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A comparison of procedures for the separation of aquatic bacteria from sediments for subsequent direct enumeration

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Summary

The effectiveness of homogenization, sonication and two chemical treatments for separating attached sedimentary bacteria from particles was examined and compared. Several criteria were considered, including the number of bacteria left on particles, the ratio of the number of attached bacteria to the number unattached, and the calculated coefficient of variation for replicates. Comparisons were made among different types of sediment for homogenization and sonication. Significant differences are obtained by the use of different techniques of dispersion. In general, homogenization and sonication yielded higher overall counts and greater ratios of unattached to attached bacteria than did either Triton X-100 or periodate treatment. It is concluded that the absolute effectiveness of a specific separation technique should not be assumed. Each sediment should be studied individually to determine the most appropriate enumeration procedure.

Key words: *Bacteria – Enumeration – Fluorescence – Sediments*

Introduction

The importance of bacteria in sedimentary environments, both as mineralizing agents and as a food source, has been widely recognized [1–3] and estimates of bacterial productivity and biomass are of considerable importance in understanding these systems. While several methods of estimating bacterial biomass are in use today, direct counts of bacterial cells have become a routine method with the widespread use of fluorochrome stains and epifluorescence microscopy [2]. Epifluorescent direct counts have the advantage of being rapid, reproducible, precise, and suitable for both pelagic and epibenthic bacteria in all freshwater, estuarine and marine environments. This method does not, however, determine bacterial viability.

Several criteria must be met for a successful direct count. First, all bacteria should be

retained by the filter used in the procedure. Secondly, all bacteria must be visible at the surface of the filter, i.e., all must lie on a single plane. Finally, the staining and optical conditions must produce high contrast between the bacteria and the background [4]. Acridine orange (AO) is the fluorochrome most often used in this procedure, and the AO technique has been so refined that the first and last criteria cited above are generally met.

The second criterion does present a problem, however, when using the AO direct count technique for sedimentary bacteria, since most cells are attached to the substrate. Bacteria may utilize electrostatic forces, mucopolysaccharide bonds, fimbriae, or a combination of these [5, 6] to adhere to particle surfaces where nutrients are concentrated [7, 8], and therefore may be hidden beneath particles or otherwise out of view, and particles themselves act to increase the working distance of the microscope objective, distorting optical quality [2]. Ideally, all bacteria should be completely removed from sediment particles and dispersed evenly throughout a given sample. Satisfaction of these requirements heightens the accuracy and precision of enumeration using the AO direct count method.

Various techniques are in use at the present time for the removal of sedimentary bacteria from particles. Most widespread in its usage is that of dilution and homogenization in a commercial blender. Dilution is necessary so that particles do not cover the majority of the microscopic field to be counted, but must not be so great as to result in too few cells for a statistically significant count [9]. Another method which has been used is dilution and sonication [10]. Alternatively, chemical or biochemical means may be used to dislodge the bacteria; these include the use of chemical surfactants or oxidizing agents which cleave the polysaccharide bonds between bacteria and particles [11].

It should be noted that a correction factor is commonly used when a large amount of particulate matter is present in the sample. This takes into account those bacteria which are hidden from view by particles [12, 13]. It is difficult to determine how accurate such a correction is for a given sample. Thus, it is desirable to use a dispersion technique which both reduces the number of particles present and effectively removes bacteria from them. The purpose of this study was to make a direct comparison between the standard removal technique of homogenization and four other methods (including sonication and chemical treatments) and to evaluate the relative effectiveness of each for the enumeration of epibenthic bacteria.

Materials and Methods

Sediment samples were obtained from an intertidal mud flat at Flax Pond salt marsh, on the north-central shore of Long Island, NY, the Carmans River, Stations 2 and 3 of Carpenter and Dunham [14], and the Hudson River, near the George Washington Bridge. The locations were chosen to encompass a range of sediment types (Table 1).

Five techniques were compared: (1) serial dilution and gentle agitation by a magnetic stirrer; (2) a chemical surfactant treatment (Triton X-100); (3) a chemical oxidant (sodium periodate), in conjunction with a chelating agent (sodium pyrophosphate); (4) homogenization; and (5) sonication. A preliminary study was necessary to determine the optimal times and frequencies of sonication, and chemical concentrations to be used in

TABLE I

SEDIMENT CHARACTERISTICS OF STUDY SITES

Location	Salinity (‰)	Porosity	Loss on ignition (%)
Flax Pond	25	0.7	7.5
Carman's River			
Station 2	14	0.8	17.0
Station 3	2	0.9	25.0
Hudson River, GWB	19	0.8	6.2

making the comparisons. The final comparisons were made using the following treatments: serial dilution to 10^4 and mixing with a magnetic stirrer; 0.1 mM Triton X-100; 50 mM sodium periodate (50 mM sodium pyrophosphate was added 5 h prior to the addition of periodate after which samples were gently shaken for at least 8 h to allow complete reaction [11]); homogenization at 20000 rpm for 1 min; and sonication using a BIOSONIK IV at 20 kHz and 900 W.

Sediment samples were processed as follows. The initial dilution was made using 1 g wet weight of surficial sediment (Flax Pond) to 100 ml of 0.2 μm filtered distilled water, and was mixed thoroughly with a magnetic stirrer. One ml of this common stock was then diluted with 99 ml of 0.2 μm filtered distilled water and treated. From this, two 20-ml subsamples were taken. After treatment, each sample was fixed with 0.2 μm filtered buffered ($NaBO_4$) formalin, resulting in a final concentration of 5% formalin. This procedure was then repeated, thereby obtaining a total of four replicate samples per treatment. Blanks of filtered distilled water used in all dilutions were also run.

Direct counts were made using the acridine orange technique of Hobbie et al. [4, 15] as modified for sediments by Robertson and Newell [13]. Nuclepore filters (0.2 μm pore size) were pre-stained with Irgalan Black for a period of greater than 5 min. Two-ml samples were then stained for 60 s with acridine orange and filtered through the previously stained 0.2 μm Nuclepore filter. The filter was then mounted on a glass slide and the bacteria counted using an Olympus epifluorescent microscope equipped with a halogen bulb, 10× eyepiece, 100×1.25 oil immersion objective and 1.25× condenser (1250× total magnification).

Data collected included the number of large particles (greater than one subsquare of the counting grid or 9 μm in size) present in the sample, the number of bacteria found attached to large particles or clumps, the number of unattached bacteria, and the total number of bacteria, represented by the sum of the bacteria dislodged and those still attached to particles. It should be noted here that the number of bacteria counted per particle may be inaccurately low since only those sides which were visible were counted. No correction factors were used to account for this effect in order to better illustrate the relative dispersive effects of each technique for the overall comparison. Ten fields per slide were counted, chosen at random, and the average number per field used to calculate the number of bacteria per gram of sediment.

The effectiveness of each technique was evaluated by three criteria: (1) comparison of

the total number of bacteria counted per gram sediment; (2) the ratio of the number of unattached bacteria to the number attached (hereafter represented as U/A); and (3) the number of bacteria per particle greater than 9 μm (or one subsquare of the counting grid) in size.

Two types of averages were calculated for unattached bacteria. Non-weighted averages are simply the arithmetic mean of the ten fields counted. Weighted averages exclude those fields containing no particles and therefore no attached bacteria for that reason alone. In this case, extra fields were counted to retain the average of ten fields counted per slide. We felt that it was most appropriate to utilize weighted averages to present a clear picture of the effectiveness of each treatment. The figures therefore present weighted averages, where applicable.

Data was tested statistically using standard *t*-Tests, and a nested anova, as applicable and according to Sokal and Rohlf [16].

Results

Preliminary studies were conducted to determine the optimal treatment times, sonication frequencies, and chemical concentrations to be used in comparisons. The effects of varying concentrations of Triton X-100 on the removal of sedimentary bacteria from particles are presented in Figure 1. Using the ratio of the number of bacteria unattached to large particles to the number of bacteria attached (U/A ratio) as a relative measure of effectiveness, it was found that 0.1 mM Triton X-100 produced higher U/A ratios than 0.01 mM, i.e., less bacteria were present as attached and more as unattached. Higher concentrations (1 mM) somehow disrupted the surficial integrity of the Nuclepore filter

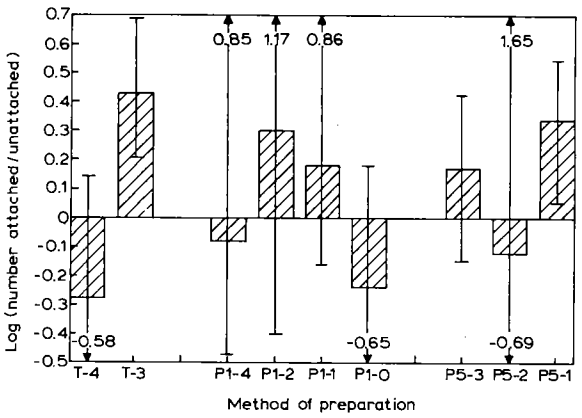

Fig. 1. The effect of various concentrations of Triton X-100 and sodium periodate on removal of sedimentary bacteria from particles expressed as the log of the ratio of unattached to attached bacteria found in the sample. (TX-4 through TX-2) indicate Triton X-100 at final concentrations of 0.01, 0.1 and 1 mM. (P1-4) through (P1-0) indicate a sodium pyrophosphate concentration of 9 mM and sodium periodate at concentrations of 0.01, 1, 9 and 50 mM, respectively; and (P5-3) through (P5-1) a sodium pyrophosphate concentration of 50 mM with sodium periodate at concentrations of 0.5, 5 and 50 mM.

used in the enumeration procedure, thus making it impossible to determine the treatment's effectiveness. The subsequent comparative study used 0.1 mM Triton X-100.

Varying concentrations of sodium periodate gave similar results. A preliminary study used 9 mM sodium pyrophosphate and concentrations of 0.01–50 mM sodium periodate, but no significant ($P<0.05$) variations in the U/A ratio could be found over this range of concentrations (Fig. 1). We then increased the level of pyrophosphate to 50 mM, and used periodate concentrations from 0.5–250 mM periodate, but again, no significant differences could be found between these concentrations up to 50 mM (Fig. 1). At 250 mM, the surface of the Nuclepore filter was disrupted, thus making it impossible to determine the effectiveness of the treatment. Both reagents were used in the subsequent comparison at final concentrations of 50 mM since this combination gave the smallest standard deviation.

Sonication was examined at 20 kHz over a range of power outputs, from a minimum of 550 W to a maximum of 1250 W. All samples were subject to sonication for 1 min. The results (Fig. 2) showed that the U/A ratio was greatly increased in going from 550 to 900 W, and that 1250 W caused cells to rupture. Another point of interest was the reduction in the amount of error present at 900 W compared with that at 550 W. On the basis of these results, 900 W was chosen for the final comparison.

A direct comparative study found clear differences among the five methods. The different methods of extraction gave very different numbers of bacteria per gram of sediment (Fig. 3). Sonication at 20 kHz, 900 W gave the highest number of bacteria per gram, 9×10^9. Homogenization yielded significantly ($P<0.001$) lower counts, with 7×10^9 bacteria per gram sediment. Both Triton X-100 and periodate gave counts which were significantly lower than both sonication and homogenization ($P<0.001$) but not significantly different from each other. Serial dilution gave a higher average number of bacteria per gram sediment than either Triton X-100 or periodate, but with a much higher variance. It was, however, significantly different than Triton X-100 and periodate ($P<0.05$), homogenization ($P<0.05$), and sonication ($P<0.001$).

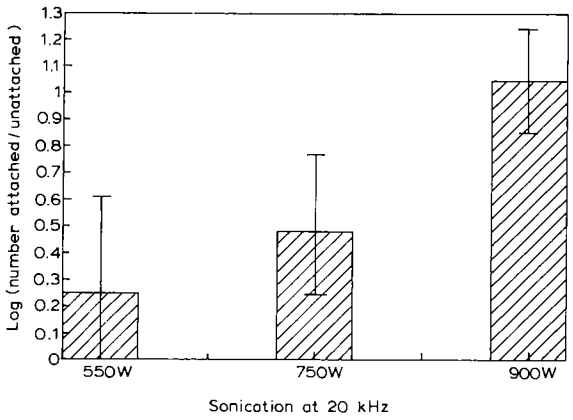

Fig. 2. The effect of sonication on separation of bacteria from particles, expressed as the log of the ratio of the number of attached to unattached bacteria in the sample. Sonication frequency was kept constant at 20 kHz, while power was varied as indicated, from 550 to 1250 W.

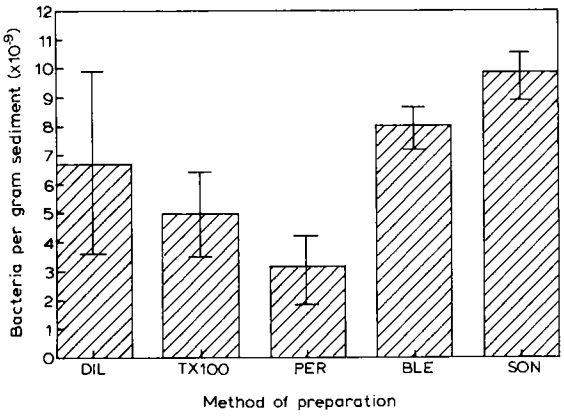

Fig. 3. Comparison of methods for the separation of bacteria from a single sediment sample. (DIL) indicates serial dilution of the sample to 10^4 and gentle agitation with a magnetic stirrer; (TX-100) indicates the use of 0.1 mM Triton X-100; (PER) indicates the use of 50 mM sodium pyrophosphate and 50 mM sodium periodate; (BLE) indicates homogenization at 20000 rpm for 1 min; and (SON) indicates sonication at 20 kHz and 900 W for 1 min.

The U/A ratio demonstrated even more clearly the relatively greater effectiveness of sonication (Fig. 4), which yielded the highest ratio. This was significantly greater ($P<0.05$) than homogenization. Lower ratios were found for Triton X-100 and periodate, both significantly different from homogenization or sonication ($P<0.001$) but not from each other. Serial dilution had the lowest ratio, significantly lower ($P<0.001$) than all other treatments. It should be noted that not only do sonication and homogenization give higher numbers; they are also more precise. Serial dilution yielded a coefficient of variation of 49%; periodate, 39%; Triton X-100, 30%; homogenization, 10.5%; and sonication, 8.5%.

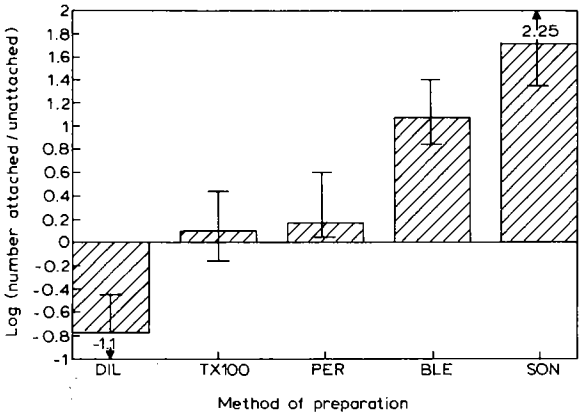

Fig. 4. Comparison of several dispersion techniques for a single sediment sample expressed as the log of the ratio of the number of unattached to attached bacteria present in the sample. Abbreviations are as for Fig. 3.

It was also found that the number of bacteria observed per particle was inversely correlated with the apparent efficiency of removal. Simple serial dilution gave the highest number of bacteria per particle, with an average of 20. Both Triton X-100 and sodium periodate yielded averages of about eight bacteria per particle. Homogenization and sonication gave the lowest averages of three and one bacteria per particle, respectively.

Statistical analyses performed on these data included the application of a nested anova. This showed that there was highly significant variance among methods ($P<0.001$) and no significant variance among slides within each method (i.e., between replicates) ($P>0.75$). Using a procedure derived for estimating variance components [16], we can express these in terms of their relative magnitudes as percentages of the sum of their variances. The results show that 48% of the variance lies between individual counts, which was expected due to the range of numbers obtained (from five per field to 104 per field), 0% from replications, and 52% due to differences in treatment. This, therefore, supported the validity of a comparison between methods.

Most enumeration procedures for epibenthic bacteria use homogenization as the method of extraction. Contention does exist, however, as to the optimal time period of the treatment [9, 10, 17]. We therefore ran a time-course for both homogenization and sonication. Figures 5 and 6 show the time courses for these treatments for three different sites (data presented is an average of replicates within a single core from each site). In most cases, the apparent number of bacteria in the sample changed significantly ($P<0.001$) over the course of 10 min of treatment. Furthermore, there were generally significant differences between homogenization and sonication over the same time course. For Flax Pond sediments, increased periods of either homogenization or sonication resulted in lower counts of unattached bacteria. Ten min of homogenization caused significantly greater loss of cells ($P<0.001$) than 10 min of sonication (Fig. 5). It is noteworthy that the patterns are quite different in the time courses for various other sediments examined (Fig. 5). For Carmans River sediments, longer periods of sonication yielded higher counts, while longer periods of blending caused loss of cells and lower counts. Hudson River sediments were the least sensitive to the time of blending or sonication.

Sediment samples taken from two different sites in the Carmans River, from the intertidal mud flat at Flax Pond, and from the George Washington Bridge site on the Hudson were further subjected to homogenization or sonication for time periods of 1 or 5 min. The results are shown in Fig. 6, and it can be seen again that all sediments do not respond similarly to the removal techniques.

There are many factors that may influence the effectiveness of any given technique including sediment size, organic content, porosity and salinity. We have studied these factors with respect to the effectiveness of sonication and blending techniques (unpublished data), but no clear trends have been established at this time.

Discussion

Many studies have been carried out on epibenthic bacteria using acridine orange direct count techniques and homogenization [9, 13, 19, 20]. Various types of sediment

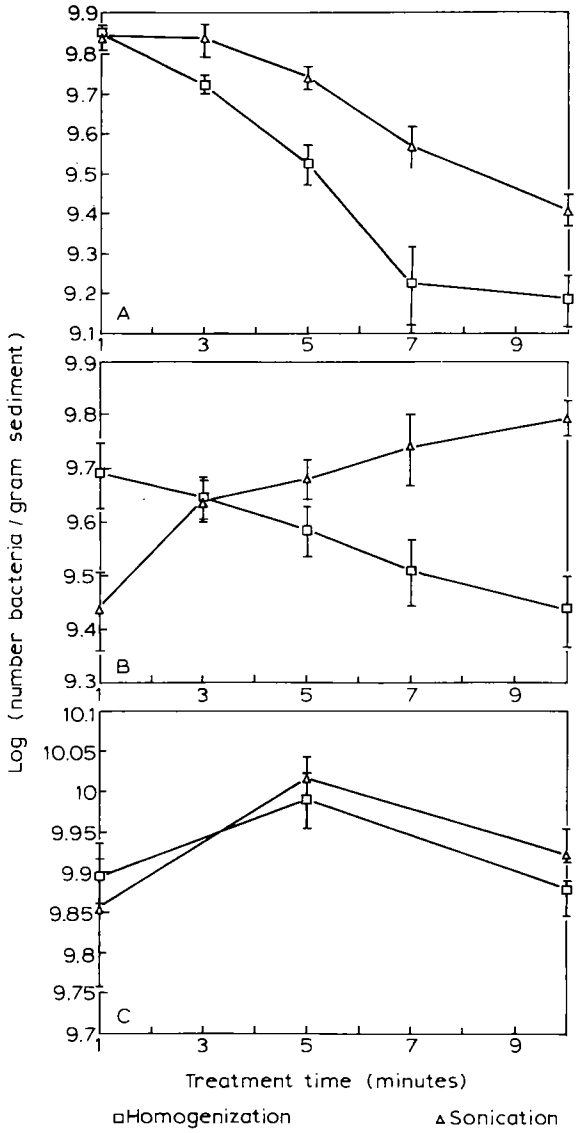

Fig. 5. Effect of treatment duration on the number of bacteria obtained from sediment taken from three different sites. Panel A shows sediment from the intertidal mud flat of Flax Pond, a north-temperate salt marsh, panel B sediments from station 3 of the Carman's River, and panel C sediments from the Hudson River, George Washington Bridge site. (□) represent homogenization at 20000 rpm and (△) represent sonication at 20 kHz and 900 W.

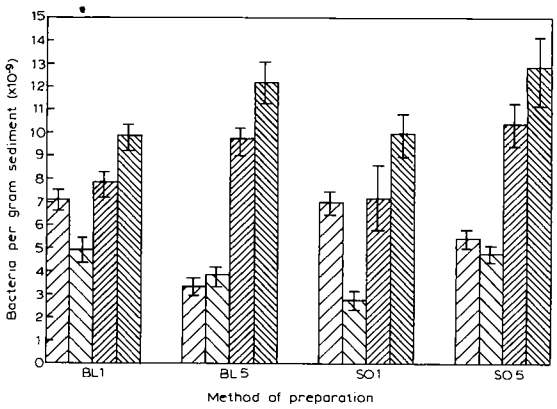

Fig. 6. Effect of treatment duration on sediments from several sites. (BL1) and (BL5) represent homogenization at 20000 rpm for 1 and 5 min, respectively; (SO1) and (SO5) sonication at 20 kHz and 900 W for 1 and 5 min, respectively. (▨) represents sediments from the intertidal mud flat at Flax Pond, NY; (▧) sediments from the Carmans River, Station 3; (▨) sediments from the Hudson, George Washington Bridge Station; and (▧) sediments from the Carmans River Station 2.

were used in these studies. Rublee and Dornseif [13] homogenized sediments for 1 min at 20000 rpm and found that sediments of a North Carolina salt marsh bacteria numbered $8.36\text{–}10.90\times10^9$ cells/cm^3 of sediment. For the same salt marsh, Rublee [18] found that over a seasonal cycle in surface sediments, bacterial numbers were highest in October (1.4×10^{10} cells/cm^3) and lowest in August (5×10^9 cells/cm^3). Mean yearly values (±1 S.D.) were found to be $8.5\pm2.1\times10^9$ cells/cm^3. Newell and Fallon [19], after homogenizing sediments for 5 min at 15000 rpm, found numbers of $6\text{–}10\times10^9$ cells/cm^3 of wet sediment at zero to 0.25 km off the Georgia (USA) coast. Cammen [20], who followed Rublee's procedure, found 7.2×10^9 cells/g sediment (no S.D. units indicated) in a mud flat located at the mouth of Cumberland Basin in the upper Bay of Fundy.

There have been previous investigations into the effects of methodology on the enumeration of sedimentary bacteria [10, 17, 21, 22]. These include: work by Meyer-Riel et al. [21], who compared shaking by hand and the use of 'Tween' 80 (a surfactant); by Dale [22], who compared homogenization, sonication, grinding, and shaking by hand with glass beads; by Dye [10] who compared sonication and homogenization; and by Ellery and Schleyer [17], who also compared sonication and homogenization. Most agree that either sonication or homogenization give maximum yield, but only recently has the importance of the effect of differences in sediment type upon the choice of method been considered [10].

The present study clearly demonstrates that the method of removal of bacterial cells from particles in sedimentary samples can have a significant effect upon the number of cells enumerated by direct count techniques.

All sediments do not respond in the same manner to variations in treatment times. For example, 5 min of homogenization results in lower overall counts for Flax Pond

sediments, but higher counts for sediments from Carmans River, Station 2. Sediments should be subject to preliminary study with respect to the most appropriate treatment and its optimal application. Destruction of bacterial cells may result over the course of treatment. This may be a result of cell lysis due to vibration or the heating of the sample caused by its agitation, and a correction factor may be applied to account for this [17]. However, this effect varies from one sample to the next, and care should be taken in its application. The temperature increase appears to have no effect on the effectiveness of the AO technique for enumerating whole cells; the only effect seen by the investigators was that as the temperature increased, so did the frequency of finding cells which were stained red instead of their characteristic green. The theory behind this is that the increase in temperature acts to denature the DNA present in cells. The dye molecules attached to the double-stranded DNA molecules fluoresce green while those attached to the single-stranded (denatured) DNA (or RNA) molecules fluoresce orange or red [23], although this has not been documented here. These cells are still easily identified as such due to their fluorescence and shape. The temperature increase may also be of interest to those doing viability or activity measurements following desorption; the temperature increases observed in 100 ml of sample are therefore presented in Table 2.

The results of our final comparative study indicate that sonication at 20 kHz and 900 W appears to be the best method of extraction for sediments from an intertidal mud flat of a north-temperate salt marsh. It reduced both the number of bacteria per particle and the number of large particles present in the sample, thus making it possible to obtain more accurate direct counts of the total number of bacteria per gram of sediment. In terms of precision, sonication gives significantly reduced variance and, hence, enhances the ability to discern significant differences between samples.

It is important to determine the best procedure for the extraction of bacteria from sediments, since different procedures can give significantly different results. The acridine orange method of direct counts is used routinely in descriptive surveys, correlative

TABLE 2

TEMPERATURE CHANGES DURING TREATMENT

Time (min)	Temperature (° C)	
	Homogenization	Sonication
0	19.5 ± 0	19.5 ± 0
1	22.3 ± 0.3	20.6 ± 0.2
2	24.8 ± 0.9	21.5 ± 0.2
3	27.2 ± 0.8	22.6 ± 0.3
4	29.4 ± 0.9	23.3 ± 0.1
5	31.8 ± 0.2	24.6 ± 0.2
6	33.8 ± 0.8	25.3 ± 0.2
7	35.3 ± 0.7	25.6 ± 0.3
8	36.9 ± 0.4	26.8 ± 0.3
9	38.0 ± 0.3	27.3 ± 0.2
10	39.3 ± 0.6	28.0 ± 0.1

studies, impact assessment activities, and experimental studies of bacterial dynamics. Epifluorescence enumeration, in conjunction with activity measurements and filter fractionation procedures, is also used for examining the productivity and nutrient uptake dynamics of native aquatic bacteria [1, 22, 24, 25]. The enumeration of bacteria in a sediment is the first step in quantitatively assessing the role of bacteria in the ecological dynamics of that particular system. Therefore, an awareness of the comparative differences in methods of separation of bacteria from sediments is of importance, since the method of separation and its particular application can have a major effect on the apparent total number counted. It is extremely important that the investigator experimentally determine the best procedure for dislodging cells for each type of sediment studied.

Acknowledgements

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