Title: The influence of deep-seabed CO_2 sequestration on small metazoan (meiofaunal) viability and community structure

Principal investigator: David Thistle
Co-principal investigator: Trisha Spears

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Recipient organization: Florida State University
Office of Sponsored Research
97 S. Woodward Avenue, 3rd floor
Tallahassee, FL 32306-4166

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Aims

Our original aims were (1) to continue in situ, small-scale, experimental exposures of deep-sea meiofauna to carbon dioxide, (2) to use a deep-sea lander to assess the dose and duration of the exposure to carbon dioxide, and (3) to improve understanding of deep-sea species' range sizes in order to better understand whether industrial-scale carbon dioxide sequestration would cause extinctions. We were able to continue our in situ experiments, but the early termination of our funding meant that (1) the equipment needed for the in situ dose-and-duration measurements could not be perfected, and (2) although we had developed the techniques necessary, we could not incorporate gene sequencing into our studies of species' ranges.

Background

Since the industrial revolution, the burning of fossil fuel has produced carbon dioxide at an increasing rate. Present atmospheric concentration is about ~1.5 times the preindustrial level and is rising. Because carbon dioxide is a greenhouse gas, its increased concentration in the atmosphere is thought to be a cause of global warming. If so, the rate of global warming could be slowed if industrial carbon dioxide were not released into the atmosphere. One suggestion has been to sequester it in the deep ocean, but theory predicts that deep-sea species will be intolerant of the increased concentrations of carbon dioxide and the increased acidity it would cause. The aim of our research was to test for consequences of carbon dioxide sequestration on deep-sea, sediment-dwelling meiofauna.

Approach

Recent technical advances allowed us to test for effects in situ at depths proposed for sequestration. The basic experimental unit was an open-topped container into which we pumped ~20 L of liquid carbon dioxide. The liquid carbon dioxide mixed with near-bottom sea water, which produced carbon dioxide-rich sea water that flowed over the near-by seabed. We did 30-

day experiments at several locations and with different numbers of carbon dioxide-filled containers. Harpacticoid copepods (Crustacea) were our test taxon.

Results

In an experiment we did during a previous grant period, we found that large numbers of individuals exposed to carbon dioxide-rich sea water had been killed (Thistle et al. 2004). During the present grant period, we analyzed the species-level data in greater detail and discovered that, although individuals of many species had been killed by exposure to carbon dioxide-rich sea water, individuals of some species had not (Thistle et al. 2005). This result suggests that seabed sequestration of carbon dioxide will not just reduce the abundance of the meiofauna but will change the composition of the community.

In another experiment, we found that some harpacticoid species swam away from an advancing front of carbon dioxide-rich sea water (Thistle et al. 2007). This result demonstrates a second way that deep-sea meiofauna react negatively to carbon dioxide-rich sea water.

Although we could not use gene-sequencing methods to assign individuals into species for our study of species' range sizes, we were able to do so using morphology. These data will be analyzed and written up in the coming months.

Summary

In summary, we used in situ experiments to show that carbon dioxide-rich sea water triggers an escape response in some harpacticoid species. It kills most individuals of most harpacticoid species that do not flee, but a few species seem to be unaffected. Proposals to reduce global warming by sequestering industrial carbon dioxide in the deep ocean should take note of these environmental consequences when pros and cons are weighed.

Graduate students trained:

Erin Easton, M.S. candidate
Linda Sedlacek, Ph.D., 2007

Post-doctoral fellows who worked on the project:

None

Publications:

Thistle, D., K. R. Carman, L. Sedlacek, P. G. Brewer, J. W. Fleeger, and J. P. Barry. 2005. Deep-ocean, sediment-dwelling animals are sensitive to sequestered CO_2. Marine Ecology Progress Series 289:1-4. (Designated a feature article for the issue.)

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Manuscripts in preparation:

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Consequences of carbon dioxide sequestration for the deep-sea-floor fauna: effects on a test taxon in different environmental settings

Linda Sedlacek[a], David Thistle[a,*], Kevin R. Carman[b], John W. Fleeger[b], and James P. Barry[c]

[a]Department of Oceanography, Florida State University, Tallahassee, Florida 32306-4320, USA

[b]Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803-1725, USA

[c]Monterey Bay Aquarium Research Institute, Sandholdt Road, Moss Landing, California 95039-9644, USA

*Corresponding author. Tel.: +1 850 644 4089.

E-mail address: thistle@ocean.fsu.edu

Abstract

As part of the effort to combat global warming, the greenhouse gas carbon dioxide may be collected and pumped into natural depressions on the seafloor below ~2600 m, where it will be liquid and denser than seawater. If sites were carefully chosen, the carbon dioxide would remain out of contact with the atmosphere for a useful period (several hundred years). As part of the evaluation of the environmental impact of this approach to carbon dioxide sequestration, we exposed the sediment-dwelling fauna at a station in Monterey Submarine Canyon (CO_2-4, 36.378° N, 122.676° W, 3262 m) and a station on the continental rise nearby (CO_2-5, 36.709° N, 123.523° W, 3607 m) to carbon dioxide-rich seawater. We chose harpacticoid copepods (Crustacea) as our test taxon and investigated seven metrics.

In experiment CO_2-4, we found that the harpacticoid fauna had been killed by exposure to carbon dioxide-rich seawater. Although we deployed more than twice as much carbon dioxide in experiment CO_2-5, the harpacticoid fauna showed no evidence of being affected. We conclude that the consequences of exposure to carbon dioxide-rich seawater for the deep-sea-floor fauna can depend on local circumstances, which will complicate evaluation of the environmental impacts of large-scale carbon dioxide sequestration on the deep-sea floor.

Keywords: Carbon dioxide sequestration; Deep sea; Ecology; Harpacticoida; Meiobenthos; USA; California; 36.378° N, 122.676° W to 36.709° N, 123.523° W

1. Introduction

Increasing concern over global warming has prompted consideration of methods for reducing carbon dioxide emissions to the atmosphere (IPCC, 2005), in particular, direct ocean carbon sequestration, which involves capturing industrial carbon dioxide at point sources (e.g., power plants) and sequestering it in the ocean such that it remains for hundreds of years (Marchetti, 1977; Ormerod et al., 2002). At the temperatures and pressures found below about 2600 m, carbon dioxide is liquid and denser than seawater (Brewer et al., 1999), so carbon dioxide injected below this depth should initially pool in place. It will dissolve into the near-bottom water producing carbon dioxide-rich seawater, which is denser than ambient seawater and would flow over the adjacent seafloor.

Laboratory studies have shown that exposure to carbon dioxide-rich seawater adversely affects the physiology of shallow-water organisms by causing hypercapnia and acidosis (Ishimatsu et al., 2004; Kurihara et al., 2004), the latter occurs because pH decreases as the concentration of carbon dioxide in seawater increases. Because natural carbon dioxide concentrations and pH are much less variable in the deep sea than in shallow water, deep-sea organisms are expected to be more sensitive to perturbations than their shallow-water counterparts (Shirayama, 1995; Seibel and Walsh, 2001, 2003).

A recent technological advance has allowed carbon dioxide-rich seawater to be created at depth (Brewer et al., 1999), and its effects on deep-sea organisms have begun to be studied ***in situ***. For example, the tests of echinoderms were partially eroded (Barry et al., 2003) and amoebas and flagellates occurred in unusually low numbers (Barry et al., 2005) in areas exposed to carbon dioxide-rich seawater.

Given the consequences of the policy decisions involved, determining whether the results of deep-sea experiments with carbon dioxide-rich seawater are replicable in different environmental settings is important. Here, we compare results from an experiment conducted at 3262 m depth in a submarine canyon (CO_2-4) with those from an experiment done at 3607 m depth on the continental rise nearby (CO_2-5, see Barry et al., 2005, for details of these experiments).

2. Materials and methods

2.1. Stations

CO_2-4 was carried out in the axis of lower Monterey Submarine Canyon (36.378° N, 122.676° W, Fig. 1) at 3262 m depth (reported as 3250 m depth by Carman et al., 2004, and Thistle et al., 2005), where the canyon was ~2 km wide. The sediment was a fine mud to a depth of many centimeters. Bottom-water temperature was 1.6°C, oxygen concentration was 60.0 to 60.5 µM, and salinity was 34.6. Average current speed 8 m above bottom

was 5.7 cm s^{-1}, net flow was 2.29 cm s^{-1} in the up-canyon direction, and average kinetic energy was 17.17 cm^{-2} s^{-2}. The surface of the seabed was featureless on scales of meters to tens of meters. At small scales, a scattering of animal tubes and tests was observed.

CO_2-5 was carried out on the continental rise off central California (36.709° N, 123.523° W, Fig. 1, Barry et al., 2005) at 3607 m depth about 84.2 km from the location of CO_2-4. The sediment was a fine-grained, clay-rich mud with a mean grain size of 4.9 μm. Bottom-water temperature was 1.55°C, oxygen concentration was 120 μM, and salinity was 34.39. Average current speed 15 m above the seafloor was 2.9 cm s^{-1}, net flow was 0.77 cm s^{-1}, and average eddy kinetic energy was 5.36 cm^{-2} s^{-2}. The surface of the seabed was featureless at scales of meters to tens of meters. At smaller scales, tubes of the ampeliscid amphipod *Haploops lodo* J. L. Barnard, 1961, formed a mat on the sediment surface. Tube lengths and widths averaged 2.0 cm and 0.75 cm respectively, but variability was high. The population averaged 382 individuals per 0.1 m^2 (Linda Khunz, personal communication).

2.2. Experimental treatment

CO_2-4 began on 11 November 2002. All manipulations on the seafloor were done with the remotely operated vehicle *Tiburon* of the Monterey Bay Aquarium Research Institute. To contain the liquid carbon dioxide, we placed three 48-cm inner diameter by 15-cm long sections of polyvinyl-chloride pipe such that their walls were perpendicular to the seabed and extended ~3 cm into

the sediment. One container was positioned at each apex of an approximately equilateral triangle ~4 m on a side (reported as ~10 m on a side by Carman et al., 2004, Fig. 2). The *Tiburon* dispensed ~20 L of liquid carbon dioxide into each container (see Barry et al., 2005, their fig. 2g) with a delivery system nearly identical to that of Brewer et al. (1999). Over time, the liquid carbon dioxide dissolved into the overlying water. On a time scale of minutes, the dissolving carbon dioxide was hydrated (Brewer et al., 2005), creating a dissolution plume of low-pH, carbon dioxide-rich seawater that was negatively buoyant and flowed over the sediment surface.

CO_2-5 began on 19 August 2003. We used the same procedures except that seven containers were placed in a circle 15 m in diameter (Fig. 3).

2.3. Test taxon

We set out to determine whether exposure to carbon dioxide-rich seawater affected small metazoans. In this paper, we report on harpacticoid copepods; Fleeger et al. and Barry et al. are preparing results from other taxa.

2.4. Sample collection and processing

2.4.1. Experiment CO_2-4

Carman et al. (2004) and Thistle et al. (2005, 2006) described the procedures. Briefly, 29 d after deployment of the carbon dioxide, we returned and collected six 7-cm-diameter sediment cores about 20 cm apart at ~2 m (= Near site) and five at ~75 m (reported as 40 m by Carman et al., 2004, and Thistle et al., 2005, 2006; = Far site) away from the source of carbon

dioxide-rich seawater (Fig. 2). Both areas lacked visible habitat heterogeneity, such as burrows or areas of disturbance, on the scale of centimeters to tens of centimeters.

During ascent (2 to 3 h), the cores warmed from ~2°C to ~11°C. On recovery, we placed them in a 4°C room and measured a pH profile using a Unisense glass microelectrode (100-µm-diameter tip, external reference electrode) connected to a Knick Portamess 913 pH meter. The pH sensor was calibrated immediately before use. We measured from ~2 mm above to ~8 mm below the sediment surface in 0.250-mm vertical steps with the aid of a micromanipulator. Cores from the Near and Far sites were processed alternately.

The water above the sediment in the core tube was aspirated off and poured through a 32-µm sieve; the sieve contents were combined with the uppermost sediment layer. A subcore (1.9-cm diameter) was placed in the center of each core for a collaborator. The remainder of the core was sliced around the subcorer into four layers: 0–5 mm, 5–10 mm, 10–20 mm, and 20–30 mm. Samples were preserved in a solution of one part formalin and nine parts artificial seawater of salinity 35 that was buffered with sodium borate.

To speed the extraction of harpacticoids, we exploited the fact that their settling velocities are slower than those of most sediment particles. The sample was swirled by hand with the colloidal silica Ludox, which had been adjusted to a specific gravity of 1.16 g cm^{-3} with deionized water, and allowed to sit for 45 min (see Somerfield and Warwick, 1996; Burgess, 2001).

Most sediment particles sank, but the harpacticoids remained suspended and were decanted off. We repeated this procedure five times. After rose bengal staining, the harpacticoids were removed from both fractions with the aid of a dissecting microscope. Adults were identified to sex and to working species. We used working species because, as is routine in the deep sea, most species we encountered were undescribed (Thistle, 1978; Seifried, 2004). The same working-species names were used in the two experiments.

2.4.2. Experiment ***CO_2-5***

Our procedures were as in CO_2-4 except in the following ways. We returned ~30 days after the treatment was established. We took four cores each at 2 m (between two adjacent containers), 4 m, 8 m, 21 m, and 75 m from the carbon dioxide source (Fig. 3). The pH measurements were made with a sensor with an internal reference electrode and were made while the cores were in a water bath. In order to use the *Tiburon*'s time efficiently, we had to take all the cores from a site on a single visit. Because the sites were not all sampled on the same day, their pH measurements could not be intermingled. pH calibrations were made before and after each profile. If the initial calibration differed by more than 5% of a pH unit from the later calibration or if the profile showed a bizarre shape, the profile was omitted.

2.5. Metrics

We used seven metrics: (1) Faunal-similarity analysis was done at the species level and was based on adults. (2) Harpacticoid abundance was the total number of adults per core.

(3) The proportion alive was the number of adults that were judged to be alive when collected divided by the total number of adults found. Briefly, individuals with internal organs in pristine condition, as viewed through the transparent body wall, were considered to have been alive when collected; see Thistle et al. (2005, 2006) for details. (4) The proportion of the copepodites that were subadult (= proportion copepodite) was calculated as the number of subadult copepodites in a core divided by the number of adult and subadult copepodites in a core. (5) The sex ratio was calculated as the number of adult males in a core divided by the total number of adults in a core. (6) The weighted mean depth in a core was calculated as:

$$X_m = \frac{\sum_{i=0}^{n} w_i x_i}{\sum_{i=0}^{n} w_i}$$

where w_i is the number of adults in the ith sediment layer, and x_i is the depth at the midpoint of the ith layer. (7) The diversity of a core was based on adults. We used the Shannon-Wiener index (H', natural logarithms); calculations were done with PRIMER version 5.2.8 (Primer-E Ltd.).

2.6. Analyses

Because the effects of carbon dioxide-rich seawater on surface and near-surface animals could be masked by relative lack of effect on animals from deeper layers if only the 0-30 mm layer was tested, for all metrics other than weighted mean depth in the sediment, we tested both the 0-5 mm and the 0-30 mm data.

To test for differences in faunal similarity between sites within stations, we calculated Bray-Curtis similarities on square-root-transformed data between all pairs of sampling sites. We used a randomization-based analysis of similarity (ANOSIM) to test for differences in faunal similarity among sampling sites. The calculations were done with the aid of PRIMER version 5.2.8.

For all other metrics, we tested for differences among sites within stations with one-way analysis of variance. We transformed variables as needed to meet the assumptions of ANOVA (abundance and weighted mean depth, $\log_{10}$; proportion alive, proportion copepodite, and sex ratio, arcsine square root).

In this exploratory study, we made no correction for multiple testing.

3. Results

3.1. pH at the end of each experiment

At the end of CO_2-4 (i.e., 29 d after initiation), no carbon dioxide could be seen in any of the containers. The pH profiles from the Near site did not overlap those from the Far site and were ~0.75 pH unit more acidic (Fig. 4).

At the end of CO_2-5 (i.e., 30 d after initiation), carbon dioxide could be seen in several containers (Barry et al., 2005). The pH profiles in cores from the different sites were intermixed and gave no indication that a pH gradient (and thus a gradient in exposure to carbon dioxide-rich seawater) remained with distance from the source of the carbon dioxide (Fig. 5).

3.2. Faunal similarity

Table 1 shows that most of the harpacticoid species present in the background fauna during CO_2-4 (i.e., at the Far site) were also present in the background fauna during CO_2-5 (i.e., at the 75-m site).

Thistle et al. (2006) tested whether the faunal similarity at the Far site differed from the Near site for the 0-10 mm layer at the end of CO_2-4. We analyzed their data for the 0-5 and 0-30 mm layers and found that the faunal similarity at the Near site did not differ significantly from that at the Far site for either layer. For CO_2-5, we found no significant difference in faunal similarity among the sites for the 0-5 mm layer. For the 0-30 mm layer, the 8-m site differed significantly from both the 4-m site (two-tailed $P = 0.029$) and the 75-m site (two-tailed $P = 0.029$).

3.3. Abundance

For CO_2-4, the average abundances of harpacticoids in the 0-5 and 0-30 mm layers at the Near site did not differ significantly from those of the Far site, and the ranges overlapped extensively for each layer (Table 2). For CO_2-5, harpacticoid abundance at no site differed significantly from that at any other site for either layer, and the ranges overlapped extensively among sites for each layer (Table 2).

3.4. Proportion alive

For CO_2-4, the proportion of harpacticoids that were alive when collected at the Far site was significantly greater than that at the Near site for the 0-5 mm layer (Thistle et al., 2005). We analyzed the data for the 0-30 mm layer and found that, for it too, the proportion of harpacticoids that were alive when collected at the Far site was significantly (two-tailed $P < 0.003$) greater than that at the Near site (Table 3). For CO_2-5, none of the sites differed significantly in this metric for either layer, and the ranges at different sites overlapped extensively for both layers (Table 3).

3.5. Proportion of subadult copepodites

For CO_2-4, the proportion of subadult copepodites (Table 4) was significantly smaller in the Near cores than in the Far cores for the 0-5 mm layer (two-tailed $P = 0.044$) and for the 0-30 mm layer (two-tailed $P = 0.001$). In CO_2-5, sites did not differ significantly in this proportion for either layer, and the sites' ranges overlapped extensively for both layers (Table 4).

3.6. Sex ratio

In CO_2-4, the average sex ratio in the Near cores did not differ significantly from that in the Far cores for either layer (Table 5). For the 0-5 mm layer, the ranges overlapped extensively; for the 0-30 mm layer, they were essentially the same. In CO_2-5, sites did not differ significantly in sex ratio

for either layer, and their ranges overlapped extensively for both layers (Table 5).

3.7. Weighted mean depth

In CO_2-4, the weighted mean depth of the adults at the Near site did not differ significantly from that at the Far site, and the ranges overlapped extensively (Table 6). In CO_2-5, the weighted mean depth at the sites did not differ significantly, and the ranges overlapped extensively (Table 6).

3.9. Diversity

For CO_2-4, H’ at the Near site did not differ significantly from H’ at the Far site for either the 0-5 or the 0-30 mm layers. The average values were nearly the same, and the ranges overlapped extensively for both layers (Table 7).

For the 0–5 mm layer of CO_2–5, the one-way analysis of variance detected a significant overall difference among sites (Table 7, Fig. 6). Tukey’s Honestly Significant Difference test revealed that the 75-m site differed from both the 2-m and the 21-m sites. For the 0–30 mm layer, the sites did not differ significantly, and their ranges overlapped extensively (Table 7).

4. Discussion

4.1. Experiment CO_2-4

In previous analyses of the harpacticoid data from the CO_2-4 experiment, Carman et al. (2004) found that the Far samples did not differ from the Near samples in per-core abundance, and

Thistle et al. (2006) found no difference in faunal similarity. In contrast, the proportion of the individuals that were collected alive at the Far site was significantly greater than that at the Near site (Thistle et al., 2005). On the basis of these results, Thistle et al. (2005) suggested that most harpacticoids at the Near site were killed in place by exposure to carbon dioxide-rich seawater but that their corpses decayed so slowly that they were still recognizable as harpacticoids in the preserved samples and were counted and identified as such.

For this report, we studied additional metrics and found that the data for sex ratio and diversity from the Near and Far sites overlapped extensively. We infer that the Near and Far sites did not differ in these metrics, a result that is consistent with Thistle et al.'s (2005) interpretation of the fate of the harpacticoids during CO_2-4.

We did find that the proportion of subadult copepodites at the Near site was significantly less than that at the Far site, but this result also supports the above interpretation because these smaller, less-calcified animals would be expected to decay more rapidly than adults and, at the time of our sampling, could have decayed sufficiently to reduce their abundance relative to adults.

4.2. Experiment CO_2-5

Our intention during CO_2-5 was to create both lethal and sublethal effects by increasing the dose of carbon dioxide-rich seawater over that in CO_2-4 and by introducing sampling sites at intermediate distances. We therefore dispensed ~140 L rather than ~60 L of liquid carbon dioxide. We placed the 2-m site in a position analogous to that of the Near site in CO_2-4, expecting it to be exposed to a greatly increased dose because carbon dioxide-rich seawater from 7 rather than 3 containers would flow over it as the near-bottom flow was rotated by the tides.

We found a significant main effect on diversity of the 0-5 mm layer and on faunal similarity of the 0-30 mm layer. In experiments such as CO_2-5 in which the fauna is exposed to a localized stress, the usual result is that diversity decreases with proximity to the source of the stress (see, e.g., Saunders and Moore, 2004). In CO_2-5, the diversity of the sites in the 0-5 mm layer did not follow this pattern (Fig. 6). In particular, the diversity of the 21-m site was comparable in its average and range to that of the 2-m site, but diversities of the 4-m and 8-m sites were higher. Therefore, we do not view these differences as arising from the effects of carbon dioxide-rich seawater.

Similarly, if the stressor were causing differences in faunal similarity, one would expect that of the 2-m site to differ from that of the 75-m site. We found for the 0-30 mm

layer that the faunal similarity of only the 8-m site differed significantly from those of the other sites; we do not view this difference as arising from the effects of carbon dioxide-rich seawater.

For none of the remaining metrics did sites differ in average values, and for each metric, the ranges at the sites overlapped extensively.

Taken together, our results suggest that during CO_2-5 the harpacticoids at the 2-m site were either not exposed to carbon dioxide-rich seawater or received exposure insufficient to kill them or even to cause detectable numbers of individuals to move away. Ongoing studies from the CO_2-5 experiment have revealed differences among sites in nematodes and polychaetes that appear to be caused by exposure to carbon dioxide-rich seawater (Fleeger and Barry, personal communications). Given that all three taxa live on and near the sediment surface, the harpacticoids must have been exposed as well, but the exposure must have been much less during CO_2-5 than during CO_2-4, despite the much larger amount of carbon dioxide we dispensed in CO_2-5.

4.3. Contrasts in environmental settings between CO_2-4 and CO_2-5

4.3.1. Differences in the near-bottom flow

Although the two stations were at similar depths and only 84.2 km apart, their environmental settings differed in ways that appear to be relevant. The average speed and turbulent

kinetic energy (see Barry et al., 2005, their fig. 6) of the near-bottom flow was much greater during CO_2-4 than during CO_2-5, and the rate at which carbon dioxide in the containers dissolved into and mixed with the near-bottom water should therefore also have been greater. As evidence of the latter, Barry et al. (2005) noted that liquid carbon dioxide remained in some containers at the end of CO_2-5 but not at the end of CO_2-4 and suggested that carbon dioxide-rich seawater was produced more slowly during CO_2-5 than during CO_2-4. It may have been produced so slowly during CO_2-5 that concentrations stressful for harpacticoids were never reached.

4.3.2. Differences in the pore-water pH

At the end of CO_2-4, the average pore-water pH at the Near site was significantly more acidic than that at the Far site. At the end of CO_2-5, no differences among sites in pore-water pH could be detected, although more carbon dioxide was delivered to the seabed during CO_2-5, and the duration of the experiment was comparable to that of CO_2-4. The cause of this difference is unclear, but its existence suggests that the local environments where the experiments were done modulated the experimental treatment and thus the ecological results.

5. Conclusions

On the basis of the results from CO_2-4, previous authors suggested that carbon dioxide-rich seawater can kill

harpacticoids and other small infaunal taxa (Thistle et al., 2005, 2006; Fleeger et al., 2006). In contrast, during CO_2-5, we do not appear to have produced exposure to carbon dioxide-rich seawater sufficiently severe to kill harpacticoids. The threshold for harpacticoid mortality therefore lies somewhere between the values produced during the two experiments. We suggest that the environmental setting in which an experiment is done can have a large influence on the effects observed. Our results also suggest that further experiments should be able to reveal the environmental circumstances under which the effects of carbon dioxide sequestration will be particularly mild or severe.

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Figure captions

Fig. 1. Chart of the area off central California showing the location of the stations at which the experiments were done.

Fig. 2. A representation (not to scale) of the CO_2-4 experiment. Large, filled circles represent the containers that received carbon dioxide; they were spaced ~4 m apart. The small, open circles represent cores. The current ellipse shows the principal axes of flow 8 m above bottom. The major and minor axes represent one standard deviation of the average current speed. The arrow indicates the average flow direction.

Fig. 3. A representation (not to scale) of the CO_2-5 experiment. Large, filled circles represent the containers that received carbon dioxide; the diameter of the circle of containers was ~15 m. The small, open circles represent cores. The current ellipse shows the principal axes of flow 15 m above bottom. The major and minor axes represent one standard deviation of the average current speed. The arrowhead indicates the average flow direction.

Fig. 4. Profiles of pH versus depth from ~2 mm above the sediment surface (dashed line) to ~8 mm into the sediment for experiment CO_2-4, showing that the pH in cores taken at the Near site was much lower (more acidic) than that at the Far site.

Fig. 5. Profiles of pH versus depth from ~2 mm above the sediment surface (dashed line) to ~8 mm into the sediment for

experiment CO_2-5, showing that the pH profiles at the sites do not indicate a pattern of increasing acidity with increasing proximity to the source of the carbon dioxide-rich seawater.

Fig. 6. For CO_2-5, the diversity (H’) of adult harpacticoids in the 0-5 mm layer, showing that, although sites differ significantly, the pattern of differences does not suggest that proximity to the source of carbon dioxide-rich seawater caused the differences observed.

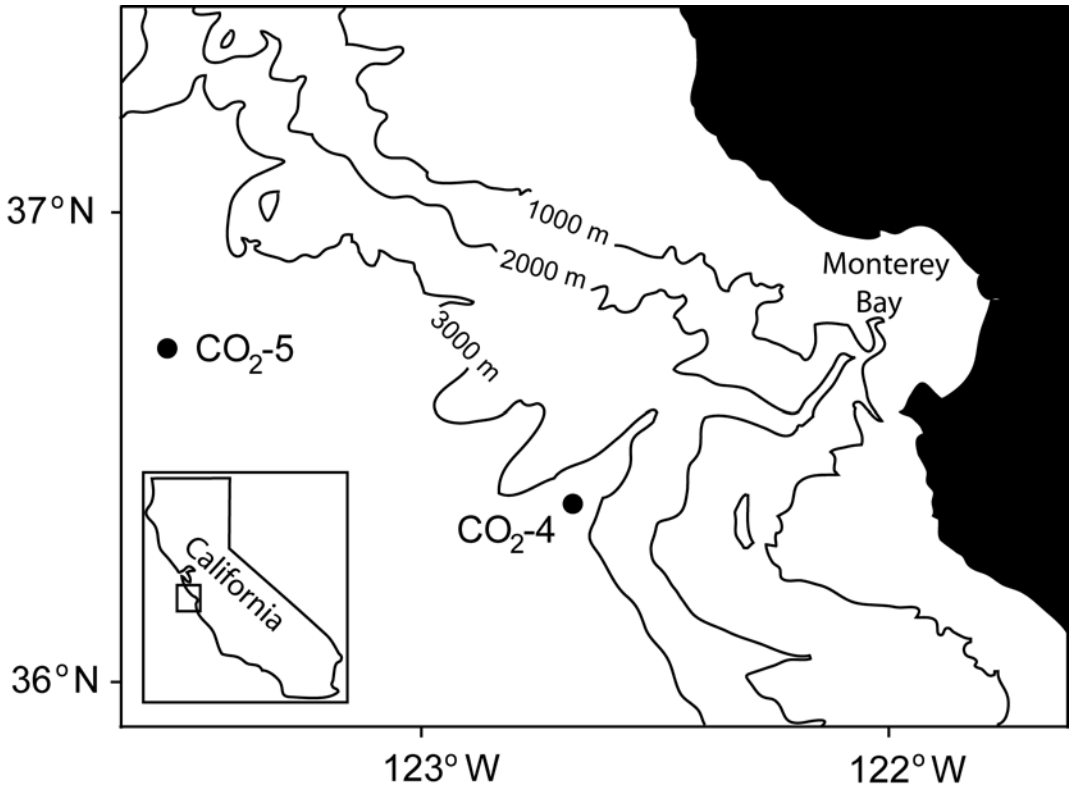
37°N
36°N
123° W
122° W
1000 m
2000 m
3000 m
Monterey
Bay
CO2-5
CO2-4
California

Figure 1.

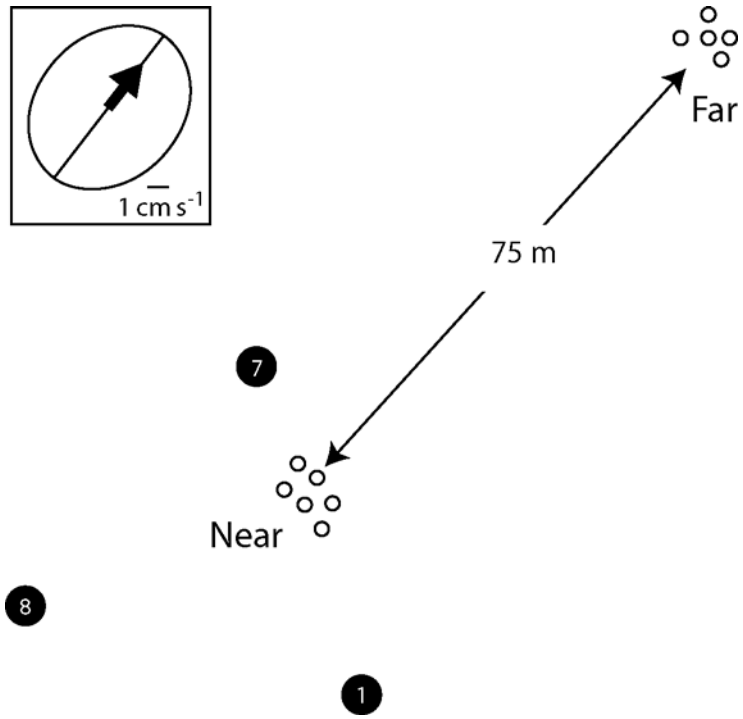

Figure 2.

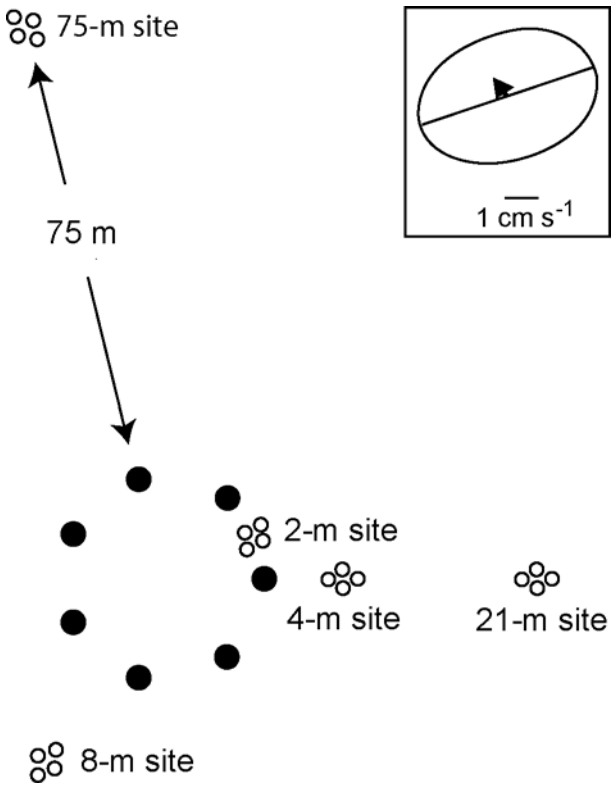

Figure 3.

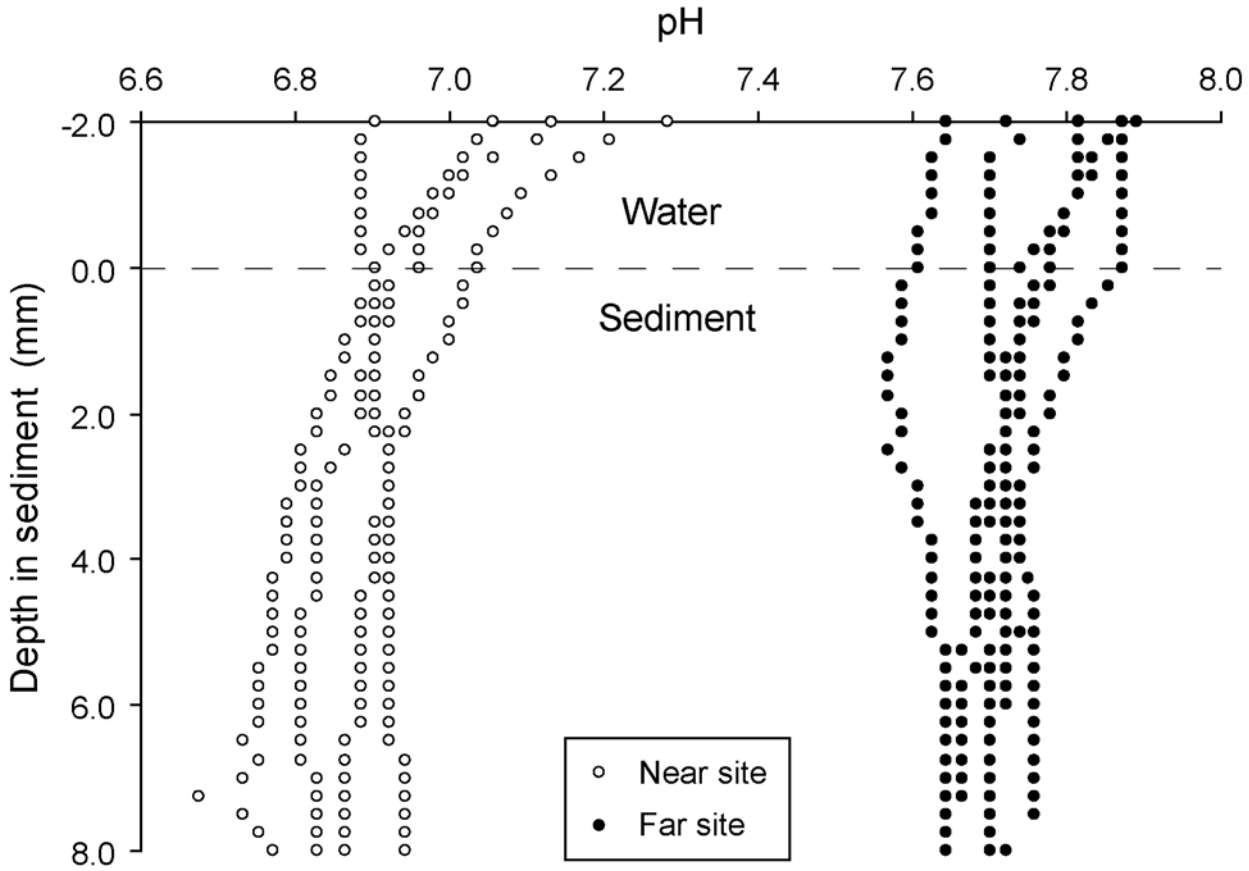
pH
6.6
6.8
7.0
7.2
7.4
7.6
7.8
8.0
-2.0
0.0
2.0
4.0
6.0
8.0
Depth in sediment (mm)
Water
Sediment
Near site
Far site

Figure 4.

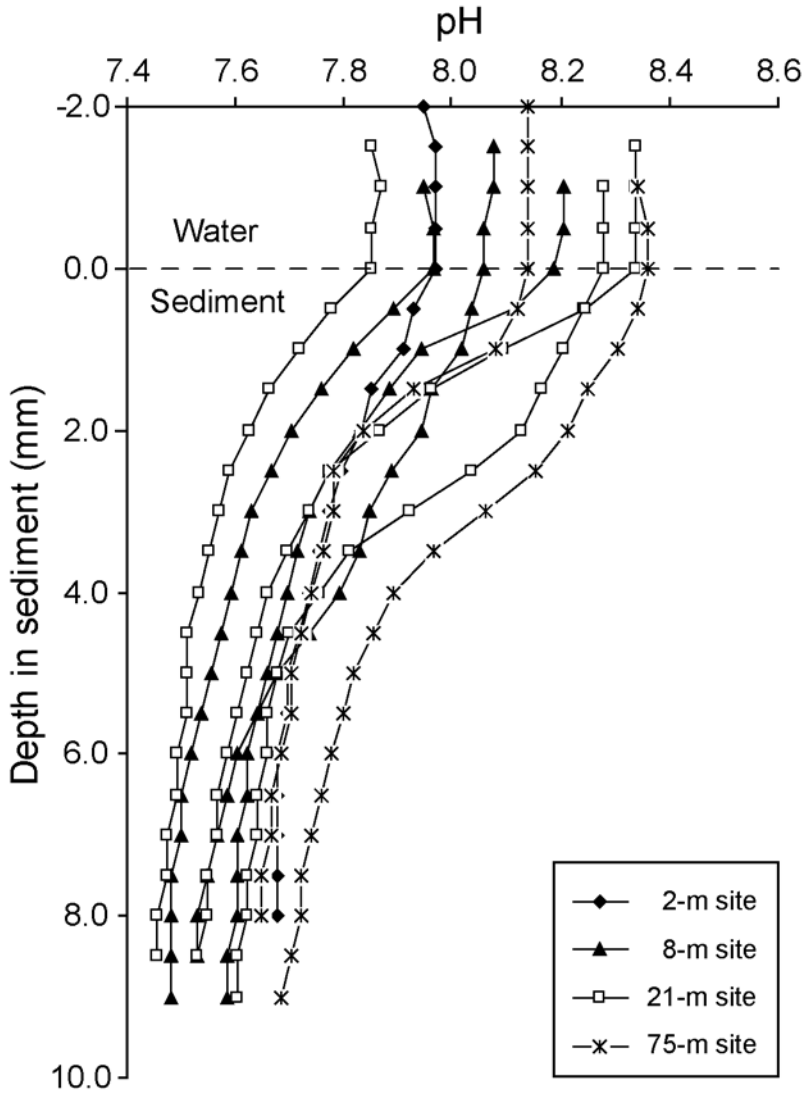
pH
7.4
7.6
7.8
8.0
8.2
8.4
8.6
-2.0
0.0
2.0
4.0
6.0
8.0
10.0
Water
Sediment
Depth in sediment (mm)
2-m site
8-m site
21-m site
75-m site

Figure 5.

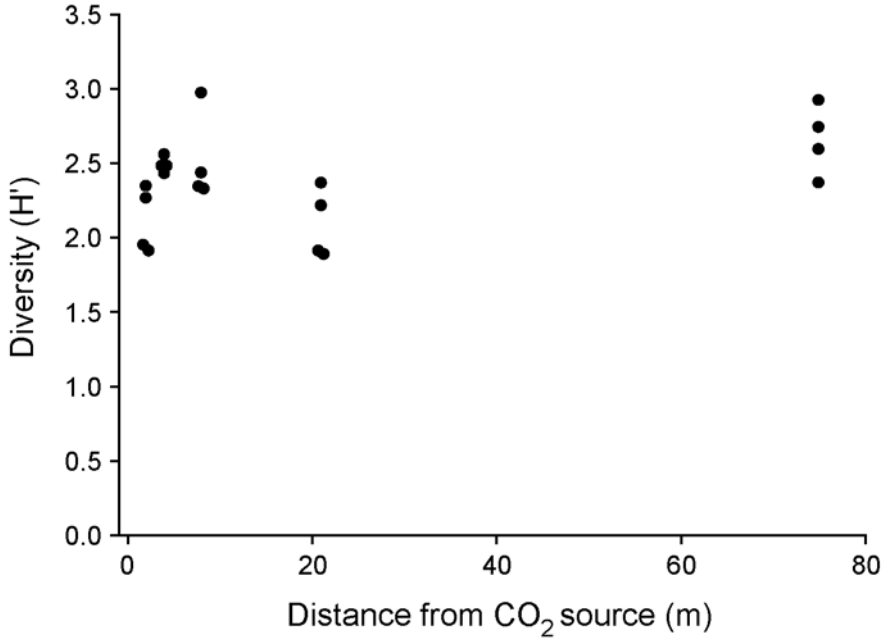
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
Diversity (H')
0
20
40
60
80
Distance from CO_2 source (m)

Figure 6.

Deep-sea epibiont ciliates from harpacticoid copepod hosts

Linda Sedlacek[a], David Thistle[a,*], Gregorio Fernandez-Leborans[b]

[a]Department of Oceanography

Florida State University

Tallahassee, FL 32306-4320, USA

thistle@ocean.fsu.edu

[b]Departmento de Zoologia

Facultad de Biologia

Pnta 9

Universidad Complutense (UCM)

28040 Madrid, Spain

greg@bio.ucm.es

[*]Corresponding author. Tel.: +1-850-644-4089, fax.: +1-850-2581

E-mail address: thistle@ocean.fsu.edu (D. Thistle)

Abstract

The biology of associations between ciliate epibionts and crustacean hosts is of interest because epibionts can stress their hosts, but these relationships are poorly known for deep-sea species. To begin to remedy this situation, we investigated associations with harpacticoid copepod hosts from the continental rise off central California. We found undescribed species of *Loricophrya*, of *Trachelolophos*, and of *Vorticella*. The *Loricophrya* species is the first of this genus found on a crustacean host and the first record of the genus in deep sea. The same was true of the *Trachelolophos* species. The *Vorticella* species is the first of this genus found on a harpacticoid host. That each species occurred significantly more often on only one or two of the more than 40 potential harpacticoid host species and epibiont individuals were significantly more abundant on adults than on juveniles of their host species suggested that host individuals were in excess. In contrast, the proportion of adult males on which ciliate epibionts occurred did not differ significantly from that of adult females. The three epibiont species differed conspicuously in the body regions of the host where individuals attached, and the degree of specialization among species paralleled trends among ciliate taxa observed in shallow water. Some ciliate epibiont species damage the

exoskeletons of their hosts, but ours did not, so whether their presence is stressful remains unclear.

Keywords: Harpacticoida; Ciliata; Symbiosis; Benthos; Continental Rise; USA; California; 36°42’N; 123°31’W

1. Introduction

Epibiotic associations are complex because they can involve costs and benefits for both the epibiont and its host. For example, the epibiont need not invest energy in locomotion, but the behavior of the host determines the locations it visits. The host may benefit from the cleansing activities of the epibiont, but the epibiont may compete with it for food.

Ciliate epibionts have been known to occur on crustacean hosts in shallow-water environments since at least the 1930's (see Chatton and Lwoff, 1935; Marshall and Orr, 1955; Herman and Mihursky, 1964; Fenchel, 1965; Morado and Small, 1995; Williams and McDermott, 2004). They also occur on crustaceans in the deep sea (Ólafsdóttir and Svavarsson, 2002; personal observation). In shallow water, ciliate epibionts can decrease the ability of their crustacean hosts to withstand stress (Xu and Burns, 1991; Puckett and Carman, 2002). They may pose similar challenges for their deep-sea hosts, so the biology of this relationship in the deep sea is of interest.

Unfortunately, because the nature of the relationship between deep-sea ciliate epibionts and their crustacean hosts is almost unknown, framing testable phylogenetic and ecological hypotheses is difficult. To begin to remedy this situation, we asked four questions about this relationship that were inspired, in part, by those of Ólafsdóttir and Svavarsson (2002). (1)

What are the taxonomic affinities of the ciliate epibiont species present at a deep-sea station? (2) Do they use a particular species, sex, or life stage of their crustacean hosts disproportionately? (3) Do they attach to particular regions of the host's body? (4) Do they damage their host's exoskeleton? Ólafsdóttir and Svavarsson (2002) investigated asellote isopods. To broaden understanding of ciliate epibionts on crustacean hosts, we chose to study a different crustacean group, the harpacticoid copepods, which we knew carried epibiont ciliates (personal observation), could be sampled in large numbers, and were phylogenetically distant from asellote isopods.

2. Materials and methods

2.1. Station

The station was located on the continental rise off central California (36° 42'N, 123° 31'W; Fig. 1) at 3607 m depth and was designated CO_2-5 by Barry ***et al.*** (2005). During our study, the bottom-water temperature and salinity were 1.55°C and 34.39. Oxygen, alkalinity, and pH were 120 µM, 2440 µm kg^{-1}, and 7.78. The average current speed 15 m above the seafloor was 2.9 cm s^{-1}. Tubes of the ampeliscid amphipod ***Haploops lodo*** J. L. Barnard were the major biological feature of the sediment surface. See Barry et al. (2005) for more information.

2.2. Sample collection

We studied the control samples taken during an investigation of the ecological consequences of carbon dioxide sequestration in the deep sea (see Barry *et al*., 2005, for details). Specifically, our data come from four 7-cm-inner-diameter cores taken on 18 September 2003 by the *ROV Tiburon* of the Monterey Bay Aquarium Research Institute; separation between adjacent cores was ~20 cm. Once the cores were on board, they were stored in an approximately 4°C cold room until processed.

2.3. Sample processing

The water in the core above the sediment was aspirated into a flask and poured through a 30-μm-aperture sieve. The contents of the sieve were placed in the sample jar that would receive the uppermost sediment layer. A subcorer (1.9-cm inner diameter) was placed in the center of each core for a collaborator. The remainder of the core was sliced around the subcorer into these layers: 0–5 mm, 5–10 mm, 10–20 mm, and 20–30 mm. Samples were preserved in a solution of one part formalin and nine parts artificial seawater of salinity 35 that was buffered with sodium borate.

In the laboratory, each sample was stained overnight with rose bengal. Harpacticoids were removed from the sediment, and adults were identified to sex and to working species (a group of individuals believed to belong to a single species that has yet to be formally described) because, as is typical in the deep sea (see, e.g., Thistle, 1978; Seifried, 2004), almost all species

were undescribed. After identification, specimens were stored in glycerin drops on microscope slides.

To discern working species of ciliate epibionts, we examined candidate individuals with light microscopy (Nikon Microphot-FX microscope with a Zeiss AxioCam digital camera attached; images captured with the program AxioVision) and scanning electron microscopy (JEOL JSM-6380). To prepare an epibiont individual for the scanning electron microscope, we placed the host individual between two pieces of sieve material (50-µm-aperture mesh) in a holder (Fig. 2). To remove the glycerin in which the epibiont and host had been stored, we placed the holder in a solution of one part ethanol and four parts deionized water, which was agitated on a shaker table for 45 min. The solution was replaced, and the holder and its fresh solution were returned to the shaker table for 45 min. These steps were repeated four times. The ciliate and its host were then dehydrated in ethanol-deionized-water solutions as follows: 30% for 10 min, 40% for 10 min, 50% for 10 min, 70% for 10 min, 90% for 10 min, 95% for 10 min, and 100% for 15 min (during which the ethanol was changed at 5-min intervals). After critical-point drying (CPD 020 Balzers Union), each specimen was placed on a carbon adhesive tab on a metal stud and coated with a mixture of gold and palladium (Emscope SC500 sputter coater). We identified the ciliate epibiont species to the lowest possible taxon.

2.4. Analyses

To determine whether a particular ciliate epibiont species occurred disproportionately on individuals of a particular

harpacticoid species, we calculated the proportion of the individuals of each harpacticoid species that had the ciliate species attached and the proportion of the remaining harpacticoid individuals in the core that had individuals of the ciliate species attached. We tested for a difference between the average of the former proportion among the cores and the average of the later (Mantel-Haenszel test, Sokal and Rohlf, 1995).

For each harpacticoid species that hosted a given ciliate epibiont species, we also asked whether the host's abundance was significantly greater than that of the average harpacticoid species at our site. To do so, we ranked the harpacticoid species in reverse order of abundance. To create an expected distribution for a given test, we noted the number of species of harpacticoids occupied by a given ciliate epibiont species. We drew that number of harpacticoid species at random and summed their ranks with the aid of the computer program Resampling Stats 5.0.2 (Simon, 1999). Following Manly (1991), we repeated the procedure 4999 times and asked whether the sum of the ranks of the host species that we observed for a given ciliate epibiont species was significantly less than expected by chance (one-tailed test), indicating that the epibiont species occurred on relatively abundant harpacticoid species. We repeated the test for each of the remaining epibiont ciliate species.

To investigate the pattern of occurrence of ciliate epibiont species among the sexes of their host species, we asked for each ciliate epibiont species whether it occurred disproportionately on one sex of each harpacticoid host species. To do so, we

calculated the proportion of the male and female individuals of a host species that harbored a particular epibiont species for each core and tested for a significant difference between these proportions (Mantel-Haenszel test, Sokal and Rohlf, 1995). This test could only be done on adults because identification of juveniles to species is not yet practicable for deep-sea harpacticoids.

To determine whether each ciliate epibiont species occurred disproportionately on juvenile (i.e., copepodite stages I to V) or adult hosts, we calculated for all harpacticoid species for each core the proportion of the adults and of the juveniles that had the epibiont species attached. We tested for a difference between the proportions of adults with epibionts and the proportion of juveniles with epibionts (Mantel-Haenszel test, Sokal and Rohlf, 1995).

In this exploratory study, we made no correction for multiple testing.

3. Results

3.1. The taxonomic affinities of the ciliate epibiont species and their hosts

Of the epibionts encountered, *Loricophrya* sp. (Fig. 3), a species tentatively placed in the genus *Trachelolophos* (Fig. 4), and *Vorticella* sp. (Fig. 5) occurred frequently enough (>3 harpacticoid individuals with a specific epibiont species attached) for further analyses. The taxonomic positions of these species are shown in Fig. 6. They are referred to hereafter by their genus names.

We recognized 45 working species of harpacticoids, which belonged to 13 families. None of the species appeared to have been described, a situation that is not unexpected in the deep sea (see for example Thistle, 1978; Seifried, 2004; Baguley et al., 2006). Sedlacek (2007) gives additional taxonomic information.

3.2. The occurrence of ciliate epibiont species on host species

Of the host individuals on which *Loricophrya* occurred, all but one was a *Nitokra* sp. Of the host individuals on which *Trachelolophos* occurred, all but one was a *Nitokra* sp. *Vorticella* occurred only once on an individual of a species other than *Ameira* sp. and *Mesocletodes* cf. *irrasus*. These qualitative indications of restricted occurrence were all statistically significant (Table 1). Sedlacek (2007) gives the data.

All of the harpacticoid species used as hosts were relatively abundant (Fig. 7). ***Ameira*** sp. was the most abundant species (average = 8.25, median = 7, and range = 4 to 15 individuals per core). ***Nitokra*** sp. was the second most abundant (average = 6.25, median = 5, range = 4 to 11 individuals per core). ***Mesocletodes*** cf. ***irrasus*** had an average of 1.25, a median of 1.5, and a range of 0 to 2 individuals per core. The host(s) for each ciliate epibiont species was significantly more abundant than the average harpacticoid species (***P*** = 0.017 for ***Loricophrya*** and ***Trachelolophos; P*** = 0.008 for ***Vorticella*** sp.).

3.3. The occurrence of ciliate epibiont species on males and females of their hosts

For none of the host-species-epibiont-species combinations tested (Table 2) did the individuals of a ciliate epibiont species occur significantly more frequently on one sex of the host species. *Mesocletodes* cf. *irrasus* was not tested because too few individuals were collected. Sedlacek (2007) gives the data.

3.4. The occurrence of ciliate epibiont species on adult and juvenile harpacticoids

We examined 327 juvenile and 546 adult harpacticoids. *Loricophrya* occurred on none of the juveniles and 15 of the adults; *Trachelolophos* occurred on none of the juveniles and 8 of the adults; *Vorticella* occurred on 1 of the juveniles and 8 of the adults. For each epibiont species, the disproportionate occurrence on adults was significant.

3.6. The attachment locations of ciliate epibionts

Loricophrya and *Trachelolophos* occurred on the antennules of their hosts and on no other locations (Fig. 8). *Vorticella* occurred in approximately equal numbers on the sides of the cephalosome, in the vicinity of the mouth parts, and on the body between the swimming legs (Fig. 8).

3.7. ***Do the ciliate epibionts damage the exoskeleton of host individuals***?

From our scanning electron micrographs, we conclude that the three ciliate epibiont species we studied do not damage the surface of the host exoskeleton. In addition, the photomicrographs revealed no evidence of bacterial growths where

the epibionts attached, which can indicate damage (Turner et al., 1979).

4. Discussion

4.1. The taxonomic affinities of the ciliate epibiont species

The abundant ciliate epibiont species on harpacticoids at our 3607 m deep site are not closely related (Fig. 6) and feed in different ways. The genus ***Loricophrya*** belongs to the suctorian ciliates, a group that uses tentacles to ingest individual prey, including food items captured by the host (Fenchel, 1965; Lynn and Small, 2000). Species of suctorian ciliates have been found on harpacticoids, e.g., *Lecanophrya drosera* on *Nitokra typica* and *Ophyodendron reversum, Dentacineta campanuliformis* and *Acineta truncata* on *Ameira* (Fernandez-Leborans and Tato-Porto, 2000b).

Species of the genus ***Loricophrya*** have not previously been reported from the deep sea, but the closely related genus *Acineta* was found on deep-sea asellote isopods (Ólafsdóttir and Svavarsson, 2002). In shallow water, species of *Loricophrya* attach themselves to inanimate objects as well as algae, polychaetes, sponges, nematodes, and echinoderms (Curds, 1987), but each species appears to attach to a single type of substrate. We report the first use of a crustacean.

The genus ***Trachelolophos*** belongs to the karyorelictid ciliates, a group that ingests individual prey (Lynn and Small, 2000). They had been known only from marine sediment particles

(Foissner and Dragesco, 1996), so we are the first to report their association with a host and their presence in the deep sea.

The genus ***Vorticella*** belongs to the peritrich ciliates of the order Sessilida, which are bacterivorous or microalgivorous filter feeders (Fenchel, 1965; Lynn and Small, 2000) that take advantage of the water currents produced by the host (Fenchel, 1965). Species of ***Vorticella*** have been found in freshwater and marine environments (including the deep sea, Ólafsdóttir and Svavarsson, 2002) on hard substrates (e.g., plants, sediment particles, and animals). They have been reported on several animal taxa (mostly in Crustacea), including cyclopoid copepods (see review by Fernandez-Leborans and Tato-Porto, 2000a). Although the peritrich *Cothurnia nitocrae* has been found on a species of harpacticoid (Precht, 1935), ours is the first report of a species of ***Vorticella*** on harpacticoids.

4.2. The occurrence of ciliate epibiont species on host species

In shallow water, ciliate species that are epibionts on crustaceans tend to be host specific (Curds, 1987). The three ciliate epibiont species we studied occurred significantly more often on one, one, and two species of the more than 40 species of potential harpacticoid hosts. We know nothing about their possible use of other hosts, but taken at face value, this result seems to show that the deep-sea ciliate epibionts we

studied followed the shallow-water pattern. In contrast, Ólafsdóttir and Svavarsson (2002) found ciliate epibionts on 15 of 32 species of deep-sea asellote isopods.

That the host species used were particularly abundant raises the question of whether the ciliate species specialized on abundant harpacticoid species or whether the harpacticoid host species were abundant because of a benefit bestowed by their epibiont ciliates. Observations of shallow-water epibionts of copepods suggest that under normal conditions ciliate epibionts neither benefit nor tax their hosts but that, under stressful conditions, the presence of epibiont individuals affects host individuals adversely (Herman and Mihursky, 1964; Turner et al., 1979; Xu and Burns, 1991; Weissman et al., 1993; Puckett and Carman, 2002). The ciliate epibionts therefore seem unlikely to be the reason that their host species are relatively abundant, so our results may indicate that selection discourages use of rare host species.

We found epibionts significantly more frequently on adults than on juveniles. Taken at face value, this result implies that the ciliate epibiont species specialize on adult harpacticoids. Alternatively, if ciliate epibiont individuals used both adult and juvenile harpacticoids roughly equally but the duration of the adult phase were much longer than the juvenile phase, then the proportion of the individuals occupied

by ciliate epibionts would be biased in favor of adults. The relative durations of the juvenile and adult phases of deep-sea harpacticoids are not known, but our result is so extreme that we feel it indicates that the epibiont species occupy adult hosts almost exclusively.

4.3. The attachment locations of ciliate epibionts

In their review, Carman and Dobbs (1997) noted that some ciliate epibionts occurred more frequently on some body regions than others (e.g., the ciliate ***Ophryodendron*** sp. occurred exclusively on the caudal ramus of the poecilostomatoid copepod ***Lichomolgus singularipes***), whereas some were found on many different areas of the body (e.g., the ciliate ***Rhabdostyla*** sp. occurred on all sides of the cephalothorax and urosome of the calanoid copepod ***Acartia hudsonica***). Henebry and Ridgeway (1979) suggested that suctorian ciliates, which are predacious, attached preferentially to the anterior portion of their host harpacticoids, where they would be exposed to the greatest number of potential prey. These authors also suggested that peritrich ciliates, which are suspension feeders, were not attachment-site specific. Our ***Loricophrya*** species is a suctorian, and we found it to be more specific to attachment sites than our species of ***Vorticella***, a peritrich ciliate, so to that extent, our results follow the trend found in shallow-water copepods.

4.4. *Do the ciliate epibionts damage the exoskeleton of host individuals*?

Individuals of none of the ciliate epibiont species that we examined appeared to have damaged the exoskeleton of their hosts, so if the epibionts have negative effects on their hosts, they must arise from a different mechanism.

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Table 1. P-values showing that *Loricophrya* and *Trachelolophos* occurred significantly more often on *Nitokra* sp. and that *Vorticella* occurred significantly more often on *Ameira* sp. and *Mesocletodes* cf. *irrasus.* The latter is abbreviated as *Mesocletodes*.

	Host species			
Ciliate species	*Nitokra*	*Ameira*	*Mesocletodes*	cf. *Nitokra*
Loricophrya	<0.001	0.569		
Trachelolophos	<0.001	0.847		
Vorticella		0.013	0.001	0.512

Table 2. P-values showing that in none of the host-ciliate combinations was one sex of the host species used more frequently than the other. N.a. is not applicable. *Mesocletodes* is *Mesocletodes cf. irrasus*.

	Host species		
Ciliate species	*Nitokra*	*Ameira*	*Mesocletodes*
Loricophrya	0.4895		
Trachelolophos	0.7528		
Vorticella		0.4078	n.a.

Figure captions

Figure 1. A chart showing the location of the station off the central California coast.

Figure 2. A diagram of the holder used during preparation for scanning electron microscopy.

Figure 3. (A) Light microscope photograph of *Loricophrya* sp. Scanning electron microscope photographs of *Loricophrya* sp. showing (B) the whole organism, (C) the attachment site, and (D) a cilium.

Figure 4. (A) Light microscope photograph of *Trachelolophos* sp. Scanning electron microscope photographs of *Trachelolophos* sp. showing (B) the whole organism, (C) the attachment site, and (D) the oral region.

Figure 5. (A) Light microscope photograph of *Vorticella* sp. Scanning electron microscope photographs of *Vorticella* sp. showing (B) the whole organism, (C) the attachment site, and (D) the oral region.

Figure 6. The taxonomic relations among the ciliate epibiont species and among their hosts. Arrows indicate ciliate epibiont-host associations.

Figure 7. A rank-order-of-abundance graph showing that host species (filled bars) are unusually abundant.

Figure 8. Ciliate attachment sites, showing that those of *Loricophrya* and *Trachelolophos* differ markedly from that of *Vorticella*. (A) *Loricophrya* and *Trachelolophos* on *Nitokra* sp. (B) *Vorticella* on *Mesocletodes* cf. *irrasus*. Arrows indicate ciliate epibionts.

Figure 1

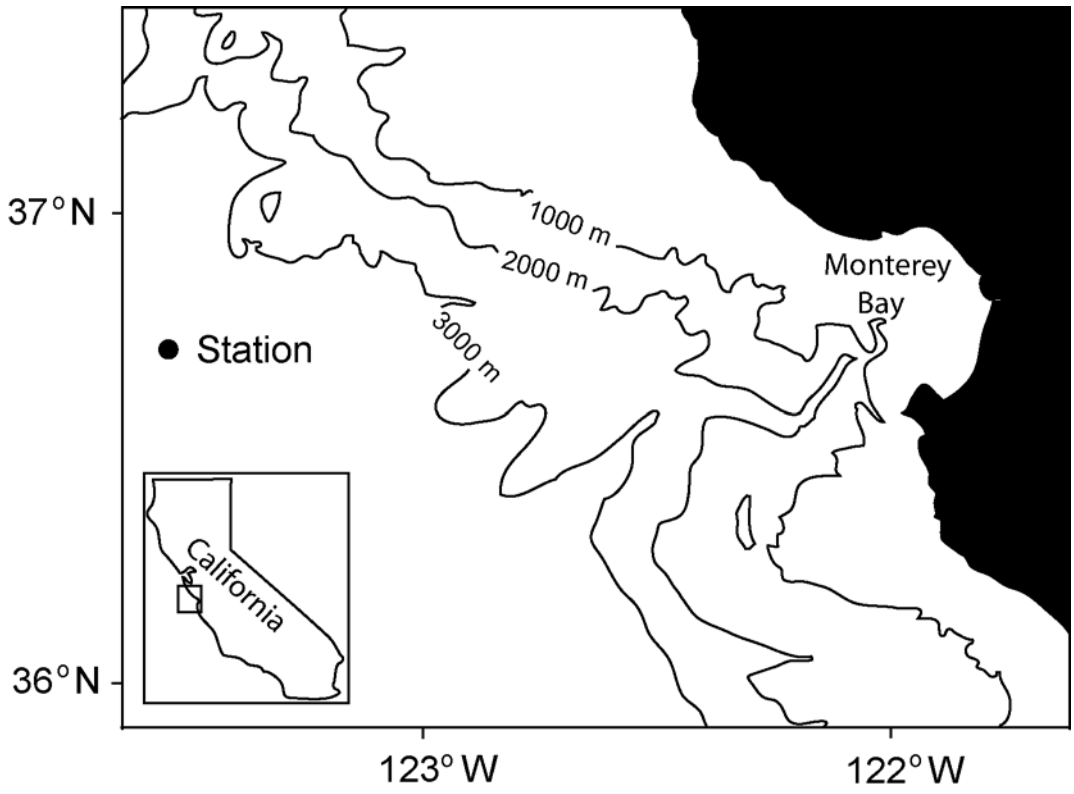
37°N
36°N
123° W
122° W
1000 m
2000 m
3000 m
Station
Monterey
Bay
California

Figure 2.

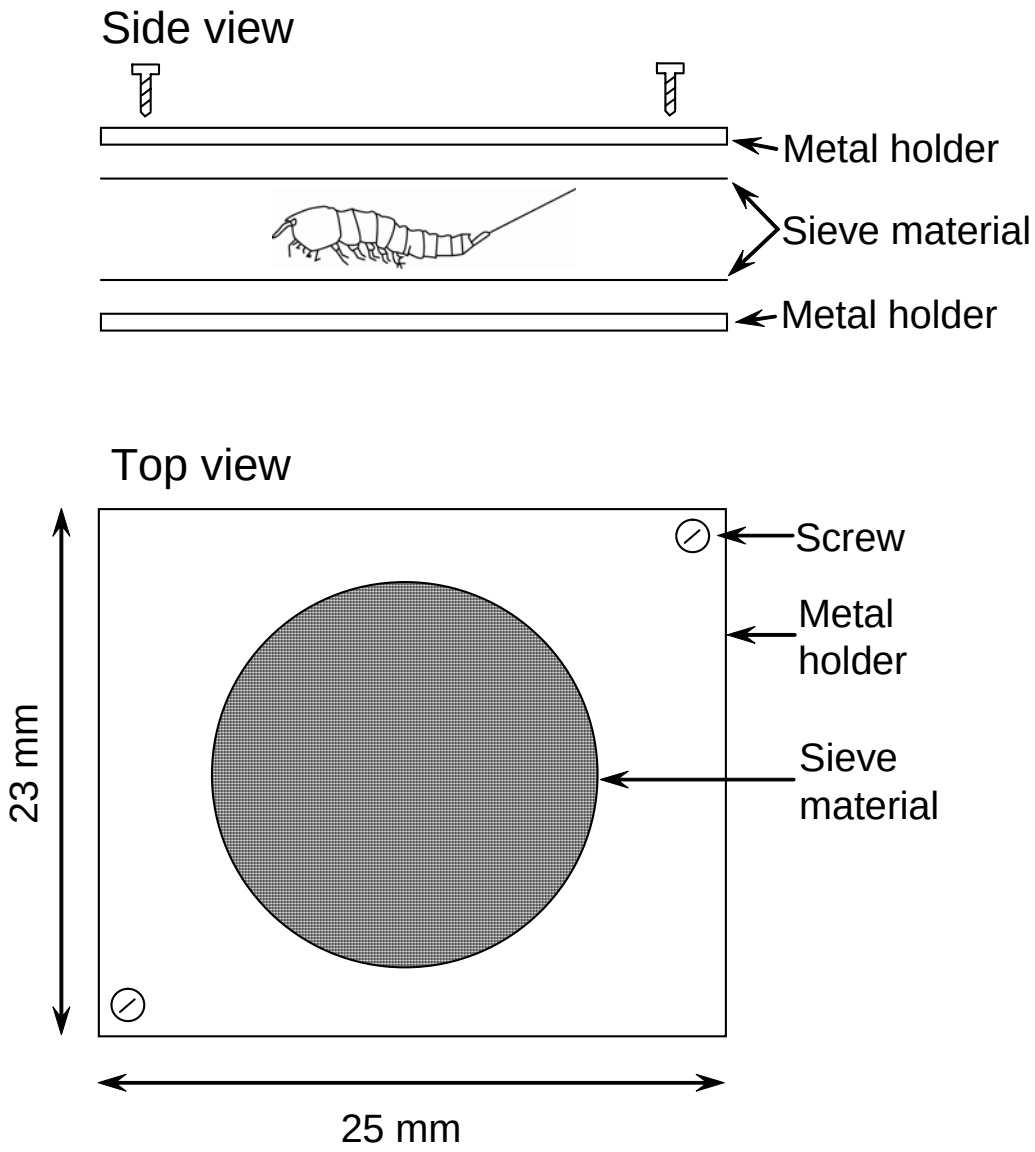
Side view
Metal holder
Sieve material
Metal holder
Top view
Screw
Metal holder
Sieve material
23 mm
25 mm

Figure 3.

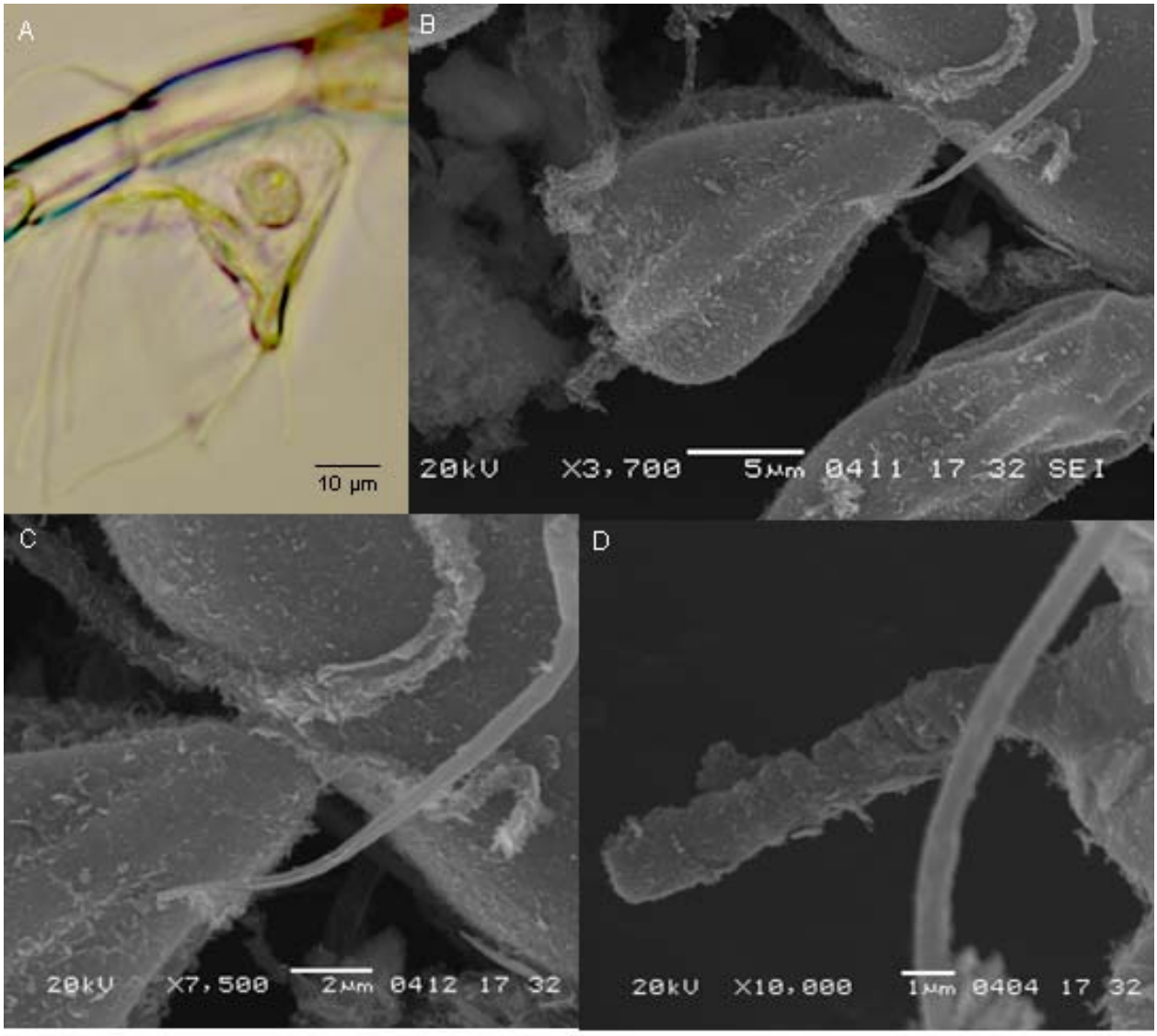
A
10 µm
B
20kV X3,700 5µm 0411 17 32 SEI
C
20kV X7,500 2µm 0412 17 32
D
20kV X10,000 1µm 0404 17 32

Figure 4.

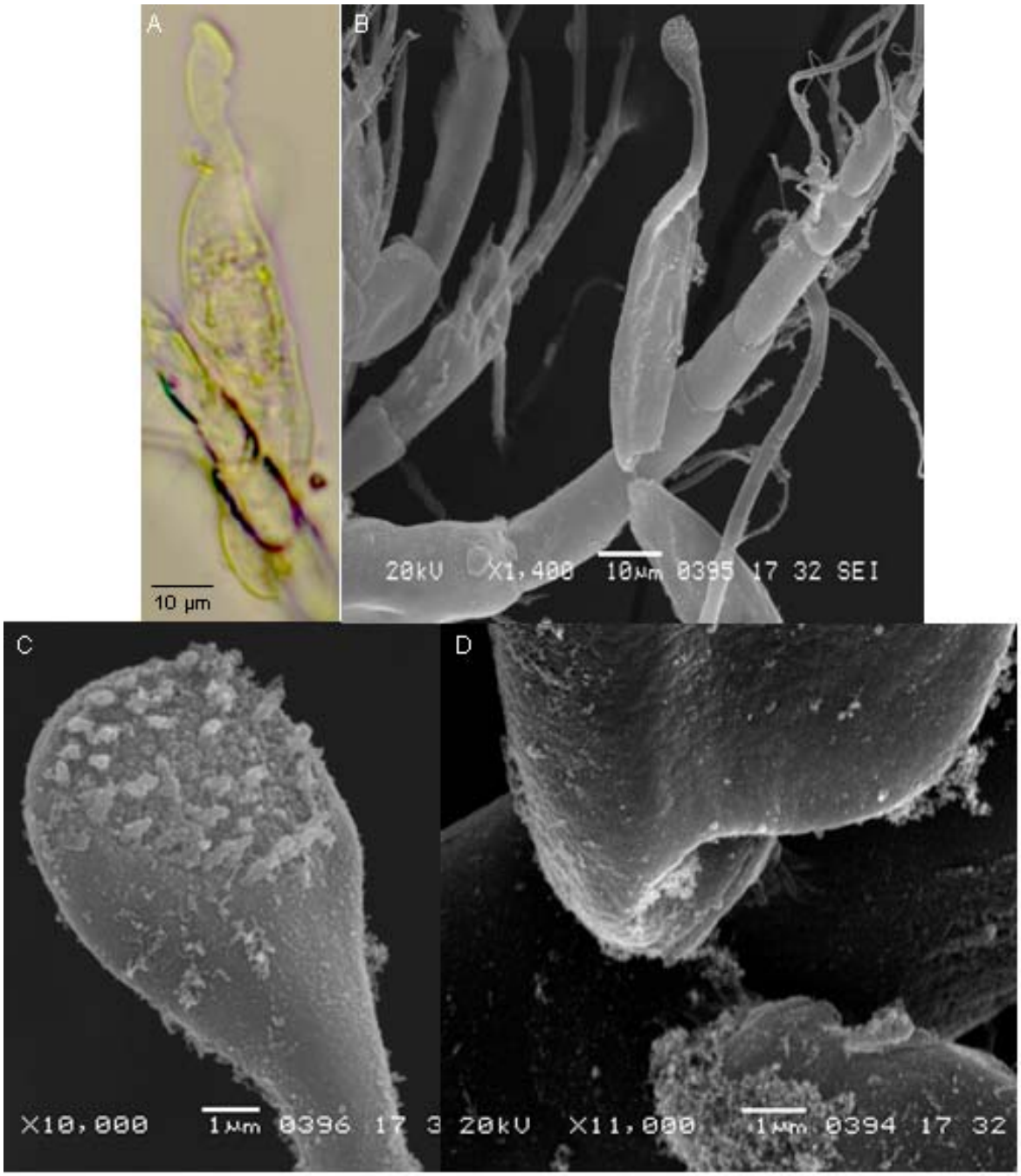
A
10 µm
B
20kU X1,400 10µm 0395 17 32 SEI
C
X10,000 1µm 0396 17 3
D
20kU X11,000 1µm 0394 17 32

Figure 5.

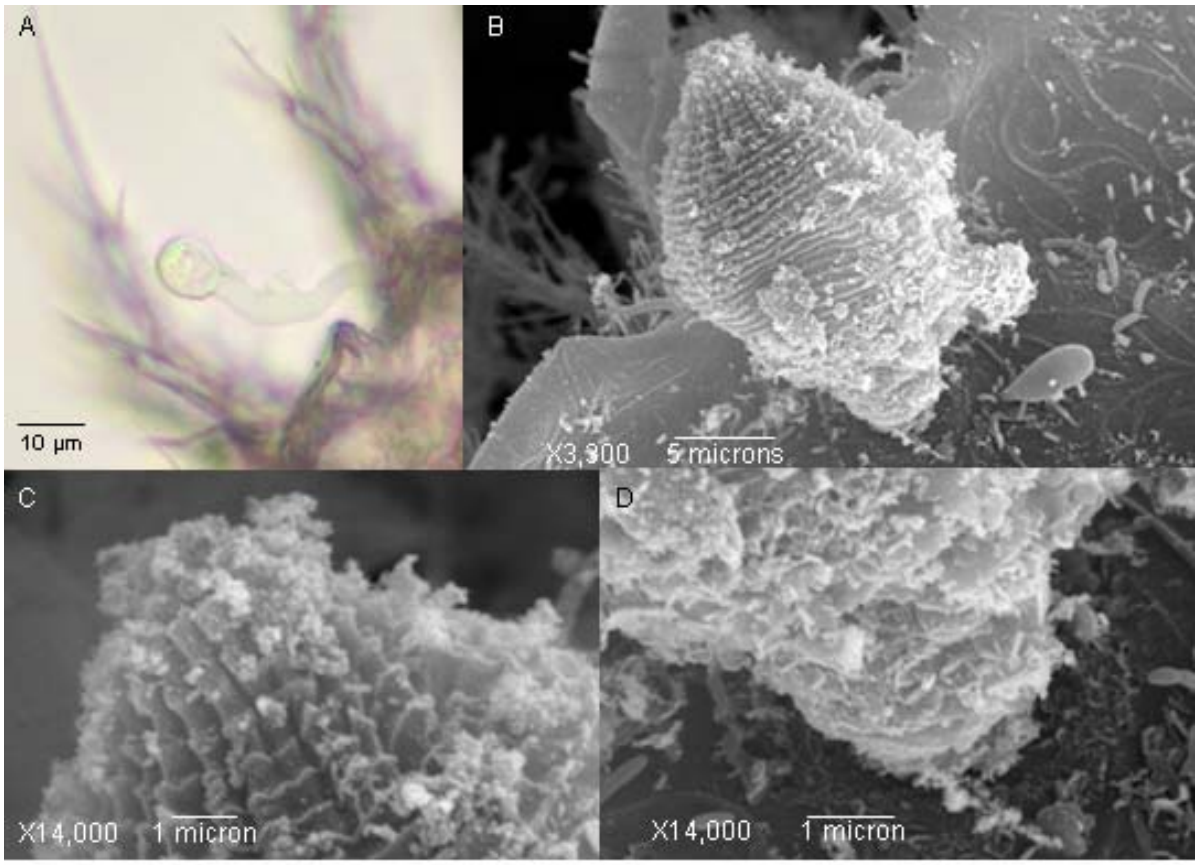

Figure 6.

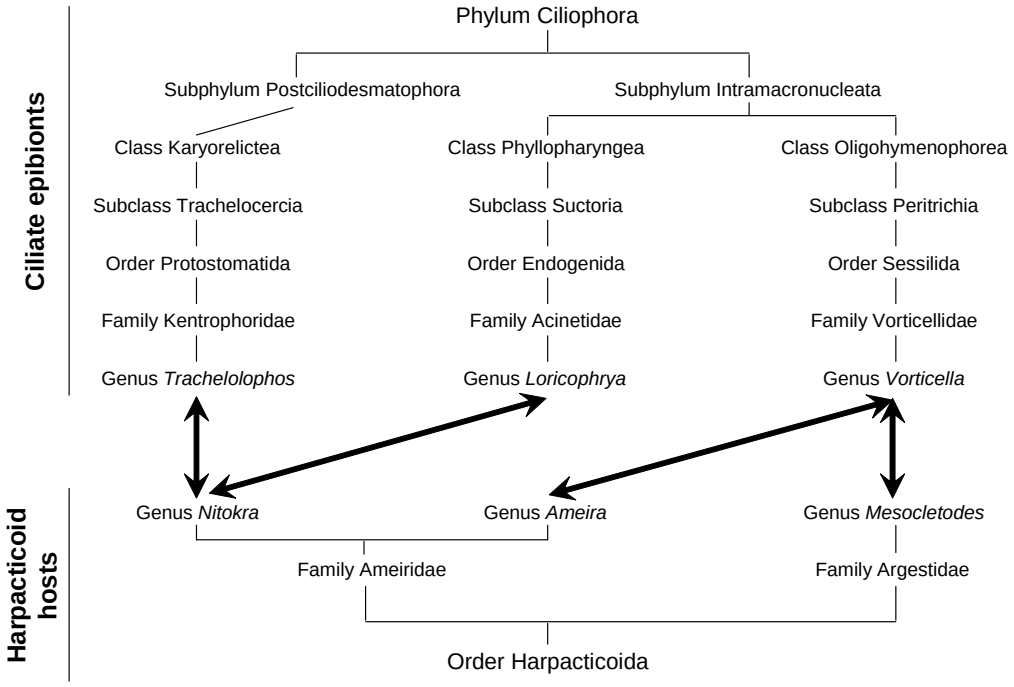
Phylum Ciliophora
Subphylum Postciliodesmatophora
Subphylum Intramacronucleata
Class Karyorelictea
Class Phyllopharyngea
Class Oligohymenophorea
Subclass Trachelocercia
Subclass Suctoria
Subclass Peritrichia
Order Protostomatida
Order Endogenida
Order Sessilida
Family Kentrophoridae
Family Acinetidae
Family Vorticellidae
Genus Trachelolophos
Genus Loricophrya
Genus Vorticella
Genus Nitokra
Genus Ameira
Genus Mesocletodes
Family Ameiridae
Family Argestidae
Order Harpacticoida
Ciliate epibionts
Harpacticoid hosts

Figure 7.

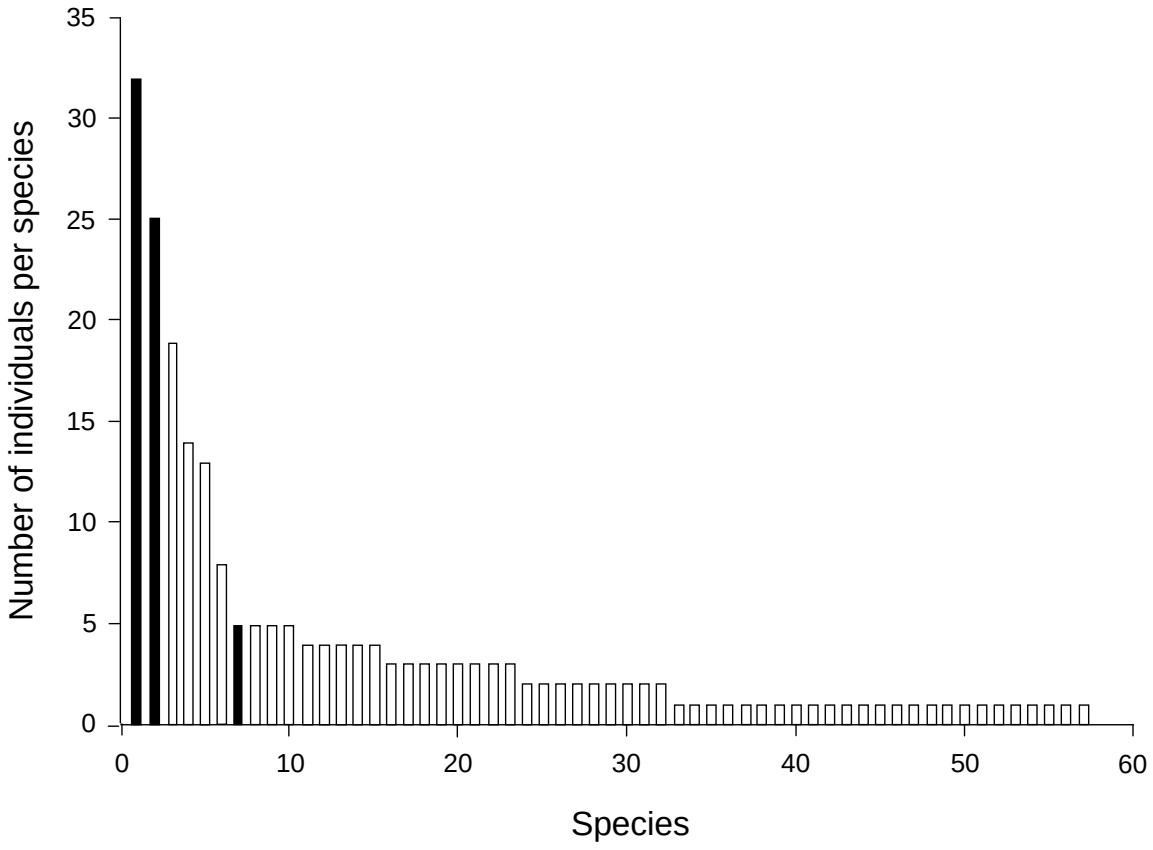
Number of individuals per species
0
5
10
15
20
25
30
35
0
10
20
30
40
50
60
Species

Figure 8.