandler 1989) and *H. ulvae* secrete mucus. During experiments with pre-enriched bacteria, controls must be performed to determine abundance of enriched bacteria stuck in the mucus secreted by grazers. Stuck bacteria modify the isotopic composition of grazers and controls are required to evaluate this bias due to non-grazing processes. In this study, freeze-killed grazer controls were used to determine this adsorption assuming that mucus post-freezing and mucus never frozen absorb bacteria at the same rate.

During grazing experiments, prey egestion from grazers may occur when chemical preservatives are used and this leads to underestimating grazing rates. Nematodes can egest a significant part of their gut contents when killed with formaldehyde (Moens et al. 1999b). In this study, grazers were frozen at -80°C to reduce this bias.

Prior to grazing experiments, enriched bacteria were frozen in liquid nitrogen to prevent spontaneous and enzymatic degradation and maintain their viability. Bacteria are commonly

330 cryopreserved during long-term storage in liquid nitrogen. Some agents like glycerol and
331 methanol can be used to enhance cryopreservation, but in the case of grazing experiments,
332 they can be toxic to grazers. Low-temperature storage enables standardised cultures, helping
333 to ensure reproducible results in a series of experiments. This storage is really useful when
334 monitoring over long periods is considered.

335 The method used allows short incubations that limit bias due to recycling. Bacterial
336 ingestion is detectable after 2 hours of incubation for 3 grazers (Fig. 3). During incubation,
337 labelled bacteria may be first ingested by grazers that are themselves preyed by studied
338 grazers. In such a situation, it is impossible to determine the part of label present in studied
339 grazers that is provided respectively by bacteria and first grazers. Even if a short incubation
340 time does not prevent this type of bias, it reduces it substantially.

341 *Demonstration of applicability*

342 Data from literature to compare with our values are scarce. First, these predator species
343 have not been systematically studied. Secondly, herbivory is more commonly studied than
344 bacterivory. Thirdly, the manner in which to report grazing rates depends on the aim of the
345 study. Studies dealing with carbon flow generally report values on a biomass basis (e.g. ngC.
346 $ind^{-1}$ $h^{-1}$). When the aim of the study is the impact of grazers on microbial community,
347 grazing rates are generally reported as rate constants (e.g. $h^{-1}$) (Montagna 1995).

348 While meiofaunal grazers are adapted to pick out specific microbial particles, macrofaunal
349 deposit feeders process large volumes of sediment. Mud snails of the genus *Hydrobia*
350 assimilate epipelic diatoms and attached bacteria (Newell 1965; Kofoed 1975; Lopez and
351 Levinton 1978; Jensen and Siegismund 1980) contained in the ingested sediment. To our
352 knowledge bacterial ingestion rates have never been determined but data is available
353 concerning algal ingestion rates. The bacterial ingestion rate found in our study is in the same

354 range as algal ingestion rates found by Forbes and Lopez (1989), Blanchard *et al.* (2000) and
355 Haubois *et al*., (2005) (Tab. 2).

356 In literature, grazing rates of nematodes are strongly variable with a range of fluctuations
357 of more than two orders of magnitude (Tab. 2). Thus, comparison of our data with literature is
358 difficult. Those discrepancies may arise from a lot of reasons such as the use of different
359 techniques or the experimental conditions. When grazing experiments are performed in
360 monoxenical conditions, nematodes are in an environment constituted by water (or agar) and
361 bacteria. Nematodes would probably present higher grazing rates in such conditions than
362 during grazing experiments where bacterial food is mixed with minerals and refractory
363 organic matter and therefore is less available. However, when our results are compared to
364 values resulting from experiments using nematodes from mudflat grazing on labelled bacteria
365 mixed with sediment, it appeared that they are in the range of grazing rates found by Epstein
366 & Shiaris (1992) but more than ten times lower than those found by Montagna (1984b).

367 Algal ingestion rates by *A. tepida* are higher (Moodley et al. 2000) than bacterial ingestion
368 rates found in our study (Tab. 2). Langezaal *et al.* (2005) used FLB in simplified microcosms
369 with one specimen of *A. beccarii* in a reduced volume of water. Their bacterial grazing rate is
370 lower than ours. This may be linked to the bacterial concentration used in microcosms (1.4
371 $10^3$cell $ml^{-1}$), which is considerably lower than benthic bacterial abundance in the natural
372 environment (c.a. $10^9$cell $ml^{-1}$) and in the present study.

## 373 Conclusion

374 The fate of benthic bacterial biomass is a topic of major importance in microbial ecology
375 and in food web studies. All various methods developed and applied to measure bacterivory
376 in natural communities possess artefacts and difficulties that make interpretation of the
377 resulting data problematic. Our experimental approach is not an exception and also presents
378 shortcomings. These bias are due principally to sediment manipulation. Labelled bacteria are

379 not available to bacterivores in the same manner as bacteria in unmanipulated sediment.

380 However, grazing experiments with $^{15}N$ pre-enriched bacteria also present several
381 advantages: (i) they can be performed *in situ* without environmental consequences, (ii) they
382 do not require long incubations, so bias due to recycling is minimized, (iii) they require quite
383 simple control tests with freezing of enriched prey, (iv) they can be performed at different
384 times under standardised conditions, (v) they can be extended to other types of sediment or
385 soil and (vi) they can be used in double-labelling experiments with $^{13}C$ enriched algae, in
386 order to simultaneously measure bacterial and algal ingestion rates.

387

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562
563
564

564 # Tables

| | Enriched Bacteria | Gastropoda *H. ulvae* | Nematod community | Foraminifera *A. tepida* |
|---|---|---|---|---|
| **% C by dry weight** | **35.2** | **33.6** | **38.2** | **5.8** |
| **% N by dry weight** | **10.2** | **8.0** | **7.4** | **0.8** |
| **Weight (g/ind)** | | **5.4E-04** | **3.0E-07** | **1.8E-05** |
| **$\delta^{15}$N living grazers** | **7068.2** | **95.0** | **20.6** | **20.0** |
| **$\delta^{15}$N dead grazers** | | **10.7** | **11.1** | **16.7** |
| **Incubation time (h)** | | **2** | **5** | **5** |
| **Ratio (enriched/non enriched bacteria) = 1.5** | | | | |
| **Ingestion rate (ngC/ind/h)** | | **1149.16** | **0.03** | **0.07** |
| **Ingestion rate ($10^{-3}$ $gC_{bacteria}$ / $gC_{grazer}$/h)** | | **6.43** | **0.23** | **0.06** |

565 Table 1. Calculation of ingestions rates of three different grazers

566

| Grazers | Grazing rate ($10^{-3}$ $gC_{bacteria}$ $h^{-1}$ $gC_{grazer}$) | Grazing rate (ngC $h^{-1}$ $ind^{-1}$) | Labelling methode | Reference |
|---|---|---|---|---|
| **Gastropoda** | | | | |
| ***Hydrobia ulvae*** | **6.43** | **1149.16** | **Stable isotope pre-enriched bacteria** | **Present study** |
| ***Hydrobia ulvae*** | | **40-2080** | **Radioactive prelabelled algae** | **(Haubois et al. 2005)** |
| ***Hydrobia ulvae*** | | **896-1064** | **Radioactive prelabelled algae** | **(Blanchard et al. 2000)** |
| ***Hydrobia truncata*** | | **506-2873** | **Radioactive prelabelled algae** | **(Forbes and Lopez 1989)** |
| **Nematode** | | | | |
| **Mudflat Nematode community** | **0.23** | **0.03** | **Stable isotope pre-enriched bacteria** | **Present study** |
| **Mudflat Nematode community** | **2.59-3.66** | | **In situ radioactive labelled bacteria** | **(Montagna 1984b)** |
| **Subtidal Nematode community** | **0.01** | | **In situ radioactive labelled bacteria** | **(Montagna et al. 1995)** |
| ***Plectrus palustris*** | | **10.54*** | **Radioactive prelabelled bacteria** | **(Duncan et al. 1974)** |
| ***Monhystera disjuncta*** | | **0.15-0.49 *** | **Radioactive prelabelled bacteria** | **(Herman and Vranken 1988)** |
| ***Diplolaimelloides meeyli*** | | **11-17** | **Radioactive prelabelled bacteria** | **(Moens and Vincx 2000)** |
| ***Pellioditis marina*** | | **55-60** | **Radioactive prelabelled bacteria** | **(Moens and Vincx 2000)** |
| **Mudflat Nematode community** | | **0.02 *** | **Fluorescent prelabelled bacteria** | **(Epstein and Shiaris 1992)** |
| **Foraminifera** | | | | |
| ***Ammonia tepida*** | **0.06** | **0.07** | **Stable isotope pre-enriched bacteria** | **Present study** |
| ***Ammonia tepida*** | **2.18** | **2.18** | **Stable isotope pre-enriched algae** | **(Moodley et al. 2000)** |
| ***Ammonia beccarii*** | | **$0.5\ 10^{-4}$ - $1{,}6\ 10^{-7}$ *** | **Fluorescent prelabelled bacteria** | **(Langezaal et al. 2005)** |

567 Table 2. Ingestion rate of bacteria observed in this study and compared with data from the literature concerning bacterivory and herbivory. *

568 ingestion rate converted with a bacterial biomass of 35 fgC $cell^{-1}$ (Theil-Nielsen and Søndergaard 1998).

569 **Figure captions**

570 Fig 1. Evolution of the percentage of CTC + cells related to total bacteria after thawing
571 cultured bacteria. Bars indicate standard deviation (n = 3).

572 Fig 2. TGGE analyses of natural sediment, first culture, subculture from the first culture and
573 subculture from the first frozen culture. The right panel shows the relating band similarity (%)
574 of bacterial communities.

575 Fig 3. Evolution of isotopic composition of three types of living (●) and dead (○) grazers
576 placed in contact with $^{15}N$ enriched bacteria. Bars indicate standard deviation (n = 3).

577

**Figure**

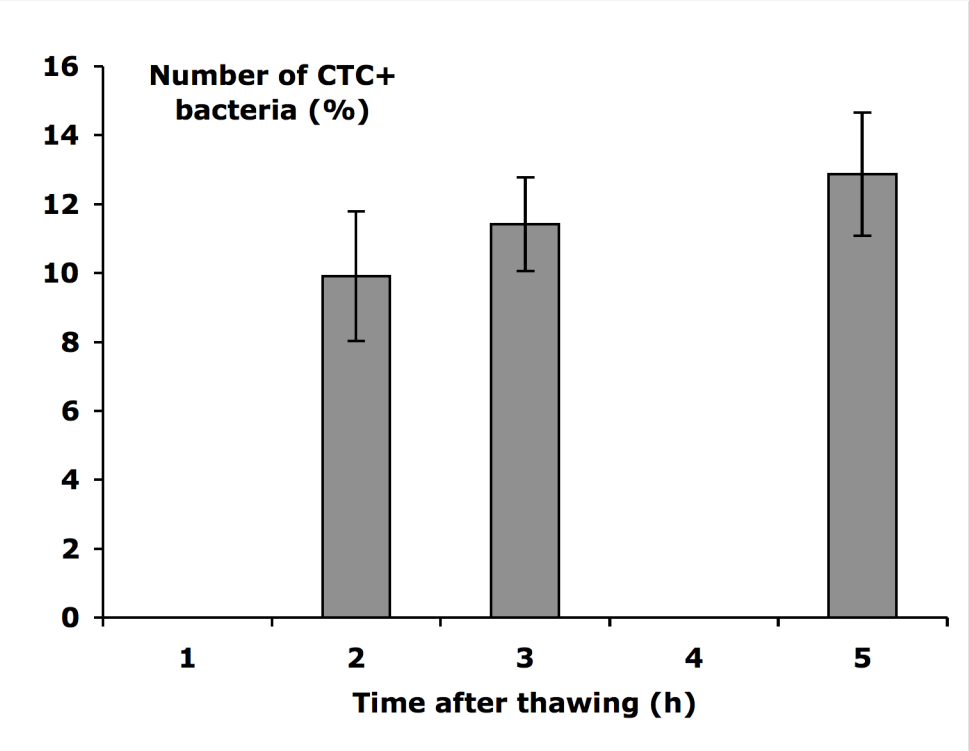
Number of CTC+ bacteria (%)
16
14
12
10
8
6
4
2
0
1
2
3
4
5
Time after thawing (h)

Fig 1.

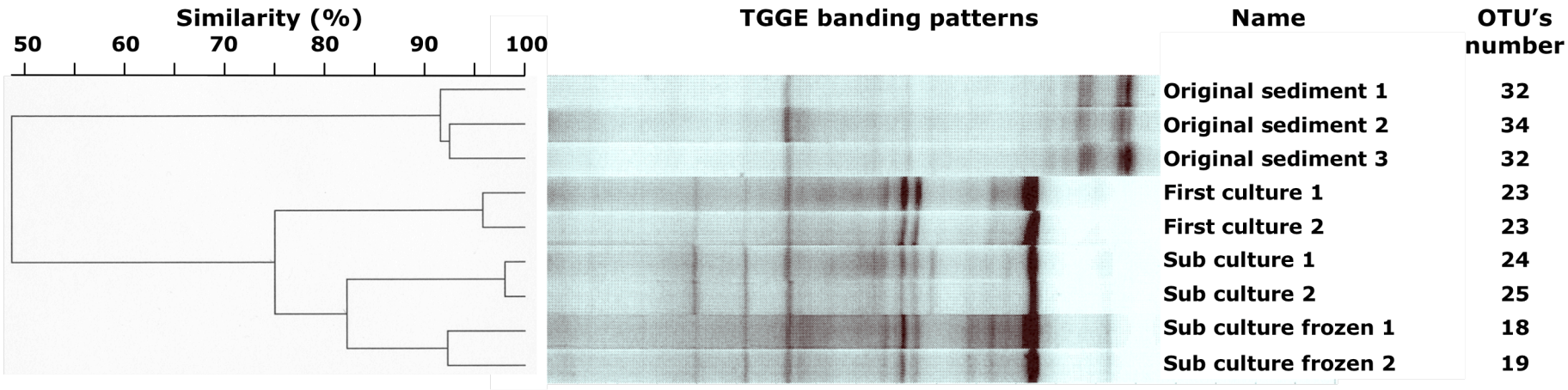
Similarity (%)
50
60
70
80
90
100
TGGE banding patterns
Name
OTU's number
Original sediment 1
32
Original sediment 2
34
Original sediment 3
32
First culture 1
23
First culture 2
23
Sub culture 1
24
Sub culture 2
25
Sub culture frozen 1
18
Sub culture frozen 2
19

Fig 2.

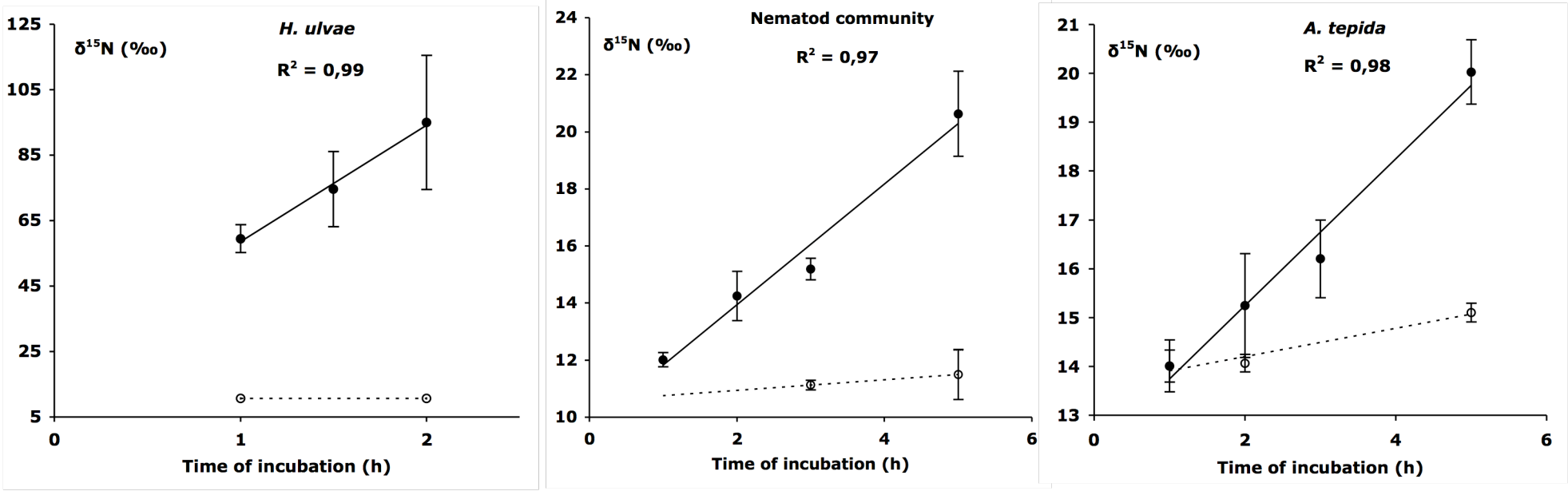
H. ulvae
δ15N (‰)
R2 = 0,99
Time of incubation (h)
Nematod community
δ15N (‰)
R2 = 0,97
Time of incubation (h)
A. tepida
δ15N (‰)
R2 = 0,98
Time of incubation (h)

Fig 3.