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Optimising the Extraction of Bacteria, Heterotrophic Protists and Diatoms, and Estimating Their Abundance and Biomass from Intertidal Sandy Sediments

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The study of microbial communities in sediments is impaired by the lack of reliable extraction methods. This study reports on the efficiency of recovery of a method for extracting bacteria, protists and diatoms from sandy sediments using a modified decant/fix method. The best extractions were achieved after fixation with a microtubule stabilising fixative and subsequent sonication for 80 seconds. We estimate that the efficiencies of recovery of bacteria and mixed heterotrophic flagellates were 96.4±3.5% and 96.9±4.6%, respectively. Diatoms were recovered with an efficiency of 38-83% and varied considerably from species to species. This study suggests that the decant/fix method is effective in extracting small cells such as bacteria and heterotrophic flagellates, and that the efficiency of recovery of the method varies due to cell length and different types of organisms. When the factors to correct inaccuracies were applied to field data from Port Botany, we found that the total microbial carbon biomass had been underestimated by up to 32%, with much of that relating to larger cells such as microalgae and ciliates. We note that the corrected abundances may be still a subset of the total numbers present.

Key words: Protists, Extraction, Decant/fix method, Sediments

INTRODUCTION

The growing attention to the significance of protists in planktonic microbial food webs (e.g., Azam *et al.*, 1983; Sherr and Sherr, 1988; Kirchman, 1994) has stimulated interest in their benthic counterparts (e.g., Hondeveld *et al.*, 1994; Starink *et al.*, 1996a, b; Epstein, 1997a; Lee and Patterson, 2002). The benthic ecosystem is less well understood than the planktonic ecosystem, because sediments are highly heterogeneous environments containing differing proportions of water, organic matter and inorganic materials; and the physical and chemical characteristics vary to a great extent (Yamamoto and Lopez, 1985; Fenchel, 1987). All components of the microbial food web are likely to be more abundant in sediments than in water columns (e.g., Patterson *et al.*, 1989; Kemp, 1990; Epstein, 1997b). The little information to date suggests that heterotrophic flagellates play an important role in benthic microbial food webs (Bak and Nieuwland, 1989; Alongi, 1991; Epstein, 1997a; Dietrich and Arndt, 2000; Lee and Patterson, 2002). Unfortunately, benthic microbial communities have not been studied as extensively as communities of the water column, and there have only been a few studies which indicate that heterotrophic flagellates are numerically important in sediments (e.g., Hondeveld *et al.*, 1994; Starink *et al.*, 1996b; Epstein, 1997a). Of those that have been conducted, most concentrate on ciliates because of their relatively large size and ease of extraction compared to other small protists such as heterotrophic flagellates (Fenchel 1967, 1968a, b, 1969; Patterson *et al.*, 1989). Difficulties in extracting and enumerating small benthic protists have impaired subsequent progress. Available estimates of benthic flagellate abundance use different extraction methods (e.g., Alongi, 1986; Bak and Nieuwland, 1989; Starink *et al*, 1996b; Epstein, 1997a, b; Tso and Taghon, 1997; Dietrich and Arndt, 2000). These estimates vary due to factors such as the methods of extraction/enumeration applied, the size ranges of organisms examined and the workers experience (Alongi, 1991). It seems likely that these results may not be accurate and may not be comparable. There have been efforts to find extraction

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methods which have a high recovery and which are consistent (Bak and Nieuwland, 1989; Alongi, 1991; Starink *et al.*, 1994; Epstein and Rossel, 1995; Epstein *et al.*, 1997). A standard method should ideally recover all microbes (bacteria, nano-, micro- and meiobenthos) with a high efficiency of recovery from the same sample on a single preparation, in order to reduce effort and to provide consistent estimates of the abundance of microbes when repeated. With a better understanding of the efficiency and the requirements for replicates, we can develop correction factors to correct experimental data.

It is little known if all microbes in sediments are extracted by the extraction methods below-mentioned. We only have knowledge of those organisms which are extracted and no information on the ones which are not extracted. Generally, free-swimming cells are expected to recover more successfully than gliding or sessile cells because they are less likely to adhere to particles. For examples, benthic diatoms and some euglenids glue themselves to the sand grains so are potentially more difficult to extract. Further experiments with species which are known to strongly adhere to particles would be necessary in order to determine their extraction efficiency.

A variety of methods have been used to extract/enumerate microbes from sediments. These include; cultivation (Mare, 1942), the coverslip method (Webb, 1956; Larsen and Patterson, 1990), dilution pipetting (Lackey, 1961), seawater ice flushing (Uhlig, 1964; Fenchel, 1967, 1970), percoll-sorbitol mixture (Alongi, 1986, 1991), flushing after fixation (Bak and Nieuwland, 1989), decant/fix with sonication (Epstein *et al.*, 1997), isopycnic centrifugation (Starink *et al.*, 1994; Epstein, 1995) and rate zonal centrifugation (Starink *et al.*, 1994). All methods may underestimate the abundance and biomass of microbes in sediments. Most of the methods have been reviewed and evaluated by several workers (e.g., Alongi, 1986, 1990; Bak and Nieuwland, 1989; Epstein, 1995).

The decant/fix method of Epstein and co-workers (Epstein *et al.*, 1997) seems to hold the greatest promise of allowing all microbes to be quickly and simultaneously counted from the same sample on a single preparation. The method involves a small amount of sediment (0.4 to 0.7 cm^3) being placed into a centrifugation tube containing 2 ml of 2 to 4% formaldehyde. It is sonicated for 80 seconds, shaken, and the supernatants are decanted into a flask. A small amount of fixative (2 ml of 2 to 4% formaldehyde) is added into the centrifugation tube and the tube is shaken. The supernatant is decanted into the flask. This extraction procedure is repeated 5 times. The supernatants are combined, diluted if required, and a subsample is stained with 4, 6-diamidino-2-phenylindol (DAPI at 5 µg/ml final concentration) for 10 to 15 min and filtered on a black filter paper to enumerate microbes.

One of the principal objectives of this study was to determine the optimal sonication times for dislodging microbes from sediments: short times tend to extract only a subset of the organisms present and long sonication periods destroy cells (Epstein *et al.*, 1997; Tso and Taghon, 1997). Underestimates caused by inadequate extraction or by damage to the cells must be considered (Ellery and Schleyer, 1984). We also sought to evaluate the efficiency of recovery of the decant/fix method and to estimate the extent of underestimates in abundance and biomass of microbes from field data of Port Botany.

MATERIALS AND METHODS

Experimental site

Six samples were collected from intertidal sandy sediments to a depth of about 1 cm from 0.25 square metre quadrat at six sampling points in Port Botany (151°12′50″ E; 33°58′00″S) of Botany Bay, New South Wales, Australia in January 1999. The surface sediments were well sorted with 99.36% sand (4.28% coarse sand, 81.06% medium sand, 14.02% fine sand, 0.38% silt, and 0.26% clay) and had a mean grain size of around 345 µm. For grain size analysis, Sieve analysis of Ingram (1971) and pipette analysis of silt and clay of Galehouse (1971) were used. The mean grain size of the sediments was calculated from the cumulative curves according to McCammon (1962).

Optimal sonication times

The samples collected from each sampling point were homogenised by mixing and then placing in each tray. Six tests (n=6) were conducted to obtain the optimal sonication times. For each test, seven subsamples (each 1 cm^3 sediment; n=7) were taken from each tray with an open syringe. The subsamples were separately poured into 50 ml centrifugation tubes (2.5 cm diameter, Starstedt) and fixed with 3 ml of a microtubule stabilising fixative (Weerakoon *et al.*, 1999) which includes 2% formaldehyde, 0.25% glutaraldehyde, 50 mM PIPES, 5 mM EGTA and

2 mM $MgSO_4$ (final concentration). Each of the subsamples was sonicated for 0s (hand shaking), 40s, 80s, 160s, 240s, 320s and 400s with an ultra-sonicator (Soniclean 40W), respectively. Sonic treatments were interrupted at intervals of 40s to protect cells from heating (Epstein *et al.*, 1997). After sonication, the subsamples were resuspended, shaken by hand and then the supernatants were decanted to 20 ml scintillation vials (Packard). Four more washes were performed, each time with 3 ml of sterile seawater and all 5 washes were combined, and then diluted to 5-10% to allow enumeration of cells under microscope. The diluted supernatants were stained with DAPI at 5 μg/ml (final concentration) on Nuclepore 0.22 μm pore-size polycarbonate black filters for 10–15 min. Microbes were counted in triplicate on randomly selected fields until 1000 bacteria (or 10 fields), 50 flagellates (or 50 fields) and 100 pennate diatoms less than 20 μm (or 50 fields) have been counted. A Leica microscope (DMR) equipped with epifluorescence was used for counts. BP 340–380 and BP 450–490 excitation filters were used for heterotrophic bacteria and flagellates, and algae, respectively.

Recovery experiment

The efficiency of recovery of the decant/fix method was evaluated by adding known numbers of the following benthic species/groups to abiotic sediments; bacteria, mixed heterotrophic flagellates, *Oxyrrhis marina*, *Peranema trichophorum*, mixed diatoms, *Amphora* sp.1, *Amphora* sp.2, *Gyrosigma macrum*, *Gyrosigma* spp., *Navicula* sp. and *Nitzschia closterium* (Table 1).

To obtain natural bacteria, heterotrophic flagellates and diatoms for these studies, sediment samples were taken from Port Botany and flushed with sterile seawater. The flushed seawater were screened with 3 μm, 20 μm and 50 μm meshes simultaneously to separate bacteria, flagellates and diatoms from larger organisms. In this procedure, a vacuum pump was not used to avoid breaking fragile cells. Extracted samples were centrifuged at 3500 rpm for 10 min to obtain a high density of cells. The heterotrophic flagellates consisted of mostly small bacterivores about 2–5 μm long and the diatoms consisted of mostly 4 species (*Amphora* sp.2, *Gyrosigma macrum*, *Gyrosigma* spp., and *Navicula* sp.) that were abundant in the sediment samples at that time. *Peranema trichophorum* was isolated from freshwater sediment samples of Engine Pond, Sydney by S. Al-Qassab and cultured with rice grains. *Oxyrrhis marina* and *Amphora* sp.1 were isolated from sediment samples of Avoca Beach (NSW) and cultured in f/2 media (Stein, 1973). *Nitzschia closterium* were obtained from J. Diemer (Brackish Water Research Station, Port Stephens, NSW Fisheries) and cultured in f/2 media. These cultures were maintained at room temperature (~20°C) and normal light conditions.

To create abiotic sediments, sediment samples collected from Port Botany were autoclaved, followed by overnight freezing at –20°C. Just before use, about 15 cm^3 of the sediments were autoclaved again, sonicated and then washed 10 times with 500 ml of sterile seawater (or freshwater) to remove or destory microbes. The known numbers of microbes (*Amphora* sp.1, *Nitzschia closterium, Oxyrrhis marina*, *Peranema trichophorum*) were inoculated into 1 cm^3 of sterile sed-

Table 1. Number of microbes added to sand for extraction experiments.

Group/Species	Size (μm)	Numbers added (cells/ml)	Subsamples (n)
Bacteria		13.5×10^6	10
Heterotrophic flagellates			
Mixed	Mostly 2–5	9.0×10^3	10
Peranema trichophorum	40–50	2.1×10^4	10
Heterotrophic dinoflagellate			
Oxyrrhis marina	25–35	1.0×10^4	10
Diatoms			
Amphora sp.1	~40	5.0×10^4	10
Amphora sp.2	40–50	1.2×10^3	5
Gyrosigma macrum	~100	1.0×10^3	5
Gyrosigma spp.	>150	0.5×10^3	5
Navicula sp.	50–65	1.3×10^3	5
Nitzschia closterium	25–35	1.2×10^6	10
Mixed	40–200	4.0×10^3	5

iments (Table 1). After 30 min, the sediments were fixed and sonicated for 80 sec, and then the microbes were extracted from the sediments using the decant /fix method described above. The combined (and diluted) supernatants were kept in a refrigerator until counted. The microbes added to sand were counted in 10 replicates using a Sedgwick-Rafter chamber using a Zeiss light microscope (Axiophot). For the recovery of efficiency the microbes were counted in duplicate from 5 to 10 subsamples of the combined supernatants.

Statistical analysis

Samples were tested for homogeneity of variances by Cochran's test (Winer *et al.*, 1991). A one-way ANOVA was used for testing differences among the numbers extracted at different durations of sonication.

RESULTS

Optimal sonication times

Statistically significant differences in the numbers of retained bacteria, heterotrophic flagellates and diatoms were found for different durations of sonication (Table 2). The maximal recovery of each type of organism was after 40 seconds (mostly at 80 seconds) of sonication. Longer periods of sonication caused numbers extracted to level off or decline (Fig. 1).

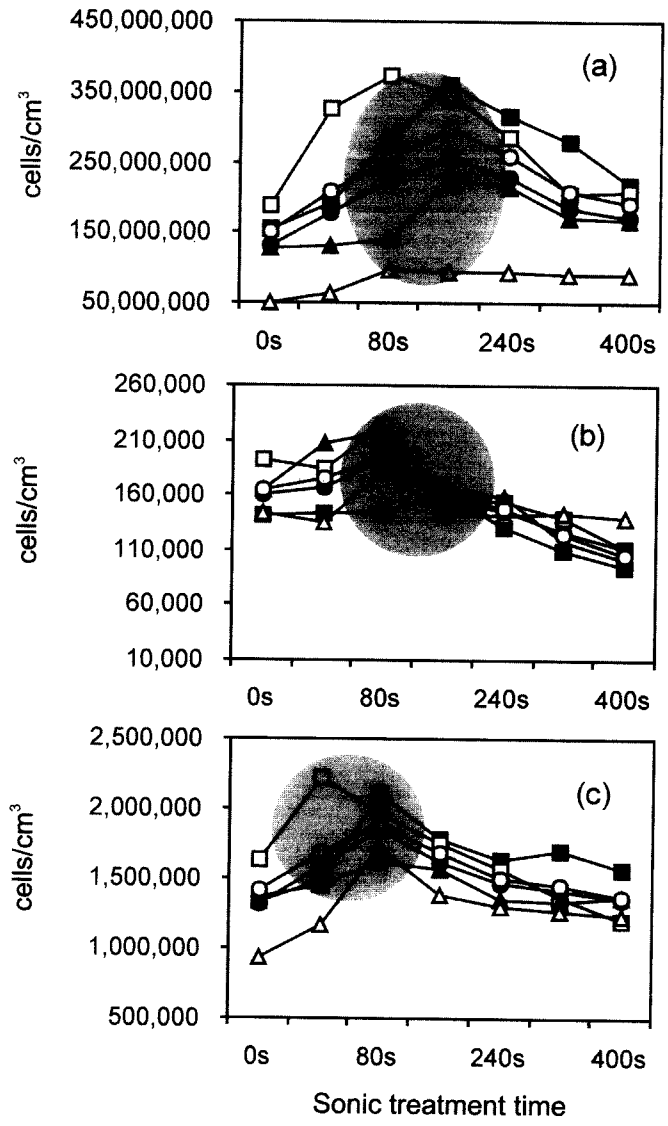

Fig. 1. Effects of sonication on the numbers of bacteria (a), flagellates (b) and diatoms (c) dislodged from sediments. Encircled areas show maximal recovery of heterotrophic flagellate added in known numbers to abiotic sediments.

Bacteria: The 6 tests suggest two different sonication times (80 and 160 seconds) are likely to give the best yields of bacteria (encircled area in Fig. 1a). The 36 counts between 80 seconds and 160 seconds were not significantly different (T=-0.60, P=0.56). The bacterial numbers extracted at 80 and 160 seconds were 1.8–2.3 times higher than at 0 second.

Flagellates: Maximal heterotrophic flagellate numbers resulted after 80 seconds sonication in five tests and from 160 seconds sonication in one test (encircled area in Fig. 1b). The flagellate numbers extracted

Table 2. One-way analysis of variance for bacteria, heterotrophic flagellates and diatoms less than 20 μm (data were untransformed; Cochran's test significant, P<0.001 for each variable; n=7 sonic treatments in each test (n=6); see text for details).

Source	DF	SS	MS	F	P
Bacteria					
Treatments	6	2.037E+17	3.396E+16	6.31	<0.001
Heterotrophic flagellates					
Treatments	6	7.841E+10	1.307E+10	28.19	<0.001
Diatoms					
Treatments	6	3.655E+12	6.091E+11	13.00	<0.001

at 80 seconds were only 1.2–1.3 times higher than at 0 second, and the yields after 0 second and 80 seconds sonication were significantly different (T=−2.24, P=0.049).

Diatoms: Maximal diatom numbers were extracted after 40 seconds sonication in one test and after 80 seconds in the other five tests (shaded area in Fig. 1c). The diatom numbers at 80 seconds were 1.2–1.8 times higher than at 0 second.

We concluded that the optimal sonication times for bacteria were found to be 80–160 seconds, and for heterotrophic flagellates and diatoms to be 80 seconds.

Recovery experiment

All samples were sonicated for 80 seconds because it was necessary to apply the same treatment for all microbes. The efficiency of recovery of the decant/fix method ranged from 38 (diatoms) to 97% (bacteria). Generally there was minor variation among replicates seen in the standard deviation, with the exception of diatoms (Fig. 2).

The efficiencies of recovery were detailed in Table 3. The efficiencies of bacteria and mixed heterotrophic flagellates were higher with 96.4±3.5% and 96.9±2.6%, respectively than those of a large heterotrophic flagellate (*Peranema trichophorum*), a heterotrophic dinoflagellate (*Oxyrrhis marina*) and diatoms (Table 3). The efficiency of mixed diatoms consisted of *Amphora* sp.2, *Gyrosigma macrum*, *Gyrosigma* spp., and *Navicula* sp. was 61.9±6.2%. The efficiency of the each component of the mixed diatoms was 73.4±11.1%, 47.5±12.4%, 37.9±3.8% and 69.3±11.1%, respectively. The cultured diatoms, *Amphora* sp.1 and *Nitzschia closterium* had 77.3±3.4% and 83.1±5.8% efficiency, respectively.

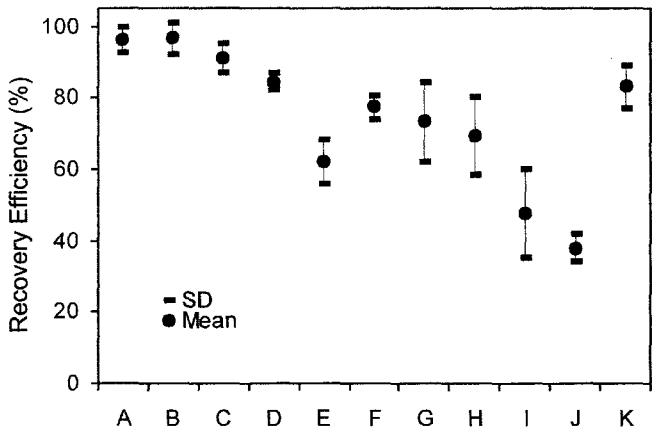

Fig. 2. Extraction efficiency of the decant/fix method applied to this study (A: bacteria, B: mixed heterotrophic flagellates, C: *Peranema trichophorum*, D: *Oxyrrhis marina*, E: mixed diatoms, F: *Amphora* sp.1, G: *Amphora* sp.2, H: *Navicula* sp., I: *Gyrosigma macrum*, J: *Gyrosigma* spp, K: *Nitzschia closterium*).

Smaller cells were recovered more successfully than large cells such as *Oxyrrhis marina*, *Peranema trichophorum* and diatoms (Table 3). The relationship between cell length and the efficiency of recovery was strongly negative (r=−0.990, P<0.001, n=6) (Fig. 3) while the relationship between cell volume and efficiency of recovery was not significant (r=−0.490, P<0.5). The efficiency of recovery of diatoms may be predicted by the regression equation (RE=94.2–0.37*CL, Fig. 3).

Application to field data from Port Botany, Botany Bay

An ecological study of heterotrophic flagellates (and other microbes) at marine sediments of Botany Bay was conducted from February 1999 to February 2000 (refer to Lee and Patterson, 2002).

The correction factors (Table 4) that were calculated using the efficiencies of recovery and the regression equations were applied to field data from Port Botany of Botany Bay (Table 5).

DISCUSSION

Optimal sonication times for microbes

It has been suggested that a sonicaton time of 60–160 seconds is best for dislodging bacteria (Epstein and Russel, 1995; Starink *et al*, 1996b; Epstein *et al.*, 1997; Tao and Taghon, 1997). There have been no recommendations for other microbes. The sonication time for dislodging bacteria obtained from this study was in the previously reported optimal range. Additionally, this study suggests the maximum yield of flagellates and diatoms will be achieved after 80 seconds sonication (Fig. 1) indicating that this time can be used for extracting other microbes.

Efficiency of recovery

Smaller cells were more effectively recovered than microbenthos (bacteria>flagellates>ciliates and diatoms) on the basis of previous and present studies (Fig. 3, Table 3).

Table 3. Extraction efficiencies (x ± SD or SE) of bacteria, flagellates, ciliates and bacteria from prior studies and this study.

Sediments Types	Species/ Group	Materials	Efficiency(%)	Approaches	References
Bacteria					
Intertidal sediments	Isotope labeled bacteria	fixed	88–98	Decant/fix with sonication	Epstein *et al.* (1997)
Sandy sediments	Bacteria assemblage	fixed	96.4±3.5	Decant/fix with sonication	Present study
Flagellate					
Freshwater sediments	*Bodo* sp	fixed	102±2.2	Rate Zonal Centrifugation	Starink *et al.* (1994a)
Freshwater sediments	*Monas* sp.	fixed	94±3.4	Isopycnic Centrifugation	Starink *et al.* (1994a)
Intertidal sediments	*Bodo* spp. & mixed flagellates	fixed	90.8±4.4	Flushing after fixation	Bak and Nieuwland (1989)
Intertidal sediments	*Bodo* spp. & mixed flagellates	fixed	68.8±10.7	Seawater-Ice	Bak and Nieuwland (1989)
Sandy sediments	*Oxyrrhis marina*	fixed	84.4±2.3	Decant/ fix with sonication	Present study
Sandy sediments	Peranema trichophorum	fixed	91.1±4.7	Decant/ fix with sonication	Present study
Sandy sediments	mixed flagellates	fixed	96.9±4.6	Decant/ fix with sonication	Present study
Mud	mixed flagellates	non fixed	85±3	Percoll-sorbitol	Alongi (1986)
Muddy sand	mixed flagellates	non fixed	91±6	Percoll-sorbitol	Alongi (1986)
Fine quartz sand	mixed flagellates	non fixed	92±3	Percoll-sorbitol	Alongi (1986)
Fine carbonate sand	mixed flagellates	non fixed	94±3	Percoll-sorbitol	Alongi (1986)
Ciliates					
Muddy sediments	*Cyclidium* sp.	fixed	65	Percoll-sorbitol	Tao and Taghom (1997)
Muddy sediments	*Euplotes* sp.	fixed	36	Percoll-sorbitol	Tao and Taghom (1997)
Mud	mixed ciliates	non fixed	85±4	Percoll-sorbitol	Alongi (1986)
Muddy sand	mixed ciliates	non fixed	95±4	Percoll-sorbitol	Alongi (1986)
Fine quartz sand	mixed ciliates	non fixed	95±3	Percoll-sorbitol	Alongi (1986)
Fine carbonate sand	mixed ciliates	non fixed	95±4	Percoll-sorbitol	Alongi (1986)
Mixed protozoa					
Freshwater sediments	mixed protozoa	fixed	101±7.0	Isopycnic centrifugation	Starink *et al.* (1994a)
Freshwater sediments	mixed protozoa	fixed	86±5.4	Rate zonal centrifugation	Starink *et al.* (1994a)
Freshwater sediments	mixed protozoa	fixed	80±8.5	Rate zonal centrifugation	Starink *et al.* (1994a)
Freshwater sediments	mixed protozoa	fixed	71±3.9	Rate zonal centrifugation	Starink *et al.* (1994a)
Diatoms					
Sandy sediments	*Amphora* sp.1	fixed	77.3±3.4	Decant/fix with sonication	Present study
Sandy sediments	*Amphora* sp.2	fixed	73.4±11.1	Decant/fix with sonication	Present study
Sandy sediments	Gyrosigma macrum	fixed	47.5±12.4	Decant/fix with sonication	Present study
Sandy sediments	*Gyrosigma* spp.	fixed	37.9±3.8	Decant/fix with sonication	Present study
Sandy sediments	*Navicula* sp.	fixed	69.3±11.1	Decant/fix with sonication	Present study
Sandy sediments	Nitzschia closterium	fixed	83.1±5.8	Decant/fix with sonication	Present study
Sandy sediments	mixed diatoms	fixed	61.9±6.2	Decant/fix with sonication	Present study
Others					
Sandy sediments	Nanobenthos	fixed	>90	Isopycnic Centrifugation	Epstein (1995)
Sandy sediments	Micro- & Meiobenthos	fixed	>95	Isopycnic Centrifugation	Epstein (1995)

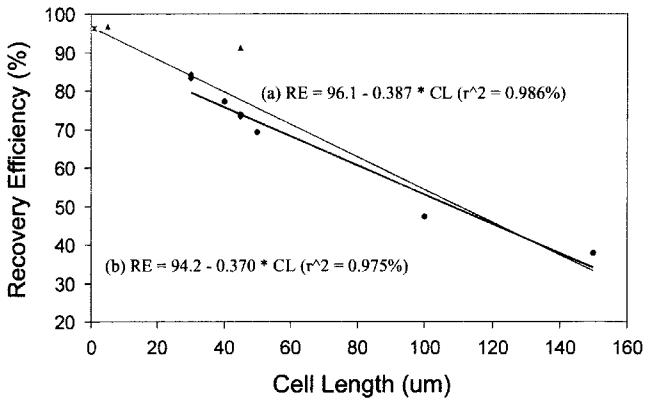

Fig. 3. Relationship between cell length (CL) and recovery efficiency (RE) (asterisks for bacteria; triangles for heterotrophic flagellates; spheres for diatoms); (a) regression equation for overall microbes used in this study, (b) regression equation for diatoms.

Table 4. Correction factors applied in this study.

Groups	Recovery Efficiency (RE)	Correction factors (CF)
Bacteria	96.4%	1.037
Heterotrophic flagellates		
-Mixed	96.9%	1.032
-Large tectic species	91.5%	1.093
Ciliates	96.1–0.39 x CL	100/RE
Dinoflagellates	84.4%	1.185
Diatoms	94.2–0.37 x CL	100/RE

Notes

- Large tectic species refers to larger euglenids such as *Peranema trichophorum* and *Notosolenus ostium*
- Correction factors=100/RE
- CL=cell length of each species or cell
- CN=cell numbers recovered by using the decant/fix method
- Corrected cell numbers=CF x CN

Table 5. Annual mean abundance and biomass of bacteria, heterotrophic flagellates, dinoflagellates, algae and ciliates at Port Botany (n=12).

	Abundance (cells/cm^3)		Biomass (μgC/cm^3)	
	Uncorrected	Corrected	Uncorrected	Corrected
Algae	2.78±1.69×10^6	2.83±1.70×10^6	54.72±22.23	66.61±27.25
Bacteria	2.95±1.30×10^8	3.06±1.34×10^8	5.85±2.56	6.09±2.67
Heterotrophic flagellates	1.84±0.90×10^5	1.90±0.92×10^5	2.37±2.33	2.44±2.40
Dinoflagellates	3.45±3.42×10^3	4.14±5.23×10^3	2.69±3.60	3.28±4.29
Ciliates	0.28±0.37×10^3	0.39±0.53×10^3	1.38+2.98	4.28+12.35

The equation (RE=94.2–0.37*CL, Fig. 3) developed to describe the efficiency by which diatoms are recovered may not apply to all species because some types of diatoms (e.g., *Cocconeis*) are extremely difficult to dislodge (Round *et al.*, 1990) and because each species may react differently to the decant/fix method even though they were fixed. The same is probably true of many tectic protists (refer to Patterson *et al.*, 1989) and bacteria. Free-swimming cells (e.g., bodonids) are recovered more successfully than gliding (e.g., euglenids and diatoms) or sessile cells (e.g., loricate choanoflagellates) which glide or adhere onto particles. Loricate choanoflagellates may not be extractable if they glue on firmly and maybe *Ploeotia*, *Petalomonas* and *Notosolenus* stick well. Therefore the numbers extracted by this method may be a subset of the total numbers present.

The composition of the microbial community varies from site to site (Lee and Patterson, 1998, 2002; Patterson and Lee, 2000), from season to season, and from sediments with differing granularities (Lee and Patterson, 2002). As underestimates are not the same for all types or sizes of microbes, there will be variation in underestimates between samples and sites. When large euglenids such as *Notosolenus ostium* and *Peranema* spp. are abundant, the biomass of heterotrophic flagellates might be underestimated to a greater extent because large cells are less well extracted than smaller cells. In Port Botany the extent of underestimates in the total microbial carbon biomass ranged from 4.1 to 31.4% with a mean of 16.8±7.4% (Table 5), with much of that relating to larger cells such as diatoms and ciliates.

Overall, the estimates of the abundance and biomass of microbes obtained by the extraction method might be underestimated. Although the estimates may be a subset of the total numbers present, we believe that more reasonable estimates can be obtained by using the correction factors. We note that the 80 seconds sonication time gives the best results, but we still don't know how many cells are not extracted, and that this would be an area which deserves further exploration.

The underestimate is possibly due to inefficient separation of microbes from fine sediment particles and detritus, or to damage to cells when dislodging microbes using a sonicator or to unsuccessful DAPI binding to DNA (especially, bacteria) at high salt concentrations (>12‰) and in formaldehyde-fixed materials (e.g., Zweifel and Hagstrom, 1995). Although the underestimate will be caused by the reasons above-mentioned, exercises such as this do provide the correction factors which can be applied to marine sandy sediments like Port Botany, and which will improve the accuracy of our descriptions of benthic microbial communities.

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