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Preservation and incubation time-induced bias in tracer-aided grazing studies on meiofauna

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Abstract A recent review suggests that meiofauna are important grazers of microphytobenthic primary production as well as of bacterial secondary production. The potential importance of meiofauna grazers may nevertheless have systematically been underestimated, since label leakage from chemically preserved animals has hitherto not been accounted for. Furthermore, a majority of studies have used relatively long incubation times and assumed, rather than proved, that label recycling over this period is negligible. In the present study we tested the influence of sample preservation on label retention in the marine nematode *Pellioditis marina* Andrassy, 1983 fed ^{3}H-labelled bacteria. Label loss from formaldehyde-preserved specimens averaged 40% after 1 h preservation and amounted to a maximum of 85% after 24 h in formaldehyde, irrespective of formaldehyde concentration; no further leakage occurred beyond 24 h. Glutaraldehyde and ethanol yielded significantly better and poorer results, respectively, but the former fixative still yielded label losses of up to 70%. A comparison of label uptake as a function of time with observations on ingestion and defecation behaviour suggest that on time scales of hours an indication of assimilation (after correction for label leakage) rather than of ingestion is obtained. When killed with formaldehyde at room temperature, *P. marina* egested a significant part of its gut contents. The sources of bias identified here may have generally led to significant underestimations of true grazing rates. The cumulative effect of label leakage, prey egestion and long incubation times, each at the highest rates observed in this study, may yield as much as a 15-fold underestimation of true food consumption. Cooling samples on ice and fixation with ice-cold formaldehyde, followed by immediate freeze-preservation, and sorting of the nematodes within 2 h after thawing, gives average values for label leakage of 50%, and hence allows the application of a proximate correction factor for label losses of 2.

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Introduction

Many laboratory and field studies have demonstrated the potential importance of bacteria and microalgae as food to nematodes and harpacticoid copepods, the dominant representatives of the metazoan meiofauna (reviews in Hicks and Coull 1983; Heip et al. 1985; Montagna 1995; Moens and Vincx 1997); yet their importance as grazers remains to be established. This is due mainly to (1) the uncertainties involved in extrapolating laboratory-obtained data to a field situation, and to (2) the many methodological difficulties with in situ experiments with the meiobenthos. The first problem (1) mainly relates to the diverse and intricate sediment–microbiota and meiofauna–microbiota interactions (Gray 1966, 1968; Gray and Johnson 1970; Riemann and Schrage 1978; Warwick 1981; Jensen 1996), which cannot accurately be mimicked in the laboratory.

Field experiments (2) with meiofauna have traditionally employed either of two approaches: either prelabelled food is added to a sediment or microcosm containing the candidate grazers – i.e. basically a two-compartment system where grazing can be calculated from the flow of label from the grazed to the grazer compartment (Haney 1971) – or tracer is added directly to the medium. The latter fits a three-compartment model where label is also present in a "free" pool (Daro 1978). Radioactive tracers have been most frequently used in meiofauna grazing experiments. Fluorescent tracers have rarely been deployed (but see Epstein and Shiaris 1992; Borchardt and Bott 1995); this is mainly due to the time-consuming sample analysis, to difficulties in counting marked cells inside grazers' guts, and to problems with grazer autofluorescence. Direct addition

of label, rather than of labelled cells, has been the method of choice in a majority of studies. From the earlier work, researchers have been duly concerned with the setup of appropriate controls correcting for non-grazing label uptake, including adsorption and absorption processes (Montagna 1983, 1984; Montagna and Bauer 1988; Carman 1990). The potential influence of preservation procedure on grazing rate estimates, although acknowledged in some studies (Montagna 1984, 1993; Blanchard 1991), has, however, remained virtually undocumented for the dominant meiobenthic groups.

A further essential assumption in the calculation of valid grazing rates from Daro's (1978) model is that during incubation, label recycling does not occur. It has generally been assumed that a validation of linear and hyperbolic uptake kinetics in the grazed and grazer compartment, respectively, provides sufficient support for this assumption. Hyperbolic uptake by nematode and harpacticoid grazers in incubations of a few hours has usually been found, though generally based on measurements of only few time points. This is surprising, given the fact that nematodes may consume several times their own body weight per day (Duncan et al. 1974; Tietjen 1980; Woombs and Laybourn-Parry 1984; Heip et al. 1985; Schiemer 1987; Herman and Vranken 1988).

Here we report on the influence of sample preservation on ^{3}H-label retention in the bacterivorous marine nematode *Pellioditis marina*, with emphasis on the impact of formaldehyde, the most commonly used fixative. The general validity of the results obtained with this species–label combination was checked in two experiments with other combinations, nematode–^{3}H and a nematode–^{14}C. Furthermore, the uptake kinetics of labelled bacterial cells by *P. marina* in a two-compartment system are discussed against the background of observations on food ingestion and defecation. The impact of our results on previously published grazing estimates is discussed.

Materials and methods

Except when noted otherwise, the nematode *Pellioditis marina* Andrassy, 1983 was used as the grazer in our experiments. This is a marine representative of an order (the Rhabditida) dominated by terrestrial, freshwater and insect-parasitic nematodes. *P. marina* is typical of organically enriched (micro) habitats, such as decaying seaweeds, worldwide. The strains TM1, isolated from *Fucus vesiculosus* stands in the Westerschelde Estuary, SW Netherlands, and TM2, isolated from a seaweed farm at Paje, east coast of Zanzibar, East Africa, were used in the present study. They were cultivated on 1% agar layers prepared in artificial seawater (ASW) (Dietrich and Kalle 1957) with a salinity of 25, and cultures were kept at 20 or 25 °C in the dark, with unidentified bacteria from the habitat as the food. Details on the isolation and cultivation of this nematode are given elsewhere (Moens and Vincx 1998).

Bacterial batch culture BPM1 consisted of a bacterial isolate from a *Pellioditis marina* culture, grown in Luria–Bertani medium (LB-medium, Sambrook et al. 1989) with a salinity of 25, and was used as the food source in the present experiments, except when otherwise stated. Observations of colony morphology of serial dilutions showed the presence of four or five different bacterial strains, two of which on average comprised more than 70 and more than 25%, respectively, of BPM1 cells.

Bacteria were grown overnight at room temperature in 30 ml of LB-medium in 250-ml aerated Erlenmeyer flasks on a rotary shaker. Cells were harvested by centrifugation and resuspended in fresh growth medium, to which either [2-^{3}H]-adenine or DL-[4,5-^{3}H]-leucine was added in final activities and concentrations of approximately 5 μCi ml^{-1} and 200 to 800 nmol, respectively. Such cultures were again allowed to grow overnight at room temperature. Cells were harvested by centrifugation of 1-ml aliquots at 8000 rpm for 5 min. Pellets were resuspended in sterile ASW. This washing procedure had to be repeated three more times for an efficient removal of non-incorporated label (Moens unpublished). Approximately 20 to 25 and 15 to 20% of the label originally added in the form of adenine and leucine, respectively, were stably incorporated by the bacteria. Label release from BPM1 cells so prepared was less than 5% during 1-h incubations at 20 to 25 °C; it remained at that level with ^{3}H-leucine as the tracer, but increased to 17% over 24 h with ^{3}H-adenine as a tracer. Since *Pellioditis marina* appears to efficiently filter the bacterial cells from the medium, the presence of a small amount of "dissolved" ^{3}H is unlikely to have biased uptake rates (Avery and Thomas 1997; Moens unpublished).

For experiments, adult nematodes were hand-picked from cultures and transferred to 450 μl of sterile ASW in a 3.5 cm diameter petri dish with a hydrophilic bottom layer. At time zero (T_0), 150 μl of a suspension of labelled BPM1 cells was added, and the total 600 μl gently agitated to form a thin water film; preliminary experiments showed that it was imperative for normal feeding activity that the nematodes were supported by a solid substrate and not suspended in a water layer. Each replicate petri dish received 50 nematodes. Although only 25 to 40 were used for final analysis, the addition of a surplus greatly reduced sorting time. A minimum of 20 *Pellioditis marina* were needed to obtain variance levels of less than 10% of the mean on average. Different numbers were required with other nematodes depending on species size and ingestion rates (Moens unpublished). Feeding experiments were terminated in any of a number of ways described below. Before analysis, nematodes were hand-picked from the experimental dishes and transferred twice through sterile ASW before the final transfer to a scintillation vial. Preliminary tests showed that each transfer step reduced the adsorbed activity – mostly in the form of bacteria attached to the nematodes' cuticle – approximately tenfold.

After rinsing, nematodes were dissolved for 24 to 48 h in 1 ml of Lumasolve (Lumac). Radioactivity was determined by liquid scintillation counting in a Beckmann LS6000 after addition of 10 ml of a compatible scintillation cocktail, here Lumasafe + (Lumac). Each sample was counted twice per run with a counting time of 10 min. Quenching was corrected for by the external standards method. Occasional samples where counting efficiency was less than 45% (90% in the experiment with ^{14}C) were rejected.

Except when noted otherwise, nematodes were allowed to graze for 1 h. Incubations were terminated by the addition of formaldehyde (Treatment 1) or glutaraldehyde to a final concentration of 2% (Treatment 2), by vol/vol dilution with reagent grade ethanol (Treatment 3), or by rapid freezing in liquid N_2 (Treatment 4). For the latter treatment, the nematode–bacteria suspension was pipetted into an Eppendorf tube and briefly immersed in liquid N_2, after which the tubes were stored frozen at −80 °C until sorting. Nematodes were sorted within 2 h after thawing. For the other treatments, nematodes were sorted 24 h after fixation. The influence of formaldehyde concentration was assessed by fixation with formaldehyde in final concentrations of 1, 2, and 4%. The first treatment (1) was repeated with heated (80 °C) and cooled (2 to 4 °C) formaldehyde. In the latter case, nematodes were first cooled on ice for 3 min before addition of cold fixative.

The influence of preservation time or time before sorting was assessed by sorting samples preserved in formaldehyde (Treatment 1) 1 h and 1, 2, and 7 d after termination of the feeding experiment. Here too, nematodes were cooled on ice before addition of the fixative. Samples were kept at room temperature until sorting.

All ingestion rates so obtained were compared to rates determined on nematodes which were hand-sorted live immediately after termination of a feeding experiment. The entire transfer and washing procedure took no longer than 15 to 20 min for each sample of 35 nematodes. In order to determine non-grazing label uptake, T_0 controls were added. These samples were preserved with 4% formaldehyde immediately upon addition of the labelled cell suspension. While previous studies have shown that – especially in experiments where tracer is added as dissolved label – T_0 controls provide inadequate correction for non-grazing uptake processes (Montagna 1983; Jarvis and Hart 1993), we did not detect any significant differences between T_0 controls and controls consisting of nematodes which had been killed beforehand, washed and incubated with labelled bacteria.

All experiments reported here used three replicates per treatment and three controls, except in the ^{14}C-trials with only two controls. All data presented have been corrected for T_0-controls or, in the *Adoncholaimus fuscus* experiment (see below) for prekilled controls, by subtracting the average control value from each experimental value. Errors, therefore, are given as the sum of the errors on control and grazing data (Peterson and Renaud 1989; Montagna 1993). Data were compared by one-way analysis of variance (ANOVA) on a set of nine $\log_{10}$-transformed hypothetical replicates per treatment, obtained by correcting each of three observed replicate values for each of three control values. Specific effects were tested through pairwise comparison of means with Tukey's honest significant differences test, using an experimentwise α-level of 0.05.

As a means to test the generality of our results for other nematodes and for other food sources, two further experiments with different nematode–tracer combinations were performed. First, the large facultatively predatory (Moens and Vincx 1997) nematode *Adoncholaimus fuscus* De Man, 1865 was incubated with unwashed aliquots of a heat-killed ^{3}H-adenine labelled BPM1 culture. Approximately 80% of the tracer pool in these incubations was present in a dissolved form – a significant part of which may have been ^3H_2O (Brittain and Karl 1990) – or associated with bacterial exudates. This nematode ingested bacterial cells at low rates, but took up significant label when offered unwashed culture aliquots (Moens et al. in preparation). Fifteen *A. fuscus* adults, isolated from freshly collected sediment of an intertidal mudflat in the Westerschelde Estuary, SW Netherlands, were incubated in 3.5 cm diameter petri dishes, bottom-covered with 1 g of sterile sediment, to which 1 ml of BPM1 culture was added. The nematodes were allowed to feed for 24 h, and were subsequently killed by the addition of formaldehyde, glutaraldehyde, or ethanol as detailed above (Treatment 1, 2 and 3). Formaldehyde-preserved samples were sorted 1, 2, or 7 d after fixation, the others all after 1 d. As controls, nematodes which had been killed beforehand with formaldehyde and had subsequently been rinsed with ASW, were incubated under the same conditions.

Second, as part of a field study of nematode grazing rates on microphytobenthos on an intertidal mudflat (Station 2, the Molenplaat, Westerschelde Estuary, for details on the biotic and abiotic environment of this site see Hamels et al. 1998), the top 1-cm horizons of a series of sediment cores were incubated with 20 μCi of $NaH^{14}CO_3$ and allowed to stand for 1 h at 20 ± 1 °C in the light. Incubations were terminated by cooling the samples on ice and adding formaldehyde (4% final concentration); samples were immediately frozen at −20 °C or kept at room temperature for 1 h, 1 d, or 7 d before sorting. Meiofauna was elutriated via centrifugation-flotation with the colloidal silicagel Ludox HS40 (DuPont) (modified after de Jonge and Bouwman 1977). Nematodes (200 per sample) were then hand-picked, rinsed and analysed by liquid scintillation counting as described above. Three replicate samples each were thawed 1, 7, and 60 d after termination of the experiment and sorted 1 d after thawing. Because of high uptake rates in dark controls, even exceeding rates in the light incubations in prolonged (2 h and more) feeding trials, the results of this experiment have been corrected only for T_0 controls; the pathways of label uptake by the meiofauna in this experiment remain to be discussed.

The influence of incubation time on ingestion rates in *Pellioditis marina* was investigated in an experimental setup as described above, with formaldehyde (4% final concentration) as the fixative after cooling of the nematodes on ice, and with sample sorting 1 d after termination of the experiment. Ingestion rates were calculated based on incubation times of 5, 10, 15, 30, 45, 60, 75, 90, 120, 240, 360, 480, and 1440 min, with reference to T_0 controls.

Results

Figure 1 depicts the results of the experiment on the time-dependence of preservation with formaldehyde on the retention of ^{3}H-label in *Pellioditis marina* after grazing on bacteria. It demonstrates that, after correction for T_0 controls, the amount of label still present after 1 d represents but a minor fraction (ca. 15%) of total label taken up (ingested and/or assimilated). Roughly 40% of the label losses occur during the first hour after preservation, and losses appear to stabilize from 1 d onwards.

No significant differences were found between uptake values (disintegrations min^{-1}, dpm) obtained after preservation with different formaldehyde concentrations (Fig. 2). Glutaraldehyde at 2% final concentration yielded uptake rates almost twice those with formaldehyde. By contrast, uptake rates calculated from ethanol-preserved samples were only half those obtained with formaldehyde. Preservation with liquid N_2 yielded uptake values superior to those with formaldehyde, but specimens were poorly preserved and many could not be sorted intact. As a consequence, sorting of particular species or genera would be seriously hampered after N_2 preservation. With the addition of formaldehyde just before freezing in liquid N_2 or immediately upon thawing, nematodes were adequately preserved and calculated average uptake rates were up to 20% higher (but $p > 0.05$) (Fig. 2).

Table 1 shows label uptake as calculated after formaldehyde fixation of *Adoncholaimus fuscus*: approximately 65% and 25% of the label was retained after

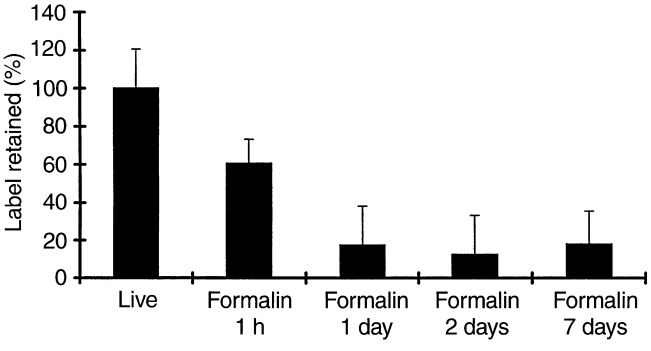

Fig. 1 *Pellioditis marina*. Label retention (%) by the nematode *P. marina* fed ^{3}H-labelled (adenine as the carrier) bacterial cells and kept in formaldehyde for different periods of time. Activity levels in nematodes not preserved with formaldehyde are given as reference. Means of three replicates ±1 SE are given

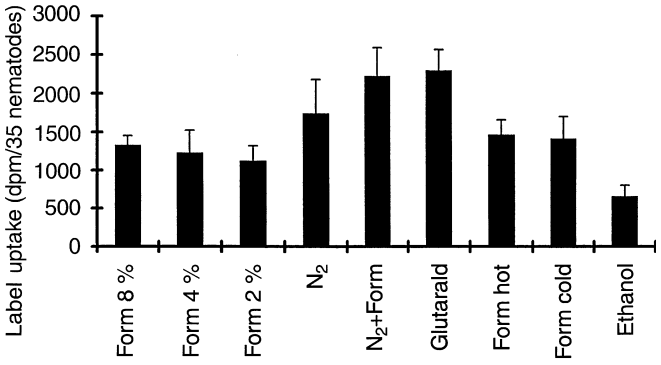

Fig. 2 *Pellioditis marina.* Radioactivity levels inside *P. marina* after feeding on ^{3}H-labelled (adenine as the carrier) bacterial cells as a function of preservation procedure. All nematodes were sorted 24 h after the feeding experiment. Frozen samples were sorted within 2 h after thawing. Means of three replicates ±1 SE are given

storage in formaldehyde for 1 h and 1 d, respectively, without a significant further decrease with time. Here too, glutaraldehyde and ethanol proved superior and inferior fixatives, respectively, to formaldehyde. With ^{14}C as the tracer and a field sample of nematodes as the grazers, label losses with formaldehyde between 1 h and 1 d were less pronounced than in the previous experiments (46.5% vs 60 and 71.5% in the experiments with *A. fuscus* and *P. marina*, respectively), while ethanol fixation yielded uptake values almost sixfold lower than did formaldehyde fixation. Prolonged storage (up to 7 d) in formaldehyde at room temperature did not result in a further label loss, and samples frozen at −20 °C for up to 60 d gave the same results as did samples sorted after 1 d (Table 1).

Figures 3 and 4 illustrate the uptake of ^{3}H in *Pellioditis marina* feeding on labelled bacterial cells over time scales of hours to minutes. Uptake is linear with time up to 8 h, after which it levels off (Fig. 3A). A linear relation is also obtained when plotting curves from measurements over 15-min intervals (Fig. 4A). While the average "ingestion" rates per quarter differ by a factor of up to three, these differences are not statistically significant ($p > 0.05$). Measurements over 5-min intervals show a small but non-significant decrease in incorporated activity during the second interval (Fig. 3B).

Discussion

Formaldehyde preservation has previously been shown to affect biochemical, biometrical and biomass characteristics of invertebrates (Kapiris et al. 1997, and references herein). The present results demonstrate that significant label loss from meiofauna occurs upon preservation with chemical fixatives. Losses occur from the moment of fixation, and almost 40% on average of the total losses upon preservation with formaldehyde transpire within the first hour. No further label losses are found beyond 1 d of incubation, in agreement with previous findings on meiofauna (Montagna 1984), but in conflict with observations on cladoceran zooplankton (Holtby and Knoechel 1981). Assuming constant label leakage from one homogeneous pool down to a maximum loss, the disappearance of label as a function of time can be described by an exponential model, $l_t = l_{\max}\ (l - e^{kt})$, where l_t and $l_{\max}$ are the loss percentage at time t and the maximum loss, respectively, and where k is a rate constant (Holtby and Knoechel 1981). Using the observed l_t at 1 h, and with an $l_{\max}$ of 85% (Fig. 1), $k = -0.636$ expressed in units of percentage per hour. In the *Adoncholaimus fuscus* experiment $k = -0.604$. These values are intermediate between loss rate constants in zooplankton fed ^{32}P-labelled yeast and preserved in ethanol or lugol's iodine, but are considerably lower than k-values obtained with ^{14}C-labelled algal food (Holtby and Knoechel 1981). Label leakage in our experiments was independent of formaldehyde concentration. Blanchard (1991) preserved samples with a final formaldehyde concentration of 0.33% to reduce label leakage, but this is not recommended because of poor preservation and slow killing of specimens. Indeed, higher formaldehyde concentrations yielded slightly higher average retention values.

To our knowledge, the label losses reported here are the highest hitherto found in comparable radioactive tracer studies on metazoans, but they are consistent with the idea that average $l_{\max}$ values exceed 50% with formaldehyde as the fixative. It should be emphasized that our interpretation of loss rates is critically dependent on the assumption that the untreated live nematodes did not lose significant label upon transfer to the

Table 1 Radioactivity levels inside nematode grazers after feeding in a ^{3}H- or ^{14}C-enriched environment: comparison of label retention with different preservation procedures (*n.d.* not determined)

Preservation procedure	*A. fuscus*–^{3}H experiment		Nematode–^{14}C experiment	
	dpm/15 ind.	±1 SE	dpm/200 ind.	±1 SE
Living nematodes	2472	255	n.d.	n.d.
4% formalin 1 h	1644	112	130	26
4% formalin 1 d	658	48	70	9
4% formalin 2 d	609	39	n.d.	n.d.
4% formalin 7 d	639	42	75	15
4% formalin 1 d + 60-d storage	n.d.	n.d.	68	17
2% glutaraldehyde 1 d	870	54	n.d.	n.d.
Ethanol 1 d	293	11	12	9

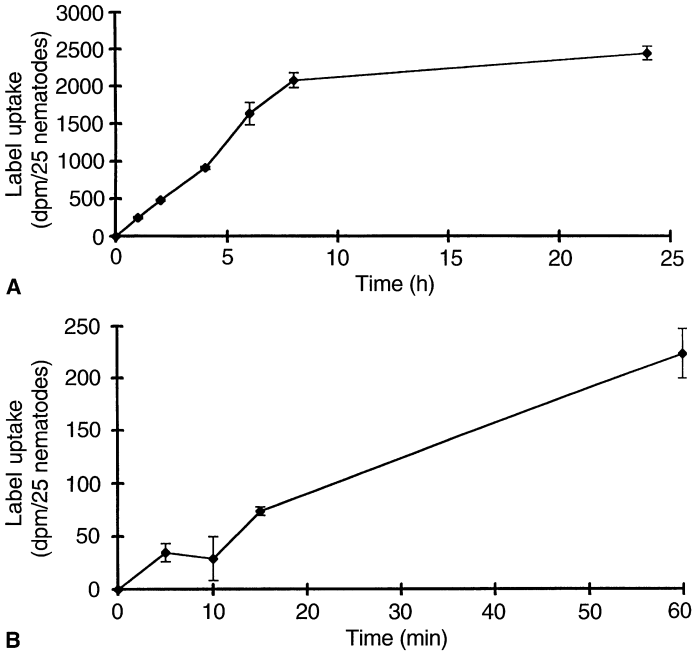

Fig. 3 *Pellioditis marina.* Radioactivity levels inside *P. marina* after feeding on ^{3}H-labelled (adenine as the carrier) bacterial cells as a function of incubation time: **A** 1-h intervals; **B** 5-min intervals. Means of three replicates ±1 SE are given

scintillation vials. It will be argued below that this assumption was probably adequately met, but our loss rates nevertheless remain conservative.

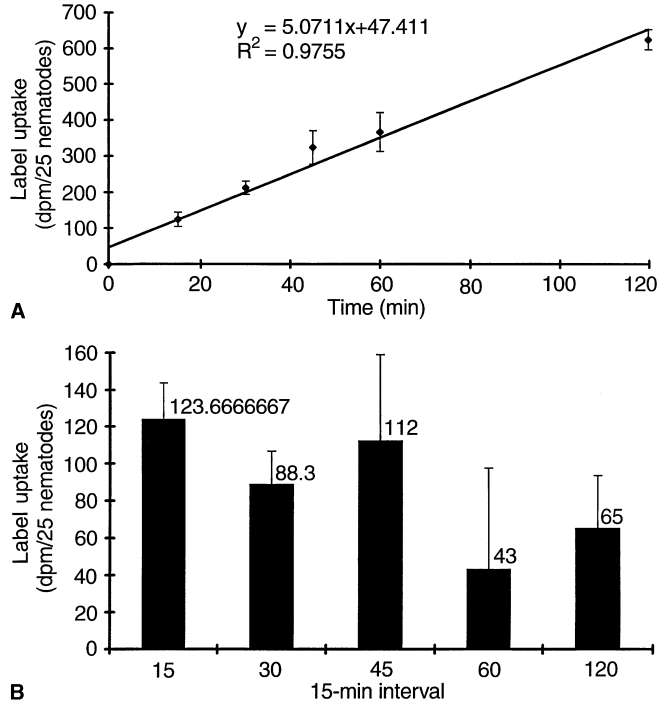

Fig. 4 *Pellioditis marina.* **A** Radioactivity levels inside *P. marina* after feeding on ^{3}H-labelled (adenine as the carrier) bacterial cells as a function of incubation time: 15-min intervals. **B** Uptake per 15-min interval. Means of three replicates ±1 SE are given

Compared to formaldehyde, glutaraldehyde yielded retention values almost twice as high (30% vs 15%). As a dialdehyde, glutaraldehyde cross-links proteins and peptides, which may prohibit leakage. Glutaraldehyde may therefore be a proper alternative to formaldehyde in studies with radioactive tracers. By contrast, ethanol gave inferior results. Ethanol has been used with varying success in zooplankton feeding studies, depending on the nature of the food offered (Holtby and Knoechel 1981; Mourelatos 1990). Fixation with liquid N_2 poorly preserved the nematodes, except when combined with a chemical preservative. In the latter case, the loss percentages were comparable to those after a 2 h preservation with that chemical only.

Although the cuticular structure and permeability vary among nematode taxa (Bird and Bird 1991), and inspite of previous reports on the relationship of label leakage to the nature of the food (see above), the results from the *Adoncholaimus fuscus*–^{3}H and from the nematode–^{14}C experiments appear to lend more general validity to our results on *Pellioditis marina*. Indeed, the species studied in this paper are taxonomically quite distant, with *P. marina* belonging to the subphylum Secernentea, all others to the Adenophorea. They also have distinct feeding modes: while *P. marina* selectively ingests bacteria (Tietjen et al. 1970; Moens unpublished), *A. fuscus* displays a variety of feeding strategies, probably including a non-selective particle ingestion and "drinking" of dissolved material (Moens and Vincx 1997), and the nematode community of Station 2 on the Molenplaat at the time of our experiment covered all but one of the traditionally recognized marine nematode feeding guilds (Wieser 1953; Moens and Vincx 1997). Mourelatos et al. (1992) also found a general food type–independence of label losses from cladoceran zooplankton upon freeze-preservation with formaldehyde.

We started from the hypothesis that initial label losses (occurring immediately upon preservation) would largely result from egestion (defecation and/or regurgitation), while further losses would result from label leakage due to increased permeability. While the magnitude of the label losses during the first hour after fixation could be interpreted in support of this idea, all data obtained from preservation after cooling of samples on ice or with instantaneous killing of the grazers point at a different conclusion.

In studies of protistan grazing, egestion of particles upon preservation is commonly blocked by putting the grazers on ice before the addition of ice-cold fixative (Sanders et al. 1989; Sherr and Sherr 1993). *Pellioditis marina* too is strongly inactivated when suddenly cooled on ice, and is therefore unlikely to egest considerable gut content amounts upon chemical preservation at <5 °C. Vice versa, instantaneously killing the nematodes by heat shock and then adding formaldehyde leaves little or no opportunity for egestion. By contrast, addition of formaldehyde at room temperature does not instantaneously kill the nematodes, and thus allows the regurgitation or defecation of ingested cells. Indeed, average label retention in *P. marina* first cooled or heat-killed after 1 h feeding is almost twice as high as with the addition of the same fixative at room temperature (ca. 28% vs 15%). These differences suggest that but a limited portion of total observed label losses is due to egestion. This portion is minimized when grazers are inactivated by cooling on ice before addition of the fixative, and by no means can it explain the bulk of label losses within the first hour of preservation.

When food is plentiful, *Pellioditis marina* feeds by a continuous ingestion and filtration (see Avery and Thomas 1997, for similar observations on the closely related *Caenorhabditis elegans*) of medium containing bacterial cells. This ingestion is visible under the light microscope as contractions of the oesophageal bulbi. Gravid females of *P. marina* (strain TM1) fed 10^9 to 10^{10} cells ml^{-1} at 20 to 25 °C, ingested food at 50 to 100 pulsations min^{-1} (Moens et al. 1996), and corresponding rates were twice as high with BPM1 cells as the food and TM2 nematodes as the grazers (Moens unpublished). This is comparable to rates reported for other rhabditid nematodes (Woombs and Laybourn-Parry 1984, and references herein). At the same time, defecation intervals under similar feeding conditions were in the order of a few to less than 1 min in other rhabditids (Mapes 1965; Duncan et al. 1974; Croll 1975; Croll et al. 1977; Thomas 1989). Preliminary observations revealed defecation intervals of 49 s to just over 6 min in three *P. marina* adults under feeding conditions identical to the ones used in the tracer experiments (Moens unpublished). Hence, linear uptake kinetics, in incubations of up to 8 h (Fig. 3A), cannot be interpreted in support of the assumption that label recycling does not occur within this period. Rather, like Schiemer (1987), we propose that they indicate assimilation instead of ingestion. Similarly, the accuracy of tritiated tracer to estimate rates of bacterivory in protozoan grazers was strongly related to the residence time of food vacuoles (Caron et al. 1993).

In support of the validity of our ingestion measurements on live nematodes, the study of Thomas (1989) can be mentioned; Thomas observed a reduction in defecation rates in *Caenorhabditis elegans* from 14 per 10 min to 0.5 per 10 min when nematodes were transferred from a bacteria-rich to a bacteria-free environment. Touch stress, as caused by the manual transfer of nematodes in our experiments, delayed rather than induced defecation in *C. elegans* (Thomas 1989). Hence, with total transfer times of less than 20 min, significant defecation probably did not occur.

If assimilation is a constant fraction of ingestion, then the kinetics of assimilation are likely to conform to those expected for ingestion from Haney's (1971) or Daro's (1978) models. Dilution of labelled cells inside *Pellioditis marina* guts with non-labelled food still present at the onset of an experiment is unlikely to have an important effect, since (1) defecation intervals are so short, and (2) mixing of gut contents is largely a passive process effected by body movements (Schiemer 1987). Bias may result from an increased ingestion rate at the start of the experiment, when nematodes starved for up to 3 h are offered food in abundance. However, this effect may be partly counteracted by the brief stress which food addition and spreading of the water film is likely to have induced in the nematodes.

The linear regression of uptake (in units of disintegrations min^{-1}, dpm) over time (expressed in minutes) conforms to an equation of the form $y = a + bx$, where b is in units of dpm min^{-1} and a is in dpm. Conceptually, a can be considered as the amount of radioactivity inside the grazers at any time during the experimental incubation and not attributable to assimilation. This portion can be conceived of as representing a "gutfull" of not yet assimilated bacterial cells, or as an approximation of the number of cells ingested during one average defecation interval. Can a values so calculated give realistic estimates of ingestion rates? Let us, e.g., consider the regression equation of Fig. 4A, where $a = 47$ dpm 25 $nematodes^{-1}$. For ease of calculation, we take a as 2 dpm $nematode^{-1}$. With 1 dpm corresponding to approximately 10^4 cells, each nematode contained 2×10^4 cells. Assuming that (1) nematodes fed and defecated continuously at the same rate, and that (2) the gut volume voided at each defecation was proportional to the amount of food ingested over an average defecation interval (see below), this corresponds to ingestion rates of 28.8×10^6 and 4.8×10^6 bacteria d^{-1} at average defecation intervals of 1 and 6 min, respectively. Tietjen et al. (1970) reported highest ingestion rates of *Pellioditis marina* on the bacteria *Pseudomonas* sp. of 43×10^6 cells ind^{-1} d^{-1}, corresponding to 15 μg wet weight. Assuming an average cell weight of 10^{-12} g for the bacteria in our experiments, each *P. marina* consumed 28.8 and 4.8 μg of bacteria d^{-1} at the shorter and longer defecation interval, respectively. The average individual wet weight of the *P. marina* used in this experiment was 1.2 μg; hence,

these values corresponded to, respectively, 26 and 4 times the nematode's body weight per day. The latter value compares well to consumption rates of 3 to 8 times body weight per day in related rhabditids under comparable conditions of temperature and food (Tietjen 1980; Woombs and Laybourn-Parry 1984; Schiemer 1987). Assuming that C is 12.4% of nematode fresh weight (Jensen 1984) and that 1 mg C equals 45.7 J (Schiemer 1987), Tietjen's (1980) energy budget for *P. marina* yields a food consumption of 3.02 times the nematodes' body weight per day.

Nematodes void but part of their gut contents upon defecation. Since defecation is pressure-related, the volume voided per average defecation is, however, likely to be proportional to the average volume of food ingested in between two defecations, and defecation intervals are not very much shorter than average gut residence times (Avery and Thomas 1997). Defecation rates in *Pellioditis marina* may not be representative of other marine nematodes. Defecation intervals of less than 4 to 43 min were observed in two actively foraging *Daptonema setosum*, and of 14 and 23 min in an adult female *Spilophorella* sp. piercing and emptying approximately 20 diatom cells per 15 min (Moens unpublished). Similarly, gut passage times of 14 to 26 min and of as short as 5 min have been found in harpacticoid copepods (Santos et al. 1995; Souza Santos 1995). Long gut residence times reported for a monhysterid (Deutsch 1978) and an oncholaimid (Lopez et al. 1979) nematode may be related to conditions of starvation.

The average difference in label retention between, respectively, grazers preserved in formaldehyde at room temperature (i.e. with significant egestion of food) and grazers preserved with the same fixative but after cooling on ice (i.e. without significant egestion), was 14.8% after a 1 h feeding incubation (Fig. 2). The *a* value – if indeed considered an adequate approximation of the amount of bacteria ingested over an average defecation interval – of Fig. 4A (also without significant egestion) corresponds to 13.5% of the activity inside grazers after 1 h feeding. Because these data stem from two different experiments, any direct comparison may be spurious; however, they do suggest that formaldehyde preservation without prior inactivation or without instantaneous killing may result in the egestion of a significant portion of the nematodes' gut contents. On this basis, the leakage rate constant *k* can be recalculated, assuming a label loss due to egestion of 15% of the total label present after a 1 h feeding period, as $-0.44183\%\ h^{-1}$; leakage then equals 70% of the total label taken up.

How can losses of 70% of assimilated label upon chemical preservation be explained if uptake rates are so high and weight losses in nematodes upon preservation with formaldehyde are relatively small (up to 24%, Jensen 1984)? It can be expected that mostly low molecular weight (LMW) compounds leak. Rivkin and DeLaca (1990) characterized the incorporation of ^{14}C from labelled algae into lipids, polysaccharides, proteins, and LMW compounds in five different grazers, and found that after a 12 h incubation up to 70% of the incorporated label in a small polychaete was present in the LMW fraction. A partial shift towards polysaccharides and proteins occurred during prolonged feeding incubations. Similarly, Nicholas and Viswanathan (1975) found that after a 24 h feeding period 30% of the carbon ingested by the terrestrial nematode *Caenorhabditis briggsae* was still present as LMW metabolites. There can be little doubt that this fraction will be much higher after feeding trials of only 1 h. If the incorporation of ^{3}H follows a similar pattern, then the high loss rates can indeed be considered as being the result of leakage of LMW metabolites.

Montagna (1995) recently reviewed published grazing estimates of meiofauna on microalgae and bacteria. While substantial differences have been found among studies and study sites, an overall system-wide conclusion suggests a broad balance of meiofauna grazing with microphytobenthic primary production as well as with bacterial secondary production. The present results illustrate that the intrepretation of these data is fraught with methodological difficulties. The sources of bias identified here should have generally resulted in a significant underestimation of true meiofauna grazing rates. The first source of bias is egestion of food. Since most studies have not cooled samples on ice before fixation or instantaneously killed the meiofauna, significant prey egestion may have generally occurred. As a consequence, most published grazing rates probably give an indication of assimilation or of a combination of assimilation and ingestion, not of ingestion per se. The second source of bias is the long incubation times ($\geq$1 h) which have generally been used and which far exceed average defecation intervals. These too suggest that the label inside the grazers reflects assimilation – after correction for label leakage (see below) – rather than ingestion. If this is true, and assuming an assimilation efficiency of 25% (Herman and Vranken 1988), this misinterpretation of label uptake alone could be responsible for as much as a fourfold underestimation of true grazing rates. The third and most important source of bias is in the high portion of assimilated label that leaks from the grazers upon chemical fixation (up to 70% with formaldehyde as the fixative); as a consequence, assimilation itself may have generally been underestimated by a factor of almost four. Whether this simple calculation can be applied to reinterpret published grazing rates depends, among other things, on the type of tracer molecule used. Adenine, e.g., is incorporated in many low molecular weight compounds, such as adenosine mono-, di- and triphosphate, and is therefore likely to yield higher leakage rates than, e.g., thymidine, which is metabolically more conservative and is mainly incorporated in nucleic acid. We preferred the use of adenine over thymidine because ^{3}H from adenine was incorporated by the bacteria at a tenfold higher rate (see Riemann et al. 1990; Brittain and Karl 1990, for similar observations on other marine and brackish-water bacteria). The similar label leakage and leakage kinetics

from nematodes fed ^{3}H-adenine- and $H^{14}CO_3^-$ -marked foods, do, however, suggest that the magnitude of the observed label losses is more generally valid.

Meanwhile, the best protocol for preservation and sorting of meiofauna after tracer-aided grazing experiments is one where (1) samples are cooled on ice before fixation, or alternatively, killed instantaneously with liquid N_2, so that egestion is kept to a minimum. This aspect is of particular relevance also to grazing studies using fluorescent rather than radioactive tracers. (2) Incubation time is kept to a minimum, which unfortunately often does not allow a sufficient label build-up in the grazers (Montagna 1993). (3) Samples are stored frozen with formaldehyde, or formaldehyde is added immediately upon thawing of the samples. And (4), the use of glutaraldehyde instead of formaldehyde may further reduce label leakage, but it may increase problems of background fluorescence when fluorescent tracers are used. Samples can be stored for more than 2 months without affecting the portion of label found inside grazers, enabling the setup of a large experiment with proper replication, even if one is to do all the time-consuming sorting alone. Each sample, then, should be sorted within 2 h of thawing. Our results suggest that under these conditions, label leakage may average 50%, and a correction factor of 2 may be applied. A similar protocol and correction factor have been proposed for cladoceran zooplankton by Mourelatos (1990).

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