Marine Biology 98, 101–110 (1988)

Marine Biology
International Journal on Life in Oceans and Coastal Waters

Partitioning radiolabeled thymidine uptake by bacteria and meiofauna using metabolic blocks and poisons in benthic feeding studies*

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Abstract

Techniques exist which allow for the measurement of in situ grazing rates of meiobenthos on sedimentary bacteria and microalgae. Radiolabeled substrates are incorporated into microbes which serve as food for meiofauna and which themselves also become labeled during feeding. However, during in situ grazing experiments, meiofauna may become radiolabeled by a variety of non-feeding processes. Proper controls to correct for these extraneous routes of labeling have been developed in the present study. The use of [methyl-^{3}H] thymidine (^{3}HTdR) in studies of meiofaunal grazing on bacteria has two unique advantages: (1) it is incorporated only into prokaryotic macromolecules, and (2) bacterial incorporation of ^{3}HTdR may be selectively blocked by several inhibitors which are non-toxic to meiofaunal grazers. Coupled with formalin-killed control treatments, the use of these inhibitors makes it possible to accurately determine the partitioning of radiolabel into meiofauna during grazing into adsorptive, absorptive and grazing components. A saturated solution of nalidixic acid and 5′-deoxythymidine was found to be most effective in inhibiting water-column bacterial uptake and incorporation of ^{3}HTdR, and had no toxic effects on meiofauna. The inhibitor was found to immediately block bacterial incorporation of ^{3}HTdR and was as effective at 20% saturation as at 100%. The presence of sediment reduced the effectiveness of this inhibitor by 50%. Solutions of the inhibitor with excess undissolved material, however, completely blocked sediment bacterial uptake of ^{3}HTdR. Employing these techniques during in situ grazing experiments showed that up to 83% of total meiofaunal uptake of ^{3}H-label may be attributable to non-grazing processes. Experiments conducted in slurried sediments yielded grazing rates which were the same as those from intact cores. Furthermore, meiofaunal grazing rates on multiple food sources (e.g. bacteria and diatoms) may be determined synoptically by adding isotopically-distinct substrates (e.g. ^{3}HTdR and $H^{14}CO_3$) to the same experimental incubation.

Introduction

There are two techniques for employing radioactive tracers to measure the flow of organic material in food webs via grazing. Microbial food is either pre-labeled (Haney 1971) or labeled while it is being grazed (Daro 1978). Both techniques have advantages and disadvantages that limit their use to either laboratory or field studies. The pre-labeling technique requires growing bacteria with a radioactive tracer, introducing the labeled bacteria to the existing microbial community, and knowing the specific activity of the food source. Such conditions are best achieved in laboratory studies. The synoptic labeling technique is more amenable to in situ studies because only the radioactive tracer is introduced, and microbial uptake of the tracer and meiofaunal grazing can be measured at the same time. However, in situ grazing studies require proper controls because meiofauna may absorb and adsorb dissolved organic matter (DOM) (Montagna 1983, 1984a, b). A properly designed in situ meiofaunal grazing experiment must consider isotopic uptake by three components: the benthic community as a whole (which includes bacteria, meiofauna, protozoans, and macrofauna), biotic absorption by bacteria and meiofauna individually, and abiotic adsorption by sediments and organisms. Proper controls for laboratory grazing studies using the pre-labeling technique would also require the measurement of these three components if recycling of the tracer by the labeled microbe were extensive.

Through the appropriate use of inhibitors and poisons, experiments can be designed that account for label uptake by several of the components in a grazing experiment. Label uptake by bacteria and meiofauna can be partitioned by selectively inhibiting bacterial metabolism through the use

* University of Texas Marine Science Contribution No. 698.

of antibiotics (Fuhrman and McManus 1984) or inhibitors of DNA synthesis (Findlay et al. 1984). Abiotic uptake may be estimated by the use of a general biological poison (e.g. formalin).

In the present study, we sought to develop and improve the technique for measuring in situ meiofaunal grazing rates on sediment bacteria using ^{3}HTdR as a tracer. We were specifically interested in determining if (1) bacterial uptake and incorporation of ^{3}HTdR could be suppressed through the use of metabolic inhibitors; (2) meiofauna could take up significant amounts of ^{3}HTdR directly; (3) meiofaunal viability is affected by the microbial inhibitors; (4) uptake of ^{3}HTdR by meiofauna could be separated into grazing and nongrazing processes; (5) intact sediment cores were necessary for in situ grazing experiments; (6) the rate of meiofaunal grazing on heterotrophic and autotrophic microbes could be measured in the same incubation; and (7) the results of the various control experiments could be used to calculate the appropriate estimates and variances of meiofaunal grazing rates.

Materials and methods

Sediments were collected from an intertidal sand flat in San Francisco Bay (SFB) at Alameda, California, USA in 1985. Sediments consisted of fine to medium-grained sands and were collected with 60 cm^3 syringe corers at low tide. SFB water was also collected in an acid-washed Nalgene bottle. Samples were processed within 1 h. Meiofauna were sorted from sediments by decantation. One additional experiment was performed using fine-sand sediments collected from coastal sediments in the Santa Barbara Channel (at a depth of 18 m).

Four microbial inhibitors were used in this study and each has unique bacteriostatic or bactericidal characteristics. Nalidixic acid inhibits the activity of the enzyme DNA gyrase, which is responsible for DNA supercoiling (Cozzarelli 1980). At low concentrations, nalidixic acid inhibits DNA replication and at higher concentrations both transcription and the amino-acyl-tRNA synthetases are inhibited (Wright et al. 1981). 5′-deoxythymidine (2′,5′-dideoxythimidine) is an inhibitor of thymine phosphorylase, which diminishes the amount of thymidine triphosphate available for DNA synthesis (B. Watkins personal communication). Benzylpenicillin inhibits cell division without significantly affecting cell numbers in seawater-bacteria cultures (Ammerman et al. 1984). Cycloheximide is a microbial protein-synthesis inhibitor that also affects 80S ribosome function and growth in eukaryotes. Cycloheximide concentrations of 100 μg ml^{-1} or below have been found to have no effect on bacterial growth or cell division (Fuhrman and McManus 1984).

Experiments were performed to examine the effects of the various inhibitors on thymidine uptake by bacteria and meiofauna and their potential use as controls for grazing experiments. A saturated solution of nalidixic acid (200 μg ml^{-1}) plus 5′deoxythymidine (2 μg ml^{-1}) (hereafter referred to as ND) was used to inhibit prokaryotic uptake of thymidine (Findlay et al. 1984). The effects of benzylpenicillin (10 μg ml^{-1}), and cycloheximide (100 μg ml^{-1}) on thymidine uptake were also investigated. All chemicals were obtained from Sigma, St. Louis, Missouri. The various inhibitor additions were compared to "no treatment" and poisoned controls treated with 2% formalin.

Inhibitor effects on bacterial uptake of ^{3}HTdR

Initially, the effects of inhibitors on microbial uptake and incorporation of ^{3}HTdR (Amersham, Elk Grove, Illinois) were tested using SFB water. Freshly collected water was filtered through a 3.0 μm membrane filter (Millipore Corp., Bedford, Massachusetts) and 10 or 20 ml subsamples were aliquoted to 50 ml sterile, plastic, centrifuge tubes. Inhibitors were added directly to the filtered samples prior to incubation. For sediment incubations, the top 2 cm of each core (12 cm^3) were extruded into a 50 cm^3 sample jar and a slurry was formed by adding 7 ml of one of the inhibitor solutions in 0.02 μm (Gelman) filtered SFB water.

In all filtered-SFB seawater experiments involving microbial or meiofaunal uptake of ^{3}HTdR, 10 ml of a sterile seawater solution containing 0.05 to 0.10 μCi (5 to 10 n*M* final conc unless otherwise noted) of ^{3}HTdR was injected to each sample. Bacterial incubations were terminated by the addition of 100 to 200 μl of a non-radiolabeled solution of thymidine (2 to 10 m*M* final concentration) and samples were chilled on ice. Once chilled, a volume equivalent to that of the initial sample of ice-cold 10% trichloroacetic acid (TCA) was added for 20 to 40 min to extract macromolecules (Fuhrman and Azam 1980, 1982, Moriarty and Pollard 1982, Bauer and Capone 1985). The extracted samples were then filtered onto 0.45 μm (Millipore) HAWP filters, rinsed three times with cold 5% TCA, placed in scintillation vials with 1 ml ethyl acetate and 15 ml Insta-Gel (Packard Instruments Downers Grove, Illinois) and counted on a Tri-Carb, Model # 4530 liquid-scintillation counter (Packard Instruments, Downers Grove, Illinois).

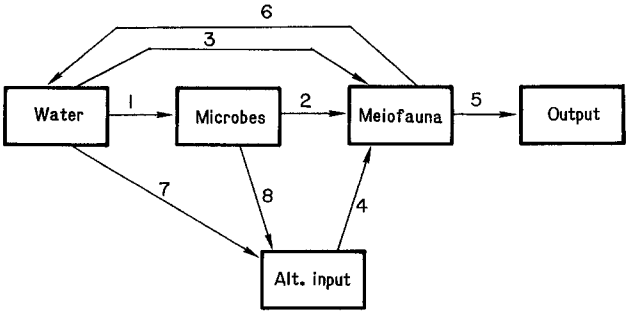

Fig. 1. Label pathways in a grazing experiment. Boxes represent potential compartments, arrows represent flow. Important flow processes include microbial uptake (1), meiofaunal grazing (2), meiofaunal recycling (5, 6), and meiofaunal direct uptake (3). Alternate input (4) includes such components as sedimentary particles, flagellates, or ciliates, which compete with the microbe of interest for label (7), and meiofaunal uptake of microbial metabolites (8)

The first of these experiments examined the effects of incubation time and varying concentrations of ND. Under the incubation conditions described above, SFB water-column bacteria were exposed to concentrations of ND ranging from 0 to 100% of saturation (200 $\mu g\ ml^{-1}$ nalidixic acid and 2 $\mu g\ ml^{-1}$ 5′-deoxythymidine). The exposure time necessary to inhibit bacteria was investigated by exposing bacterial populations to ND for up to 2 h before adding label and then assaying for ^{3}HTdR incorporation. The effectiveness of ND was also compared to other antibiotic agents for both water-column and sediment bacteria.

Inhibitor effects on nematode uptake of ^{3}HTdR

There are eight possible pathways for radioisotopic label to follow during an in situ grazing experiment (Fig. 1). In the present study we were interested in investigating the kinetics of ^{3}H-label appearance in meiofauna via pathways other than grazing. When ^{3}HTdR is used to label sediment bacteria without inhibitors, a fraction of the isotope is taken up by the meiofauna due to (1) epicuticular or gut bacteria, (2) active absorption by the invertebrate, or (3) passive adsorption (Fig. 1). When meiofauna are incubated in sterile seawater without sediments, separation of each of the three processes is achieved by comparing uptake in killed, inhibited, and live (non-treated) samples. Formalin treatments (killed controls) result in uptake by abiotic or passive adsorption only. Bacterial inhibitors (inhibited controls) result in uptake by means of both meiofaunal absorption and adsorption, but inhibit uptake by bacteria associated with meiofauna. These three processes can be calculated using the following formulas (DPM = dis/min):

$$\text{Meiofaunal adsorption} = \text{DPM in formalin treatment.} \quad (1)$$

$$\text{Meiofaunal absorption} = (\text{DPM in inhibitor treatment}) - (\text{DPM in formalin treatment}). \quad (2)$$

$$\text{Associated bacteria} = (\text{DPM in no treatment}) - (\text{DPM in inhibitor treatment}). \quad (3)$$

The effects of ND on nematodes (and any attached bacteria) were examined in a second series of experiments. Fifty nematodes were sorted from sediments by decantation and placed in 10 ml of sterile 0.2 μm-filtered SFB water with or without ND added. ^{3}HTdR was then injected into the samples, which were incubated for varying periods up to 4 h. ^{3}HTdR uptake by nematodes was determined with varying thymidine concentrations (from 1 nM to 10 mM) as well.

When meiofauna were incubated in sterile seawater without sediments, procedures identical to those for bacteria above were used except that TCA extraction was not performed. Meiofauna were simply collected onto 3 μm polycarbonate filters (Nuclepore Corp., Pleasanton, California), dissolved overnight in 200 μl Soluene tissue solubilizer (Packard Corp., Downers Grove, Illinois), and counted in 15 ml Insta-Gel.

To check for the effects of the various microbial inhibitors on meiofaunal viability, 10 to 20 nematodes and polychaetes were sorted and placed in sterile solutions of one of the inhibitors (ND, benzylpenicillin, benzylpenicillin + ND, or cycloheximide) and the number still viable after 66 h was determined.

Use of inhibitors in grazing experiments

A two-way factorial experiment was performed to examine the effectiveness of ND and the effect of slurried sediment on in situ meiofaunal grazing rates. Treatment solutions consisted of either 7 ml of sterile SFB water, ND, or 4% formalin (5 replicates of each treatment). Solutions were added either to the top 2 cm (12 cm^3) of 60 cm^3 intact sediment cores, or to slurries of cores. Two microcuries of ^{3}HTdR in a 10 μl sterile solution were added to the slurries or intact cores, and samples were incubated for 2 h. Incubations were terminated by adding 2% formalin. Preliminary experiments found that subsampling slurries to measure ^{3}HTdR incorporation by sediment bacteria gave highly variable results. This was probably due to the small volume of slurry (1 ml) subsampled. Therefore, in all subsequent experiments, slurry incubations for determination of incorporated ^{3}H-label by sediment bacteria were run in parallel with grazing incubations and the entire sample was cold TCA-extracted.

Meiofauna were separated from sediments by diluting samples with 2% formalin, swirling to suspend the animals, and decanting them and the supernate onto 63 μm Nitex screen filters. Meiofauna were then rinsed into jars and kept in refrigerated 2% formalin until sorting (1 to 2 d). Sorting was performed under a dissecting microscope, and meiofauna were sorted by taxa into scintillation vials containing 1 ml distilled water. After sorting, meiofauna were dried at 60 °C and solubilized in 100 μl Soluene tissue solubilizer for 24 h. Samples were counted by liquid-scintillation spectrophotometry in 15 ml Insta-Gel.

Use of dual labels and controls in grazing experiments

In situ grazing experiments that incorporate all proper controls are highly labor-intensive. To measure grazing on heterotrophs and autotrophs four synoptic incubations with replication are necessary. As a result of this large sample number, the investigator must either sacrifice replication or sort meiofauna for extended periods of time (during which the label can move in and out of meiofauna, causing erroneous results). However, by labeling both heterotrophs and autotrophs in the same incubation, the number of samples may be halved. To design a more efficient protocol for estimating meiofaunal grazing rates on bacteria and/or algae, meiofauna were incubated in slurries with one or both of the radiolabeled substrates ^{3}HTdR and $H^{14}CO_3^-$, and the differences in grazing rates were compared.

Two microcuries of $H^{14}CO_3^-$ in sterile solution were added either individually or simultaneously with 2 μCi ^{3}HTdR to slurried samples. Five replicate slurries each received either ^{3}HTdR, $H^{14}CO_3^-$, or both. The proper live

controls for this experiment consisted of 5 replicate slurries with both ^{3}HTdR and $H^{14}CO_3^-$ added, and which received ND and were incubated in the dark to inhibit photosynthetic fixation of CO_2.

After 2 h incubation, bacterial slurries were extracted as above and a 1 ml subsample was withdrawn from the individual or dual isotope slurries. The subsample was filtered onto a 0.45 μm HAWP and rinsed three times with filtered seawater to estimate uptake of $H^{14}CO_3^-$ by microalgae. The subsample was dispersed and suspended in 5 ml distilled water and 15 ml Insta-Gel for liquid-scintillation counting. Meiofauna were extracted from the remainder of the sample, and incorporation of ^{3}H and ^{14}C label was determined as previously described.

Because both ^{3}H and ^{14}C were potentially present in all experimental treatments receiving both ^{3}HTdR and $H^{14}CO_3^-$, those subsamples for bacteria, algae, and meiofauna were counted using dual-channel liquid-scintillation counting. Corrections for isotopic crossover into each channel were made using the following algorithms (from the Instruments Manual):

$$\mathrm{DPM}_{^3\mathrm{H}} = \frac{A - (E_2 \cdot \mathrm{DPM}_{^{14}\mathrm{C}})}{E_1}, \tag{4}$$

$$\mathrm{DPM}_{^{14}\mathrm{C}} = \frac{B}{E_3}, \tag{5}$$

where E_1 = efficiency of counting ^{3}H in lower energy region, E_2 = efficiency of counting ^{14}C in lower energy region, E_3 = efficiency of counting ^{14}C in higher energy region, A = total counts/min in lower energy region, and B = total counts/min in higher energy region.

By setting the lower limit of the higher energy region at or above the endpoint of the ^{3}H spectrum, the counting efficiency of ^{3}H in the higher energy region reduces to zero.

Calculation of grazing rate estimates and variances

Meiofaunal grazing rates on bacteria and microphototrophs were estimated by the model proposed by Daro (1978) and modified by Roman and Rublee (1981) and Montagna (1984b). The meiofaunal grazing rate (G) is the proportion of material flowing from the donor (or food) compartment to the recipient (or predator) compartment per hour. G is expressed in units of h^{-1} and is calculated as follows (Montagna, 1984b):

$$G = 2F/t, \tag{6}$$

$$F = M/B, \tag{7}$$

where F is the fraction of DPM in meiofauna (M) relative to bacteria (B) at time t. In this discussion, B could be any microbe. Since $2/t$ is a constant, variability in G is only due to variability in M and B.

There are two ways in which to obtain values of M and B. Either each core is subsampled to obtain a value for B and the rest of the core is sorted to obtain M, or M and B can be estimated independently. For each case, the mean of the fraction ($\bar{F}$), and the variance of the fraction S_F^2, is calculated in different ways. When F is measured by subsampling for M and B:

$$\bar{F} = \sum \frac{F_i}{n}, \tag{8}$$

$$S_F^2 = \sum \frac{(F_i - \bar{F})^2}{n-1}. \tag{9}$$

When estimating F by independently measuring M and B:

$$\bar{F} = \frac{\bar{M}}{\bar{B}}, \tag{10}$$

$$S_F^2 = \frac{S_M^2 + \left(\frac{\bar{M}}{\bar{B}}\right)^2 \cdot S_B^2}{(\bar{B})^2}. \tag{11}$$

The independent sampling method is more accurate, but better precision is attained by the subsampling technique.

Since grazing rates must be measured as a function of two estimates, i.e., experimental and control values, S_F^2 is also a function of the variances of the two values (Kempthorne and Allmaras 1965). There are two different ways grazing rates can be corrected using control values, and each has different implications for calculating the precision of the grazing rate. Control or correction values are subscripted with a c below. Since,

$$\frac{M}{B} - \frac{M_c}{B_c} = \frac{MB_c - M_cB}{B \cdot B_c}, \tag{12}$$

and does not equal

$$\frac{M - M_c}{B - B_c}, \tag{13}$$

the investigator must choose between Eq. (12) or Eq. (13) as the proper correction for grazing rates. It is necessary to correct meiofauna for non-grazing uptake of label and bacteria for adsorption. M and B must be reduced before F_c, the corrected grazing fraction, is calculated. Therefore, Eq. (13) is most applicable and is used in all subsequent calculations. The two ways in which corrected grazing-rate fractions (F_c) can be measured is either by subsampling parallel, control incubations, or by conducting four completely independent experiments. When using parallel controls with subsampling to obtain M and B, F_c is calculated by:

$$\bar{F}_c = \frac{\left(\frac{M_i - M_c}{B_i - B_c}\right)}{n}, \tag{14}$$

$$S_{F_c}^2 = \text{unknown}. \tag{15}$$

When subsampling, it is not clear which replicate of the control values to use to correct any given experimental value. Most often the mean control value, $\bar{M}_c$ or $\bar{B}_c$, is used to correct all experimental values. However, it is not known how the variance of F_c should be calculated if this is done.

Most often Eq. (9) is used, and the problem of the variance of the control value (Eq. 15) is ignored. When four independent measurements of M, B, M_c, and B_c are made, F_c is calculated by:

$$\bar{F}_c = \frac{\bar{M} - \bar{M}_c}{\bar{B} - \bar{B}_c}, \tag{16}$$

$$S^2_{\bar{F}_c} = \frac{(S^2_M + S^2_{M_c}) + \left(\frac{\bar{M} + \bar{M}_c}{\bar{B} + \bar{B}_c}\right)\cdot(S^2_B + S^2_{B_c})}{(\bar{B} + \bar{B}_c)^2}. \tag{17}$$

If all samples are independent, Eq. (16) is appropriate, and the variance of F_c can be calculated (Eq. 17). Due to the multiplicative nature of the calculations, the variance is very high.

Results

Microbial inhibition

The prokaryotic inhibitors benzylpenicillin, ND (nalidixic acid + 5′-deoxythymidine), and formalin all had the same effectiveness in inhibiting microbial incorporation of ^{3}HTdR in SFB water (Table 1). All three treatments inhibited ^{3}HTdR incorporation to levels which were 2 to 3% of control (no treatment) values. Cycloheximide also significantly inhibited activity by about one-third over non-treated controls.

In contrast, when sediments were treated with the inhibitors or combinations of them, much lower inhibition of ^{3}HTdR incorporation was noted (Table 2). Benzylpenicillin, cycloheximide, and their combinations showed lower mean amounts of ^{3}HTdR incorporation than controls, but these differences were not significant ($P \geqslant 0.05$). Conversely, addition of ND (and its combinations) resulted in significantly lower incorporation rates than non-treated controls. The ND treatment, which apparently inhibited ^{3}HTdR incorporation by only 50%, was not significantly different from formalin-killed samples ($P \geqslant 0.05$).

Incorporation of ^{3}HTdR was linear for varying degrees of saturation of ND during 2 h incubations (Fig. 2). Inhibition of incorporation was observed beginning at 1% of ND and was complete at 40 to 100% saturation. More extensive examination of a wider range of inhibitor concentrations shows that below 1% saturation of ND there was no effect or slight stimulation of ^{3}HTdR incorporation by SFB-water bacteria (Fig. 3). However, complete or nearly complete inhibition of ^{3}HTdR incorporation was apparent at 20 to 100% saturation of ND.

ND was immediately effective in inhibiting ^{3}HTdR incorporation. No difference could be found in the rates of incorporation when SFB-water bacteria were exposed to ND momentarily or for 60 min prior to measuring ^{3}HTdR incorporation (Fig. 4). Furthermore, inhibition was still complete after up to 3 h of exposure to ND.

On the basis of these results it is hypothesized that, in sediments, the effectiveness of inhibitors in stopping ^{3}HTdR incorporation may be a function of their bioavailability. The inhibitors themselves may be adsorbed onto sediments and associated organic matter (both dissolved and particulate). To investigate this possibility, a supersaturated solution (200%) of ND was added to sediments and compared to sediments receiving no treatment or formalin. This supersaturated treatment was successful in inhibiting ^{3}HTdR uptake as effectively as formalin treatment (Fig. 5).

Table 1. Uptake and incorporation of [methyl-^{3}H] thymidine (^{3}HTdR) by San Francisco Bay (SFB)-water bacteria incubated with inhibitors. All treatments consisted of triplicate 10 ml samples of 3 μm filtrates incubated with 5 nM ^{3}HTdR and the inhibitors for 1 h. Values are mean dis/min ml^{-1} h^{-1}. Underlining joins means that are not significantly different (Tukey multiple-comparison test, $P \geqslant 0.05$). ND: nalidixic acid + 5′-deoxythymidine

Inhibitor				
None	Cyclo-heximide	Benzyl-penicillin	ND	Formalin
15 746	11 610	461	194	104

Table 2. Uptake and incorporation of ^{3}HTdR by sediment-bacteria incubated with inhibitors. All treatments consisted of triplicate samples (2 cm^3 surface sediment mixed with 5 ml of sterile inhibitor solutions) incubated with 5 nM ^{3}HTdR for 2 h. Values are mean dis/min cm^{-3} h^{-1}. Underlining joins means that are not significantly different (Tukey multiple-comparison test, $P \geqslant 0.05$). N: no treatment; P: benzylpenicillin; C: cycloheximide: ND: nalidixic acid + 5′-deoxythymidine; F: formalin

Inhibitor								
N	P	C	P+C	ND	ND+P+C	ND+P	ND+C	F
2539	2180	2066	1960	1103	1082	1017	936	380

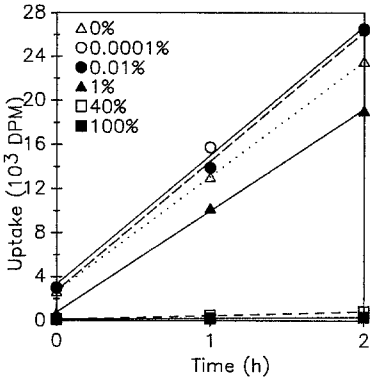

Fig. 2. Effect of incubation time on different concentrations of DNA-synthesis inhibitor (nalidixic acid + 5′-deoxythymidine, ND) on [methyl-^{3}H] thymidine (^{3}HTdR) uptake by water-column bacteria. 3 μm San Francisco Bay (SFB)-water filtrates, with diluted block added, were incubated with 5 nM ^{3}HTdR for 2 h

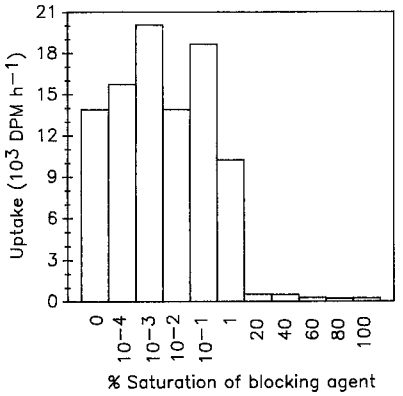

Fig. 3. Effects of DNA-synthesis inhibitor (ND) concentration on ^{3}HTdR uptake by water-column bacteria. 3.0 μm SFB filtrates, with diluted ND added, were incubated with 5 n*M* ^{3}HTdR for 2 h

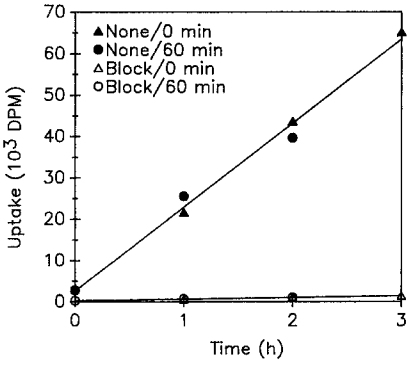

Fig. 4. Effect of incubation time and pre-treatment period of DNA-synthesis inhibitor (ND) on ^{3}HTdR uptake by water-column bacteria. 3.0 μm SFB filtrates received 5 n*M* ^{3}HTdR either immediately or 60 min following no addition or addition of ND

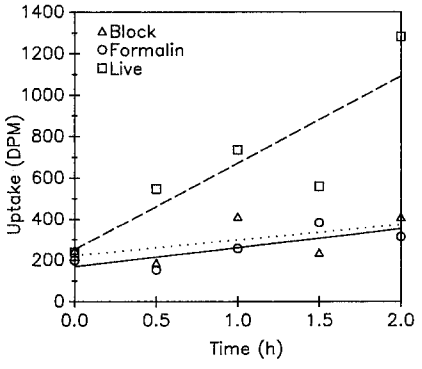

Fig. 5. Effect of adding double-saturated ND to sediments. This experiment was performed on Santa Barbara coastal sediments which were incubated with ^{3}HTdR incubated for 2 h

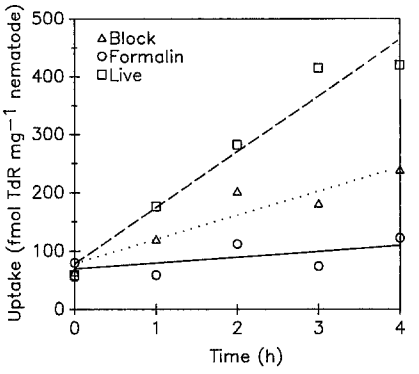

Fig. 6. Effect of incubation time on uptake of ^{3}HTdR by nematodes. Nematodes were incubated in sterile solutions of ND, no treatment or formalin with 10 n*M* ^{3}HTdR. Uptake expressed as fmol TdR mg^{-1} nematode dry wt

Table 3. Toxicity of selected microbial inhibitors to meiofaunal nematodes and polychaetes. Fifteen nematodes and polychaetes were placed in 10 ml of sterile solutions of seawater (None), 200 μg ml^{-1} nalidixic acid + 2 μg ml^{-1} 3'-deoxythymidine (ND), 10 μg ml^{-1} benzylpenicillin (P), ND + benzylpenicillin (ND + P), or 100 μg ml^{-1} cycloheximide; and the number viable after 66 h was determined

Treatment	% alive after 66 h	
	Nematodes	Polychaetes
None	87	100
ND	93	100
P	27	67
ND + P	73	100
Cycloheximide	87	14

Thymidine uptake by nematodes

Neither nematodes nor polychaetes were affected by ND after 66 h of exposure (Table 3). However, both groups exhibited some sensitivity to the other inhibitors. Nematodes were most sensitive to benzylpenicillin (27% viable after 66 h), while polychaetes were most sensitive to cycloheximide (14% viable). When ND and benzylpenicillin were added together there was no effect on either nematode or polychaete viability.

^{3}HTdR uptake was linear over 4 h when nematodes in sterile seawater were exposed to ND, formalin, or nothing (Fig. 6). Non-treated controls had the highest rates of uptake, followed by those nematodes exposed to ND and those exposed to formalin, respectively. This suggests that total meiofaunal uptake of ^{3}H-label is comprised of three distinct and separable pools.

^{3}HTdR uptake by nematodes was linear over a wide range of TdR concentrations in 2 h incubations (Fig. 7). There was a parallel response in ^{3}HTdR uptake across the concentration ranges for all treatments (none, ND, and formalin). Between 1 n*M* and 10 m*M* TdR, adsorption accounted for less than 10% of the total ^{3}H-label taken up by nematodes, 35 to 40% of uptake was attributable to associated bacteria, and about 57% was due to direct nematode absorption (Fig. 8). The partitioning of ^{3}HTdR uptake was not constant over time (Fig. 9). At Time 0, bacteria had not taken up any measurable ^{3}H; however, meiofaunal absorption and adsorption had already occurred to some extent. After incubation for 1 h, the various pools of ^{3}H appeared to be in equilibrium, and this was maintained for up to 4 h.

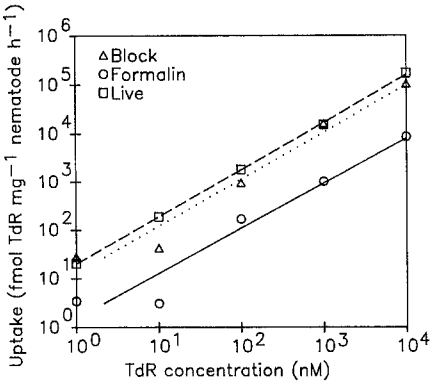

Fig. 7. Effect of thymidine concentration on uptake of ^{3}HTdR by nematodes. Nematodes were incubated in sterile solutions of 1 to 10^4 n*M* ^{3}HTdR with no treatment, ND, or formalin. Uptake expressed as fmol TdR mg^{-1} nematode dry wt

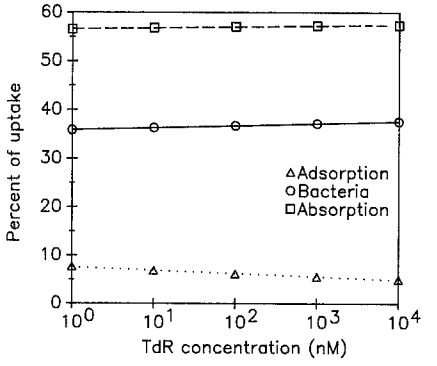

Fig. 8. Effect of thymidine concentration on percent uptake of ^{3}HTdR due to either absorption, adsorption, or bacteria attached to nematodes

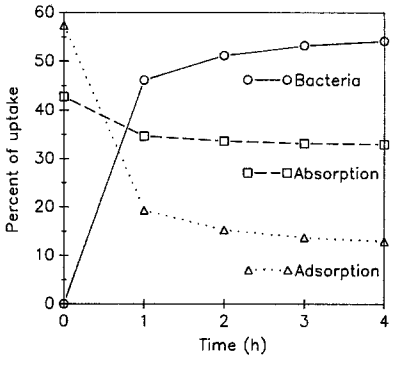

Fig. 9. Effect of incubation time on percent uptake of ^{3}HTdR by nematodes, due to absorption, adsorption, and associated bacteria

Meiofaunal grazing rates

There was no significant difference between grazing rates from intact sediment cores and slurries of sediment cores (Tukey multiple-comparison tests, Table 4). However, the total dis/min in bacteria and meiofauna in slurries (from which the grazing rate was calculated) was twice as great as in intact cores. ND was again only partially effective, and grazing rates were not significantly different from either live or formalin-killed treatments. None of the individual meiofaunal taxa were found to have significantly different grazing rates except for polychaetes, which exhibited 3- to 10-fold greater rates of grazing than other taxa on bacteria ($P \geqslant 0.05$).

When the partitioning of ^{3}H-label derived from ^{3}HTdR was calculated for the course of a 2 h grazing experiment, 2% of the total label taken up by an intact core was attributable to meiofauna (Table 5). Of this 2%, only 17% was due to actual meiofaunal grazing while 54 and 29% were due to absorptive and adsorptive processes, respectively. We found 51% of the label in the sediment bacterially-incorporated fraction, and 10% in the sedimentary-adsorbed fraction. Finally, up to 37% of the label taken up by a given sediment core could not be accounted for in any of the defined pools.

No significant differences were found when meiofaunal grazing rates were determined by injecting ^{3}HTdR and $H^{14}CO_3^-$ either individually or in the same incubation (Table 6). The mean grazing rate (expressed as % label removed h^{-1}) on ^{3}H-labeled microorganisms was four times greater than on ^{14}C-labeled microorganisms.

Table 4. Effects of core treatment, inhibitors, and taxa on meiofaunal grazing rates. All rates are mean grazing rates (% removed h^{-1}, and were determined from dis/min of ^{3}H incorporated into meiofauna after incubation of surface sediments (5.5 cm^2 core) with ^{3}HTdR. Underlining joins means that are not significantly different (Tukey multiple-comparison test, $P \geqslant 0.05$). Poly: Polychaeta; Biva: Bivalvia; Nema: Nematoda; Harp: Harpacticoida; Ostr: Ostracoda; Fora: Foraminifera

Source			Tukey multiple-comparison test			
Core			0.22 Slurry		0.17 Intact	
Inhibitor			0.26 None		0.20 ND	0.10 Formalin
Taxa	0.57 Poly	0.17 Biva	0.13 Nema	0.13 Harp	0.10 Ostr	0.06 Fora

Table 5. Partitioning of ^{3}HTdR by sedimentary compartments. Sediment slurries were incubated with ^{3}HTdR and extracted for bacterial or meiofaunal incorporation of ^{3}HTdR. Three experimental treatments (none, ND or formalin) enabled partitioning of ^{3}H-label

Benthos	DPM[a]	% Label
Bacteria (none-ND)	26655	51
Abiotic (formalin)	5750	10
Meiofauna Absorption: 485 (54%) (ND-formalin) Adsorption: 255 (29%) (formalin) Grazing: 155 (17%) (none-ND)	895	2
Unaccounted for (ND-formalin)	20450	37

[a] Means of 3 relicate samples. Values are dis/min and % of total label attributable to each process

Table 6. Effect of individual and dual labeling on meiofaunal grazing (% label removed h^{-1} individual^{-1}). Sediment slurries were incubated with ^{3}HTdR, $H^{14}CO_3^-$, or both, and meiofauna grazing rate was determined. No significant differences in grazing rates for a given isotope were found ($P \geq 0.05$)

Label	Treatment	
	Alone	Together
^{3}HTdR	0.029	0.026
$H^{14}CO_3^-$	0.008	0.007

Discussion

Benthic bacteria (Zobell and Feltham 1938) and microalgae (Leach 1970) have long been hypothesized as major food and carbon sources for benthic invertebrates. Diatoms and bacteria are eaten by meiofaunal taxa such as nematodes (Jensen 1982, Romeyn and Bouwman 1983) and harpacticoid copepods (Sellner 1976, Rieper 1978, 1982, 1984). Although trophic relationships have been investigated, few of these studies measured in situ rates of meiofaunal grazing on natural microbial populations (Montagna 1984b).

Radiolabeled substrates have proven to be very useful tools in studies of food-chain dynamics in planktonic systems (Conover and Francis 1973, Hollibaugh et al. 1980, Ducklow et al. 1986). In particular, uptake of [methyl-^{3}H] thymidine (^{3}HTdR) has been used for estimating both bacterial production (Fuhrman and Azam 1980, Moriarty and Pollard 1982) and the transfer of bacterial carbon to higher trophic levels (Hollibaugh et al. 1980, Roman and Rublee 1981). The use of ^{3}HTdR for food-web studies is attractive because it is taken up and incorporated into macromolecules exclusively by prokaryotes (Hollibaugh et al. 1980, Moriarty and Pollard 1982), and there is a potential to measure bacterial carbon production and fate in the same experiment.

A variety of related methodological problems associated with conducting proper meiofaunal grazing experiments have been addressed in this study. Past studies of planktonic (Conover and Francis 1973) or meiofaunal (Carman and Thistle 1985) grazing have frequently made erroneous assumptions concerning controls or completely ignored controls altogether.

Our choice of a combination of nalidixic acid plus 5′-deoxythymidine (ND) as an inhibitor of bacterial ^{3}HTdR uptake (Findlay et al. 1984) was justified due to the absence of toxic effects on meiofaunal organisms (Table 3). Inhibitors that affect meiofaunal physiology and viability would obviously be poor choices and would probably give low estimates of grazing if feeding behavior and ingestion rate were altered.

The ND was found to be an effective inhibitor of bacterial uptake and incorporation of ^{3}HTdR in the water column. The inhibitor's effect was apparent immediately and it could be used in a range of concentrations (from 20 to 100% of saturation in seawater). However, in sediments, ND was not as effective in stopping uptake and incorporation of ^{3}HTdR as it was in seawater. ^{3}HTdR incorporation was inhibited by only 43% (1103/2539) when sediments were incubated in slurries of a saturated solution of ND (Table 2) and resulted in 37% of sediment incorporation being unaccountable for (Table 5). Possible reasons for the lack of biological action of the ND in sediments are (1) inactivation of nalidixic acid and/or 5′-deoxythymidine due to complexation with macromolecular dissolved organic matter in pore waters (Carlson et al. 1985); (2) ionic binding with mineral complexes; and (3) lower sediment microbial sensitivity to ND.

Other investigators have also found limited effectiveness of antimicrobial agents in inhibiting the activities of naturally occurring microbes (Fallon et al. 1983). Jensen (1984) found that uptake and respiration of amino acids by lakewater bacteria were inhibited by less than 50% when a variety of antibiotics were used. Interestingly, $^{14}CO_2$ fixation by lake algae and extracellular organic carbon release were found to be most affected by antibiotics. Findlay et al. (1984) observed that while saturated solutions of nalidixic acid and 5′-deoxythymidine inhibited ^{3}HTdR uptake and incorporation of macrophyte-associated bacteria by 90%, sediment bacteria were inhibited by only 50%. Jensen (1984) suggested that when such inhibitors are used, corrections for incomplete bacterial inhibition must be made. As pointed out by Yetka and Wiebe (1974), antibiotics may only be effective on rapidly growing microbial populations. The low growth-rates characteristic of sediment bacteria may account for the ineffectiveness of ND on these populations. However the single experiment conducted using supersaturated solutions of ND showed that ND was effective in blocking ^{3}H-label incorporation by sediment bacteria (Fig. 5). This suggests that chemical binding of ND with sediment complexes accounts for the ineffectiveness of saturated-ND solutions.

Several criteria exist for the choice of a metabolic or growth inhibitor for microbes (i.e., as a live control) in ecological studies. Since radiotracer may appear in a grazer by direct grazing, abiotic adsorption, active transport or uptake by enteric or epicuticular bacteria, corrections must be made for all of these possible routes. Montagna (1983) clearly showed that live, non-feeding meiofauna incorporate more radiolabel in a grazing experiment than their formalin-killed counterparts. These sources of label in meiofauna will result in overestimates of grazing if the rates are not corrected.

When the total uptake of ^{3}H-label by various meiofaunal compartments was partitioned, the results were surprising (Figs. 7 and 8). Apparently, enteric or epicuticular bacteria associated with meiofauna were responsible for the majority ($\geq$ 50%) of "nongrazed" label appearing in live nematodes. Contributions by absorption and adsorption, while still significant, were of secondary importance in comparison to this fraction. It would be of interest to know if these associated bacteria are of some nutritional importance to meiofauna. Such partitioning would be impossible without the use of selective inhibitors.

In their studies of meiofaunal copepod grazing on sediment bacteria and microalgae, Carman and Thistle (1985) used formalin-treated controls only and concluded that neither absorption nor adsorption were important. Howev-

er, without live controls it is impossible to discern whether or not uptake of label by bacteria associated with meiofauna or active transport was significant and hence, whether reported feeding rates were accurate. As demonstrated in the present study, these non-grazing forms of meiofaunal uptake of radiolabel could actually account for the major fraction of label in live meiofauna (83%, cf. Table 5).

Grazing experiments may be facilitated by employing slurries rather than whole cores. Previous reports (Findlay and White 1983) have indicated that certain microbial activities may be enhanced or depressed in a slurry environment.

The ultimate purpose of employing the correct types of live and killed controls in meiofaunal grazing experiments is to obtain accurate rates of cropping of bacterial and microalgal populations. Mean meiofaunal grazing rates in the dual-label experiment were $0.28 \times 10^{-3}\ h^{-1}$ for bacteria and $0.08 \times 10^{-3}\ h^{-1}$ for microalgae for sandy SFB sediments. In comparison, Montagna (1984b) found nematode grazing rates of 0.217×10^{-3} and $1.44 \times 10^{-3}\ h^{-1}$ on bacteria and algae, respectively, in natural sediments from a South Carolina mudflat. Feeding-dish studies have shown rates of $0.032 \times 10^{-3}\ h^{-1}$ (Duncan et al. 1974) and $3.6 \times 10^{-3}\ h^{-1}$ (Admiraal et al. 1983) for nematodes grazing on bacteria and algae, respectively. In our experiment in which grazing rates by individual taxa were determined (Table 4), nematodes grazed bacteria at $1.3 \times 10^{-3}\ h^{-1}$. This rate was not significantly different from that for all other taxa except for polychaetes ($5.7 \times 10^{-3}\ h^{-1}$).

The apparent preference of meiofauna for bacteria over microalgae in the dual-labeling experiment is consistent with findings of Brown and Sibert (1977), who found harpacticoids to select bacterial carbon. In contrast, Montagna (1984b) found that diatom carbon was preferentially ingested, while Van den Berghe and Bergmans (1981) found no preference among *Tisbe* sp. Guidi (1984) found that food composition had no effect on ingestion rates of *Tisbe cucumariae*. The present study did not determine bacterial and microalgal biomasses, and large differences between these two pools of microbial carbon would be reflected in the selection of these microbes.

The results and conclusions of the present study lead to several recommendations for accurately estimating meiofaunal grazing rates in sediments using radiolabeled substrates. First, slurries appear to be an adequate medium for conducting grazing experiments, and slurries increase the precision of measuring meiofaunal accumulation of label because label is homogeneously distributed. Note that this may not be true for concurrently determining microbial productivity; such activities have been found to be greatly enhanced in slurries (Bauer and Montagna, unpublished data). Second, a proper, living meiofaunal (and microbially inhibited) control must be employed to correct for radiolabel uptake by meiofauna by a variety of routes. Finally, streamlining and simplification of a grazing experiment may be achieved by using dual radiotracers and dual live controls (i.e., addition of a bacterial inhibitor and incubation in the dark to inhibit photosynthesis) to simultaneously measure grazing on bacterial and microautotrophic populations.

Acknowledgements. This work was supported by the U.S. Department of Interior, Minerals Management Service, Pacific Outer Continental Shelf Office, under Contract 14-12-0001-30159 to Kinnetic Laboratories, Inc.

Literature cited

Admiraal, W., Bouwman, L. A., Hoekstra, L., Romeyn, K. (1983). Qualitative and quantitative interactions between microphytobenthos and herbivorous meiofauna on a brackish intertidal mudflat. Int. Revue ges. Hydrobiol. 68: 175–191

Ammerman, J. W., Fuhrman, J. A., Hagström, Å., Azam, F. (1984). Bacterioplankton growth in seawater: I. Growth kinetics and cellular characteristics in seawater cultures. Mar. Ecol. Prog. Ser. 18: 31–39.

Bauer, J. E., Capone, D. G. (1985). Effects of four aromatic organic pollutants on microbial glucose metabolism and thymidine incorporation in marine sediments. Appl. envirl Microbiol. 49: 828–835

Brown, T. J., Sibert, J. R. (1977). The food of some benthic harpacticoid copepods. J. Fish. Res. Bd Can. 34: 1028–1031

Carlson, D. J., Mayer, L. M., Brann, M. L., Mague, T. H. (1985). Binding of monomeric organic compounds to macromolecular dissolved organic matter in seawater. Mar. Chem. 16: 141–153

Carman, K. R., Thistle, D. (1985). Microbial food partitioning by three species of benthic copepods. Mar. Biol. 88: 143–148

Conover, R. J., Francis, V. (1973). The use of radioactive isotopes to measure the transfer of materials in aquatic food chains. Mar. Biol. 18: 272–283

Cozzarelli, N. R. (1980). DNA gyrase and the supercoiling of DNA. Science, N.Y. 207: 953–960

Daro, M. H. (1978). A simplified ^{14}C method for grazing measurements on natural planktonic populations. Helgoländer wiss. Meeresunters. 31: 241–248

Ducklow, H. W., Purdie, D. A., Williams LeB., P. J. (1986). Bacterioplankton: a sink for carbon in a coastal marine plankton community. Science, N.Y. 232: 865–867

Duncan, A., Schiemer, F., Klekowski, R. Z. (1974). A preliminary study of feeding rates on bacterial food by adult females of a benthic nematode, *Plectus palustris* DeMan, 1880. Polskie Archwm Hydrobiol. 21: 249–258

Fallon, R. D., Newell, S. Y., Hopkinson, C. S. (1983). Bacterial production in marine sediments: will cell-specific measures agree with whole-system metabolism? Mar. Ecol. Prog. Ser. 11: 119–127

Findlay, S., Meyer, J. L., Smith, P. J. (1984). Significance of bacterial biomass in the nutrition of a freshwater isopod (*Lirceus* sp.). Oecologia 63: 38–42

Findlay, R. H., White, D. C. (1983). Assay of polymeric betahydroxyalkanoates from environmental samples and *Bacillus megaterium*. Appl. envir. Microbiol. 45: 71–78

Fuhrman, J. A., Azam, F. (1980). Bacterial secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. envirl Microbiol. 39: 1085–1095

Fuhrman, J. A., Azam, F. (1982). Thymidine incorporation as a measure of heterotrophic bacterioplankton production in marine surface waters: evaluation and field results. Mar. Biol. 66: 109–120

Fuhrman, J. A., McManus, G. B. (1984). Do bacteria-sized marine eukaryotes consume significant bacterial production? Science, N.Y. 224: 1257–1260

Guidi, L. (1984). The effect of food consumption on ingestion, development, and survival of a harpacticoid copepod, *Tisbe cucumariae* Humes. J. exp. mar. Biol. Ecol. 84: 101–110

Haney, J. F. (1971). An in situ method for measurement of zooplankton grazing rates. Limnol. Oceanogr. 16: 970–977

Hollibaugh, J. T., Fuhrman, J. A,, Azam, F. (1980). Radioactive labeling of natural assemblages of bacterioplankton for use in trophic studies. Limnol. Oceanogr. 25: 172–181

Jensen, L. M. (1984). Antimicrobial action of antibiotics on bacterial and algal carbon metabolism: on the use of antibiotics to estimate bacterial uptake of algal extracellular products. Arch. Hydrobiol. 99: 423–432

Jensen, P. (1982). Diatom-feeding behaviour of the free-living marine nematode *Chromodorita tenuis*. Nematologica 28: 71–76

Kempthorne, O., Allmaras, R. R. (1965). Errors in observation. In: C. A. Black (ed.) Methods in soil analysis. Part 1. Physical and minerological properties, including statistics of measurement and sampling. American Society of Agronomy, Madison, p. 1–23

Leach, J. H. (1970). Epibenthic algal production in an intertidal mudflat. Limnol. Oceanogr. 15: 514–521

Montagna, P. A. (1983). Live controls for radioisotope food chain experiments using meiofauna. Mar. Ecol. Prog. Ser. 12: 43–46

Montagna, P. A. (1984a). Competition for dissolved glucose between meiobenthos and sediment microbes. J. exp. mar. Biol. Ecol. 76: 177–190

Montagna, P. A. (1984b). In situ measurement of meiobenthic grazing rates on sediment bacteria and edaphic diatoms. Mar. Ecol. Prog. Ser. 18: 119–130

Moriarty, D. J. W., Pollard, P. C. (1982). Diel variation of bacterial productivity in seagrass (*Zostera capricorni*) beds measured by the rate of thymidine incorporation into DNA. Mar. Biol. 72: 165–173

Rieper, M. (1978). Bacteria as food for marine harpacticoid copepods. Mar. Biol. 45: 337–345

Rieper, M. (1982). Feeding preferences of marine harpacticoid copepods for various species of bacteria. Mar. Ecol. Prog. Ser. 7: 303–307

Rieper, M. (1984). Relationships between bacteria and marine copepods. In: University Provence (eds.) Bacteriologie Marine. CNRS, Paris, p. 169–172

Roman, M. R., Rublee, P. A. (1981). A method to determine *in situ* zooplankton grazing rates on natural particle assemblages. Mar. Biol. 65: 303–309

Romeyn, K., Bouwman, L. A. (1983). Food selection and consumption by estuarine nematodes. Hydrobiol. Bull. 17: 103–109

Sellner, B. W. (1976). Survival and metabolism of the harpacticoid copepod *Thompsonula hyaenae* (Thompson) fed on different diatoms. Hydrobiologia 50: 233–238

Van den Berghe, W., Bergmans, M. (1981). Differential food preferences in three co-occurring species of *Tisbe* (Copepoda, Harpacticoida). Mar. Ecol. Prog. Ser. 4: 213–219

Wright, H. T., Nurse, Y. C., Goldstein, D. J. (1981). Nalidixic acid, oxolinic acid and novobiocin inhibit yeast glycyl- and leucyl-transfer RNA synthetases. Science, N.Y. 213: 455–456

Yetka, J. E., Wiebe, W. J. (1974). Ecological application of antibiotics as respiratory inhibitors of bacterial populations. Appl. Microbiol. 28: 1033–1039

Zobell, C. E., Feltham, C. B. (1938). Bacteria as food for certain marine invertebrates. J. mar. Res. 1: 312–327

Date of final manuscript acceptance: February 2, 1988.
Communicated by R. S. Carney, Baton Rouge