IMPROVED MICROSCOPIC ENUMERATION OF ATTACHED AQUATIC BACTERIA FOLLOWING DEFLOCCULANT AND ULTRASOUND TREATMENT

by

M. Iqubal Velji

B. Sc., University of Alberta, 1978

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF

THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

in the Department

of

Biological Sciences

SIMON FRASER UNIVERSITY

November 1983

Approval

Name: Mohamed Iqubal Velji

Degree: Master of Science

Title of Thesis: Improved microscopic enumeration of attached aquatic bacteria following deflocculant and ultrasound treatment

Examining Committee

Chairman: Dr. G. R. Lister

Dr. L. J. Albright, Senior Supervisor

Dr. L. D. Druehl

Dr. James E. Rahe, Public Examiner

Date approved May 22, 1984

PARTIAL COPYRIGHT LICENSE

I hereby grant to Simon Fraser University the right to lend my thesis, project or extended essay (the title of which is shown below) to users of the Simon Fraser University Library, and to make partial or single copies only for such users or in response to a request from the library of any other university, or other educational institution, on its own behalf or for one of its users. I further agree that permission for multiple copying of this work for scholarly purposes may be granted by me or the Dean of Graduate Studies. It is understood that copying or publication of this work for financial gain shall not be allowed without my written permission.

Title of Thesis/Project/Extended Essay

Improved microscopic enumeration of attached aquatic bacteria following deflocculant and ultrasound treatment

Author:

(signature)

Mohamed Iqubal Velji

(name)

May 22, 1984

(date)

ABSTRACT

A technique has been developed to disperse bacteria from their attachment surfaces and the matrices in which they were embedded. The bacterial cells within and on surfaces were first strengthened by fixation with 3.7 % formaldehyde (v/v). Tetrasodium pyrophosphate, a sequestering and a deflocculating agent, was then added to a final concentration of 0.001 M for water samples, and 0.01 M in 0.44 M sodium chloride solution for sediment, fecal material of sea cucumber, Parastichopus californicus, and blade disc samples of kelp, Macrocystis integrifolia, for 15 to 30 min. All samples were then sonicated at 100 W power level for 30 to 45 s. Bacterial cells dispersed in this manner were then stained with 4', 6-diamino-2-phenylindole (DAPI) or Hoescht 33258, which produced minimal background fluorescence. This technique resulted in (1) a random distribution of bacteria on filters, (2) lower variance within and similar variances between subsamples, and (3) lower number of microscopic grids to be enumerated since the coefficient of variation (CV) stabilized after counting bacterial cells in 10 randomly chosen microscopic fields as compared to untreated samples. Bacterial populations in and on sediment and old kelp blades which were previously difficult or impossible to count can now be determined. A mechanism is proposed to explain the increase in dispersion of bacterial cells resulting from this technique.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. Introduction

Bacteria are ubiquitous within the aquatic environment. They occur in and on sediments (Dale, 1974; Weise and Rheinheimer, 1978), on plants, including seaweeds (Cundell et al., 1977; Roland, 1980), in and on animals (Sieburth, 1979), attached to rocks (Geesey et al., 1977) and as a major component of detritus (Fenchel and Jorgensen, 1977). A relatively small proportion of the bacterial population within the aquatic environment is free living; most of these unattached cells occur in the water column.

The current method of determining water and sediment bacterial numbers is by staining the cells with a fluorochrome (usually acridine orange), filtering them onto a 0.2 um pore size polycarbonate membrane (Nuclepore filter), and examining them by epifluorescent microscopy (Francisco et al., 1973; Hobbie et al.; 1977, Daley, 1979). This approach works well with unattached bacteria but bacteria which are attached to surfaces and embedded in colloidal matrices are difficult and often impossible to observe. This is due to (1) fluorochrome usually binding non-specifically to the bacteria as well as to the substrate surfaces, (2) bacteria often being obscured and hidden by overlying particles and other cells and (3) the fact that bacteria tend to grow in microcolonies and hence are unevenly distributed on attachment surfaces. Bacterial distribution is

frequently non-random in water (Daley, 1979; El-Shaarawi et al., 1981; Kirchman et al., 1982), sediments (Montagna, 1982) and on surfaces of plants (Hossel and Baker, 1979b) requiring special statistical treatments or homogenization (Daley, 1979). Bacterial enumeration would be more accurate and precise if the bacteria were randomly distributed (Cassell, 1965).

Bacteria possess an ionogenic surface of mainly acidic (carboxyl and phosphoryl) and a few basic (amino) groups and generally have a relatively constant net negative charge between the pH values of 3.0 and 8.0. At pH values greater than 8.0 the net negative charge of bacterial surfaces further increases. At pH 2.0 and lower, the surface will have a net positive charge (Marshall, 1973).

Daniels (1980) has hypothesized that a balance between London-Van der Waals forces and the electrostatic forces of the electrical double layer is involved in the initial stage of attachment of bacteria to surfaces. This is the reversible phase. Following the initial sorption of the cell to a surface, irreversible adhesion occurs by means of bacterial extracellular polysaccharide material (Marshall and Cruickshank, 1973). Attachment of bacterial cells to a surface is considered to be irreversible if the cells cannot be released by washing with 2.5 % sodium chloride solution (Corpe, 1974). The outer cell walls and membranes of plants and animals are of specific polysaccharide composition and are generally negatively charged. The polysaccharide fibers of bacteria attach to these surfaces

by forming either specific lectin(s) or cationic bridges between acidic groups (Costerton et al., 1978; Fletcher, 1980), and they may also form a tangled mat between the bacterial, animal or plant surfaces (Costerton et al., 1978).

Based upon these observations bacterial adsorption to surfaces can be prevented or reversibly adsorbed bacteria can be detached by increasing the thickness of the electrical double layer between the cell and other surfaces. Various bacterial isolates have been shown to attach to surfaces such as soil particles, chitin and plankton at low pH but are repelled at higher pH due to the increase in negative charge on the surfaces (Santoro and Stotzky, 1968; Hattori, 1970; Marshall, 1973 , Kaneko and Colwell, 1975). A decrease in the degree of aggregation of soil particles and the attachment of bacteria to clay and glass surfaces occurs when univalent cations compared to di-and tri-valent cations are used (Peele, 1936 cited in Marshall 1980b; Santoro and Stotzky, 1968) or the concentration of monovalent cations, such as sodium, is increased (Kaneko and Colwell, 1975). Prevention of bacterial attachment has been shown to occur by the use of tetrasodium pyrophosphate (Fletcher, 1980). Condensed polyphosphates have been used for sequestering calcium and magnesium cations in hard water. This prevents the polyvalent cations from binding to the surfactant and soil components such as lipid residues and clays thereby improving the efficiency of cleansing. The sequestering agents prevent redeposition of soils removed from the surface being

cleaned (Considine and Considine, 1983).

In summary,there are five ways to cause bacterial disaggregation and detachment: (1) by increasing the pH of the suspending medium (2) by the use of monovalent rather than polyvalent cations in the suspending medium (3) by removal of excess polyvalent cations (e.g. by a sequestering agent) (4)by oxidizing or physically disrupting tangled bacterial polysaccharide fibres and (5) by prevention of reattachment of bacteria to surfaces once they have been detached.

Various investigators have attempted to disperse bacteria in water, sediments, soil, sludge and plant surfaces using blender homogenization, sonication and chemical treatments. Homogenization of water samples from several British Columbia lakes did not increase bacterial counts (Daley, 1979) and sediment samples required extensive treatment before interference from detrital particles and background fluorescence was eliminated (Montagna, 1982). Homogenization of plant tissue followed by a viable plate count has also been used (Scotten, 1971; Laycock, 1974; Mazure and Field, 1980).

Ultrasound has been used to examine the bacterial component of activated sludge (Pike et al., 1972; Banks and Walker, 1977), soil samples (Zvyagintsev and Galkina, 1967; Bingle, 1980), sand grains (Weise and Rheinheimer, 1978) and for the removal of epiphytic contaminants of seaweeds in culture (Polne et al., 1980). This technique was only partially successful since bacterial removal was not complete.

Sonication also lyses viable bacterial cells (Pike et al., 1972; Coakley et al., 1977; Paul and Myers, 1982), Gram negative bacteria being more susceptible to ultrasound than Gram positive types and rod shaped cells more susceptible than coccoid forms (Coakley et al. 1977). Most aquatic bacteria are Gram negative rods while some Gram positive cells are found within and on sediments (Sieburth, 1979). Bacterial lysis may occur in sediment with prolonged homogenization (Montagna, 1982). Susceptibility of bacterial cells to homogenization or sonication is an important consideration if either is used to aid in the disaggregation of bacteria.

While chemical reagents have not yet been utilized for dispersing bacteria in natural aquatic samples, Marshall (1973) found that in laboratory experiments, fresh water and marine bacteria attached to glass slides could be removed by treatment with tetrasodium pyrophosphate followed by sodium periodate. Corpe (1974) examined the use of acids, bases, chelators and oxidizing agents to detach cells of a marine pseudomonad irreversibly attached by extracellular polysaccharide material to glass slides. The role of some of these reagents needs further examination in terms of their effectiveness in dispersing bacteria attached to natural substrates.

The combination of chemical reagents (e. g. sodium tripolyphosphate and tetrasodium pyrophosphate) with ultrasound has proven to be more successful than ultrasound alone for dispersing bacteria present in flocs in activated sludge (Pike

et al, 1972; Gayford and Richards, 1970; Banks and Walker, 1977). The sequestering and deflocculating effects of these reagents may aid in disaggregation and detachment of bacteria from various surfaces.

I have developed a method of dispersing bacteria from surfaces to which they are attached into a suspension medium prior to epifluorescence microscopic counting. This useful technique allows for (1) quantitative evaluation of bacterial numbers within niches hitherto inaccessible (e.g. bacterial epiphytes on senescent kelp blades), (2) reduction in variability within bacterial samples and (3) a reduction in the number of microscopic fields to be examined and counted.

This technique was used to examine bacterial dispersion in aquatic samples of subsurface and epibenthic waters, and sediment and fecal material from Parastichopus californicus Stimpson (Holothuroida, Echinodermata). In addition, different aged blades of the lesser giant kelp, Macrocystis integrifolia Bory (Laminariales, Phaeophyceae), which had densities of bacteria, ranging from low on the young blades to very high on the older blades, were also examined.

II. Materials and Methods

1. Sample collection

Water, sediment, Parastichopus californicus and Macrocystis integrifolia samples were collected by SCUBA in January, 1983 from a kelp bed in Bamfield Inlet, Bamfield, Vancover Island, British Columbia. The site was approximately 10 m from shore and 4.5 m in depth. The sea floor consisted of rocks and cobbles interspersed with high organic content sediments and sand.

Subsurface (0.1 m depth) and epibenthic (4 m) water samples were collected with sterile 1 L polypropylene bottles. Water salinities were 22 % and 26 %, respectively. The pH was 8.2 for both water layers. A sediment sample was taken with a hand held sterile 50 mL syringe corer, the end of which was immediately capped.

The sea cucumber, P. californicus, had a contracted length of 20 cm and width of 10 cm. A fecal pellet sample was removed from the posterior digestive tract by aseptic dissection.

A M. integrifolia frond with 29 blades and total length of 4.1 m was collected. The blades were numbered from the frond apical meristem and 4 blades were sampled. Blade 1 was the first new blade free from the apical meristem while blade 22 was the oldest and closest to the holdfast. Blade 4 was the longest complete blade while blade 10 was chosen as the middle aged

blade. For logistical reasons all samples were transported to Simon Fraser University on wet ice-dry ice mixture. The samples were preserved with 3.7 % formaldehyde (v/v) in sea water. The final pH of the sample after fixation was 7.8.

2. Enumeration of bacteria

A Zeiss Standard WL microscope fitted with an epifluorescence light condenser and an ultra-violet (UV) source (HBO-50 high pressure mercury lamp) was used to enumerate bacterial cells from the different marine habitats. A scored grid reticule (5x5 units) placed in the ocular lens (x12.5) housing delineated an area of 2500 um^2 with a neofluor oil immersion objective lens of x100 (Zeiss).

The four fluorochromes used for staining were acridine orange (AO, Sigma; Hobbie et al. 1977), fluorescein isothiocynate (FITC, Sigma; Babiuk and Paul, 1970), 4', 6-diamidino-2-phenylindole (DAPI,Sigma; Porter and Feig, 1980) and Hoechst 33258 (Hoechst, Riedel-DeHaan AG Seetze, Hanover; Cowden and Curtis, 1981; Paul, 1982).

The Zeiss filter set 48 77 09 (BP 450-490, FT510 and LP 520) was used for the longer wavelength emission of AO and FITC while set 48 77 01 (BP 365/10, FT 310 and LP 395) was used for the shorter wavelength emission from DAPI and Hoechst stained samples. Acridine orange is a non-specific flurochrome since it binds to DNA, RNA, mucopolysaccharides (Albert, 1966), sulphated

and acidic polysaccharides (Burns et al., 1982), while FITC binds to amino groups in general (Fliermans and Schmidt, 1975). The most useful stains for the bacteria on different substrate types examined were DAPI and Hoechst. The bacterial samples had low background fluoresence since these stains specifically bind to adenine-thymine regions of DNA (Coleman, 1980; Porter and Feig, 1980; Cowden and Curtis, 1981).

The direct count method (Hobbie et al. 1977) as modified for DAPI by Porter and Feig (1980) was used for staining the bacteria, except that the final DAPI concentration used was 1.0 instead of 0.01 ug mL^{-1}. The lower concentration did not adequately stain the cells. The contents of each storage container were throughly mixed before randomly chosen samples were taken. After the specified treatment (section 3) the samples were incubated with DAPI for 10 min and mixed with a Vortex Genie (Fisher Scientific) for 15 s before being filtered on to pre-dyed (Irgalan Black, Ciba-Geigy Corp.) 0.2 uM pore size, 25 mm diameter Nuclepore filters (Nuclepore Corp.) and enumerated with epifluorescence microscopy.

The volume of sample filtered was adjusted so that there were between 10 to 30 fluorescing bacterial cells per grid and 20 to 30 randomly chosen microscopic fields were enumerated. The volume of subsurface and epibenthic water filtered for bacterial enumeration was 5 mL. One hundred ul of the sample sediment and fecal material was suspended in 5 mL of filter sterilized artificial sea water (section 3.1). After treatment two

subsamples of 1 mL each were stained and made up to 5 mL before filtration. The kelp blade was sampled with a sterilized 4 mm cork borer. The discs were stained with DAPI, rinsed with 26 % (0.44 M) sodium chloride solution (w/v) and placed on a glass slide. Warm glycerol gelatin was then poured over each disc and a glass coverslip placed on top. After the glycerol had solidified the slide was examined using oil immersion. Discs treated with tetrasodium pyrophosphate and ultrasound (section 3.2) resulted in a dispersed mixture of bacteria, kelp cells and mucilage. This mixture was stained and filtered in a similar manner as the sediment or fecal material suspensions. The maximum volume of the mixture that could be filtered without clogging the filter was 3 mL from a total of 5 mL. For discs from blades 1, 4 and 10, 3 mL were filtered, while for blade 22, 2 mL was filtered due to the much higher density of bacteria encountered per grid.

3. Effect of chemical reagents and ultrasound

There were three stages to this study. The first entailed qualitative microscopic examination of the effect of chemical reagents, ultrasound and the combination of the two on bacterial and sediment aggregation and on epiphytic bacteria on blade 10 of the kelp. The second aspect concerned the tolerance of isolated and natural bacterial cells to sonication after fixation with formaldehyde. The final stage was an examination

of quantitative changes in distribution and bacterial numbers using the treatments that were found to be useful for dispersion in the qualitative part of the experiments on subsurface and epibenthic water, sediment, fecal material and blades 1, 4, 10 and 22 of the kelp frond.

3.1 Chemical reagents

Several reagents (Table 1) were examined for use as dispersants of sediment and kelp-attached bacteria that had been fixed with 3.7 % formaldehyde (v/v). Sodium chloride solution (ASW - artificial sea water) of the same salinity as the epibenthic water sample (26 %) was used as suspending medium for these samples since sea water and synthetic sea water (Rila Marine Mix, Teaneck, N.J.) foamed on application of ultrasound and formed a precipitate in the presence of sodium carbonate and tetrasodium pyrophosphate. Sediment and kelp disc samples from blade 10 were suspended in specified concentrations of reagent (Table 1) made up in ASW. The samples thus treated were thoroughly mixed with a vortex-mixer (setting 4) for 1 min, incubated for 15 min at 22° C with intermittent mixing after every 5 min. The kelp disc samples were rinsed with ASW after treatment and stained. The sediment samples were filtered onto a Nuclepore filter, rinsed with 5 ml of ASW and then stained with DAPI while still on the filtration stand. The treated samples were qualitatively examined under an epifluorescense microscope

Table 1. Chemical reagents examined for dispersent properties on different bacterial habitats.

Reagents	Concentration (M)	Habitat Type[1] M	S	F	SW
Hydrochloric acid	0.001,0.005, 0.01	+	+	-	-[2]
Sodium carbonate	0.001,0.005, 0.01	+	+	-	-
Sodium hydroxide	0.001,0.005, 0.01	+	+	-	-
Sodium periodate	0.001,0.005, 0.01	+	+	-	+
Tetrasodium pyrophosphate	0.0001,0.001, 0.01,0.05,0.1	+	+	+	+

[1]Habitat Types: M = Macrocystis integrifolia blade
S = Sediment
F = Fecal Material
SW = Sea Water.

[2]+ reagent used.
-reagent not used.

for gross changes in clumping or attachment of bacteria.

3.2 Ultrasound

A Biosonik II generator with a high power level ultrasonic probe (Model BP-II) and a standard 1.27 cm diameter titanium tip (Bronwill Scientific, N.Y.) was used for the ultrasound experiments. The power level could be varied from 12.5 watts (W) to a maximum of 125 W. The volume of all samples or sample plus suspending medium was kept constant at 5 mL in sterile disposable tubes, and the probe tip was kept 1 cm from the bottom of the tube. The sample tubes were precooled to 0° C and packed in crushed ice during sonication to dissipate heat. Treatment of samples which required sonication for periods greater than 15 s were carried out in bursts of 15 s sonication followed by 45 s of cooling in crushed ice before subsequent sonication.

The tolerance of bacterial cells to sonication was examined. A Gram negative rod shaped bacterium was isolated from the sea water supply at Simon Fraser University using Difco marine agar 2216. Two 500 mL cultures of the isolate were grown in 2 L Erylenmeyer flasks at 15° C using a reciprocating incubator with a shake rate of 50 strokes/min. The growth medium consisted of polypeptone, 5.0 g; yeast extract, 3.0 g; glucose, 1.0 g; Rila Marine Mix, 6.0 g and sodium chloride, 20.0 g dissolved in 1 L distilled water and adjusted to pH 7.3. The

exponential growth phase of the culture was between 2 to 25 h. Eight 5 mL aliquots were sampled at 18 h from each flask. Four of the aliquots were fixed with 3.7 % formaldehyde (v/v) for 30 min. The tolerance of fixed and unfixed isolate cells to sonication for durations ranging from 0 to 90 s at a power level of 125 W was assessed by examining the change in absorbance and bacterial counts of the samples. The light absorbance of two 2.5 mL samples was measured at 540 nm using a Carl Zeiss PMQ II spectophotometer, and bacterial counts were carried out using two 100 uL subsamples from each of the two samples for each of the sonication periods.

Preliminary experiments with sonication of sediment samples had indicated that a power level of less than 75 W required more than a 1 min treatment before there was a noticeable decrease in aggregation of bacterial cells and organic material. The tolerance to sonication of 3.7 % formaldehyde fixed bacterial cells in water and sediment samples (v/v) was investigated. Two 5 mL epibenthic water samples and two 100 uL sediment samples suspended in 5 mL of ASW were sonicated for time periods ranging from 0 to 60 s and at power levels of 100 and 125 W, except where noted. The samples were enumerated for bacterial cells before and after treatment.

3.3 Chemical reagents followed by ultrasound

Sediment and kelp disc samples were treated with chemical reagents as in section 3.2 and then subjected to sonication at a power level of 100 W for 15 s. The samples were similarly examined for qualitiative changes in clumping or attachment of bacteria.

Since sodium pyrophosphate followed by sonication treatment had the greatest effect on dispersion of bacteria in the sediment and kelp disc samples, the tolerance of the bacterial cells to the combination treatment was examined. Replicate 5 mL subsurface and epibenthic water samples were incubated with 0.001 M sodium pyrophosphate for 15 min and sonicated at 100 W power level for time periods ranging from 0 to 60 s. Replicate 100 uL sediment samples were treated with 0.01 M pyrophosphate and sonicated at 75 to 125 W power level for duration up to 60 s. The samples were then examined for changes in bacterial numbers.

4. Statistical analysis of bacterial distribution from different habitats

The effect of sonication in the presence and absence of pyrophosphate on the distribution and variation of the bacterial counts in subsurface and epibenthic water, sediment, fecal material and different aged kelp blades was investigated. The

samples from each habitat type were divided into three : (1) untreated; (2) sonicated at a power level of 100 W for 30 s and (3) incubated in 0.01 M pyrophosphate for 15 min prior to sonication, except where noted. For water samples, 0.001 M pyrophosphate was used while for kelp discs the incubation time in 0.01 M pyrophosphate was for 30 min followed by sonication for a 45 s period.

Bacterial samples were randomly chosen from each habitat type and 20 randomly chosen microscopic fields were enumerated in the water, sediment and fecal material samples, while 30 fields were enumerated for kelp blades due to the very low bacterial numbers encountered on the young blades. For the subsurface and the epibenthic water four 5 mL samples were used, for sediment and fecal material four 100 ul aliquots were made up to 5 mL with appropriate treatment suspension medium and after treatment 2 1 mL subsamples were enumerated. Six kelp disc samples from each of the blades 1, 4, 10 and 22 were used per treatment.

Statistical tests used in this analysis were from Sokal and Rohlf (1969). The coefficient of dispersion (CD=Variance/Mean) was used as a preliminary test to examine whether the observed bacterial spatial distribution was a Poisson (CD=1), clumped (i.e. contagious, CD>1) or regular (CD<1). The assumptions for the use of analysis of variance (ANOVA) were checked before comparisons between treatments were carried out. The non-parametric Kolmogrov-Smirnov test for goodness of fit was

used to determine if the observed bacterial frequency was uniform, Poisson or normally distributed. Equality of variances was examined by Bartlett's test for homogeneity of variances. If the assumptions for ANOVA were not fulfilled the bacterial frequencies were transformed and then checked again. The difference between the treatment means was tested by the non-parametric Kruskal-Wallis test when ANOVA could not be used. Relative variability within and between samples in the different treatments was checked by comparison of coefficient of variation (CV= (Standard deviation/Mean)x 100). The CV was also used to examine the variation in counting of bacteria using 5, 10, 20 and 30 grid fields per sample in the different treatments. The statistical computer packages SPSS and SPSS update 7-9 (Nie et al., 1975; Hull and Nie, 1981) were used for the analyses.

III. Results and Discussion

1. Effect of chemical reagents and ultrasound

1.1 Effect of chemical reagents

Microscopic observations of the marine sediment sample revealed the major component to be amorphous organic material interspersed with sand grains, shell and algal debris. Most of the bacteria were within and on the organic material, attached to the sand grains, shell fragments and algal debris and aggregated into microcolonies (Fig. 1). A small number of bacteria were single and unattached. Vortex mixing of the sediment suspended in ASW was not sufficient to cause dispersion of the bacteria and particulate aggregates. This was consistent with the reports by Zvyagintev and Galkina (1966) for soil samples.

Discs from blade 10 of M. integrifolia had relatively more epiphytes than blades 1 and 4 but less than blade 22 (Fig. 2). Bacteria, diatoms and detrital particles were present in several optical planes in the mucilage layer, above as well as on the meristoderm layer. This made observation of bacteria tedious and time consuming. Treatment of the discs suspended in ASW with

Fig. 1. Photomicrograph of DAPI stained bacteria from untreated sediment sample : (a) single bacterial cell, (b) aggregated bacterial cells and organic matter enmeshed by colloidal material, (d) organic material. Magnification x1200.

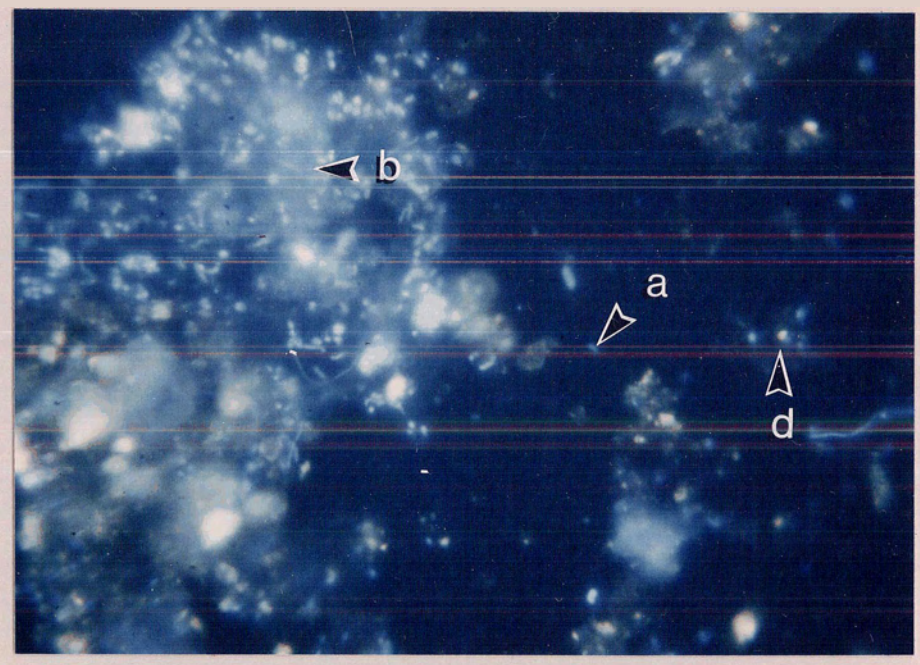
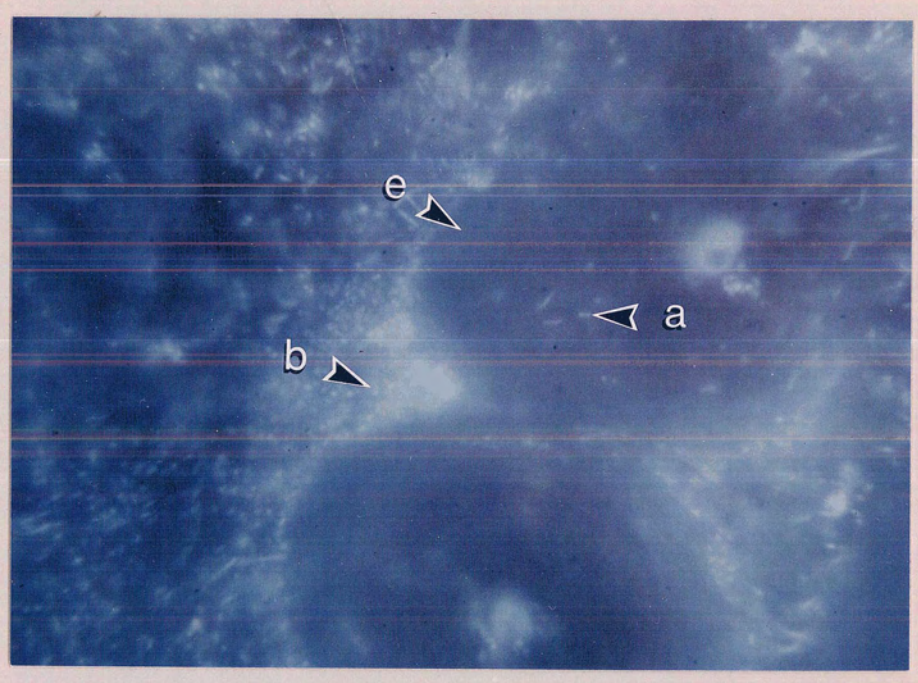

b
a
d

Fig.2. Photomicrograph of DAPI stained epiphytes on blade 22 of Macrocystis integrifolia frond: (a) single bacterial cell, (b) clumped bacterial cells, (e) diatom.


e
a
b

vortex mixing did not cause the majority of epiphytic bacteria to detach from the blade surface.

Treatment of sediment and kelp discs with hydrochloric acid, sodium hydroxide, sodium carbonate, tetrasodium pyrophosphate and sodium periodate, respectively, followed by vortex-mixing, under the experimental conditions, did not result in bacterial and sediment disaggregation or bacterial detachment from the kelp discs. In addition, sodium carbonate precipitation of cations from the sediment interfered with the observation of bacteria. Corpe (1974) showed that marine Pseudomonas cells, irreversibly attached to glass slides after 18 h at 25° C, could not be removed by treatment with distilled water or 0.1 M hydrochloric acid for 5 min, while 0.1 M sodium hydroxide was found to lyse bacterial cells.

The bacterial cells attached to sediment and kelp blade surfaces were irreversibly attached since they could not be detached with (1) 26 % sodium chloride solution, (2) an increase in pH which would have increased the net negative charge on the surface of bacteria and other surfaces resulting in repulsion and (3) substitution of the polyvalent cations present in the seawater with the use of monovalent sodium cation in the ASW, which would have neutralized the negative charge on the bacterial and attaching surfaces at the basic pH used.

Pyrophosphate formed precipitates with divalent cations at concentrations ≥ 0.003 M in sea water and > 0.05 M in ASW containing sediment. Concentration of < 0.05 M pyrophosphate did

not appear to cause dispersal of bacterial and sediment aggregates or release of epiphytic bacteria on blade 10. Sodium pyrophosphate has been reported to release bacteria attached to glass slides (Fletcher, 1980). Marshall and Cruickshank (1973) showed that polysaccharide fibrils responsible for attachment of bacteria to glass surfaces were periodate sensitive. Treatment of sediment and kelp tissue with sodium periodate was not effective since most of the bacterial and sediment aggregates and the epiphytic bacteria on the blade were still present. In addition, sodium periodate rapidly quenched the fluorescence of stained bacteria.

There are several reasons which may serve to explain the differences in the results of various experiments dealing with the effect of chemical reagents on detachment of bacteria from artifical substrates as compared with natural habitats. The heterogeneous nature of sediment and kelp surfaces may have more firmly attached and embedded bacterial cells as a result of their longer attachment periods in their natural environment. Attachment of pure bacterial isolates to glass surfaces in laboratory experiments was examined over relatively shorter time periods, usually less than 24 h (Marshall and Cruickshank, 1973; Corpe, 1974; Fletcher, 1980). One of the effects of the longer time period in a natural environment may be the production of a greater quantity and/or different quality of polysaccharides by the microorganisms and macrophytes than occurs in 24 h on glass surfaces. There may also be a problem of the reagents not being

able to reach bacterial cells so that changes in the surface charge, sequestration of cations, or oxidation of polysaccharides may not have taken place under the experimental conditions used here.

1.2 Effect of ultrasound

Unfixed cells of the Gram negative rod shaped bacterial isolate were very sensitive to sonication. Untreated cells were observed to be single or clumped in small aggregates. Within 5 s of treatment at 125 W power levels, the bacterial count declined by 46 % and after 90 s treatment, only 1 % of the original numbers remained (Fig. 3). Light absorbance measurement of the unfixed samples after treatment with sonication showed a similar trend (Fig. 3). The susceptibility of bacterial cells to sonication has also been reported by Pike et al. (1972); Banks and Walker, (1977) and Coakley et al. (1977).

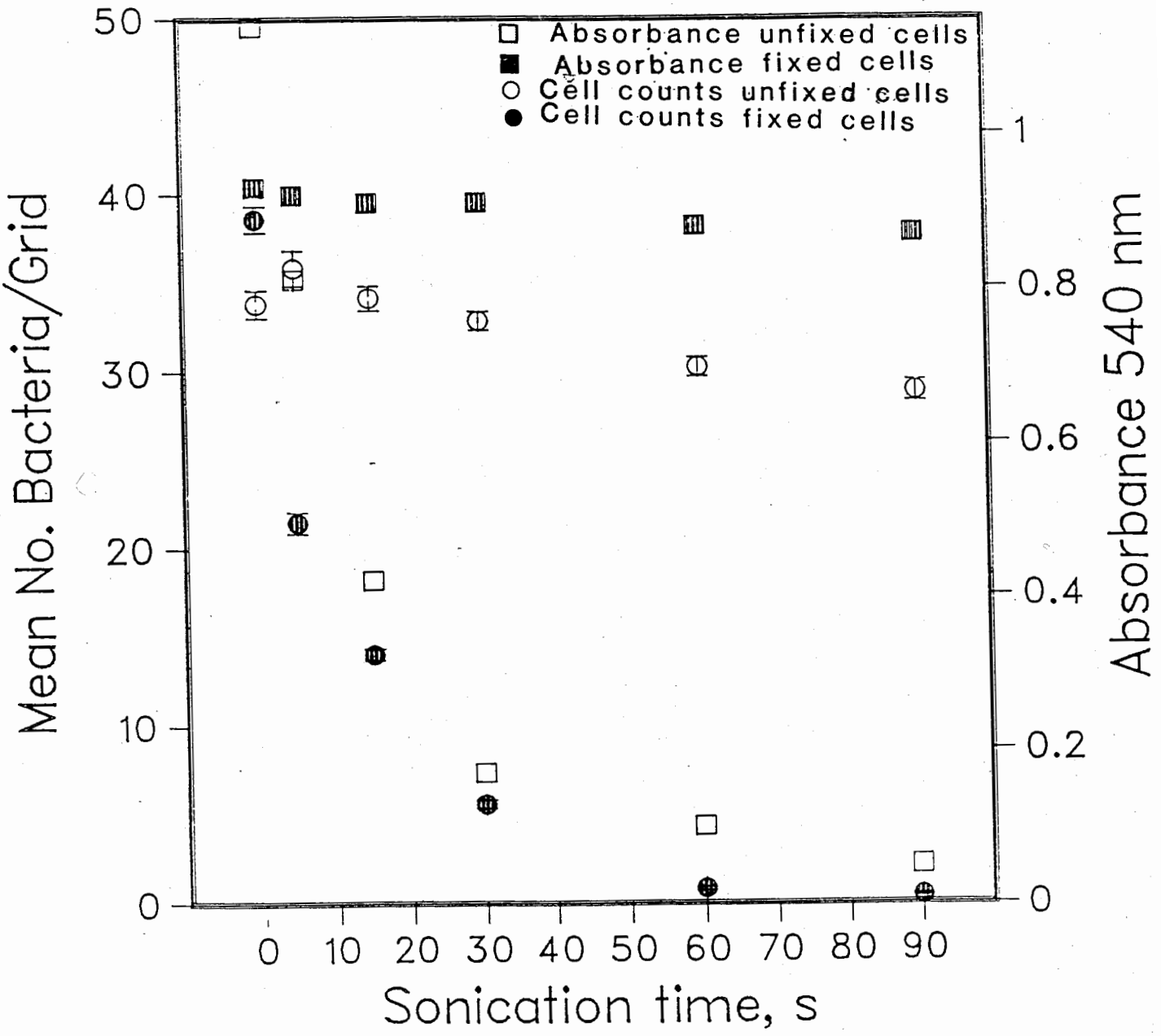
Many microorganisms treated with sonication have survival curves which show a negative exponential form (Coakley et al., 1977). A Gram negative and rod shaped bacterium was chosen since it represented the most sensitive cell type to sonication (Coakley et al., 1977). When the bacterial samples were fixed with 3.7 % formaldehyde (v/v) for 30 min there was an increase in tolerance of bacterial cells to sonication. After 5 s sonication at 125 W there was a small increase in bacterial numbers (6 %) probably due to disaggregation of bacterial

clumps. This was followed by a slow decline in numbers with time of sonication until at 90 s, 85 % of the original bacterial numbers remained (Fig. 3).

Absorbances of the sonicated samples were relatively constant for up to 30 s treatment and then showed a small decline after 60 and 90 s treatments (Fig. 3) confirming the increased tolerance of formaldehyde fixed cells to sonication. Bingle (1980) observed that there was a decline in viable count of mixed soil bacteria after 5 min of sonication at power levels of 50 to 300 W (treatment of less than 5 min was not examined). Since soil contains both Gram negative and Gram positive bacterial cells as well as rod and coccoid forms (Alexander, 1977), the tolerance of soil bacteria to 5 min of sonication probably reflects the differential survival of bacterial forms such as Gram positive or coccoid forms which have stronger cell walls than Gram negative or rod shaped cells (Coakley et al., 1977).

Aldehyde fixatives form inter- and intra-molecular crosslinks with proteins (Hayat, 1981) and hence strengthen the cell components, including the cell walls, which results in an increased tolerance of these cells to sonication. The fixation of proteins by formaldehyde has been attributed to formation of methylene bridges between amino, imino or peptide groups, which contain an active hydrogen atom (Hayat, 1981). Accordingly all water, sediment, fecal material and kelp blade samples were fixed with formaldehyde (3.7 %) for at least 30 min prior to

Fig.3. Tolerance of Gram negative bacterial isolate cells to sonication before and after fixation with formaldehyde. Bacterial means ((±SE) and absorbance at 540 nm for n=2 and number of microscopic grids (g)=20 (SE smaller than the symbols are not represented).


Absorbance unfixed cells
Absorbance fixed cells
Cell counts unfixed cells
Cell counts fixed cells
Mean No. Bacteria/Grid
Absorbance 540 nm
Sonication time, s
0
10
20
30
40
50
60
70
80
90
0
0.2
0.4
0.6
0.8
1

sonication.

It was impossible to assess the tolerance of bacteria in fecal material, sediment and on kelp blades to sonication since the numbers of bacteria present before treatment could not be accurately enumerated due to interference from the other components in the sample. Therefore to assess bacterial tolerance to sonication in a natural environment an epibenthic water sample was investigated. This sample had lower detrital and particulate content (Fig. 4). Treatment of fixed epibenthic water samples with sonication, at a power level of 100 W for up to 90 s, did not cause a decline in bacterial counts (Fig. 5) when compared to untreated samples (0 s, Fig. 5). However, sonication at 125 W power level for longer than 15 s caused a decline in bacterial numbers (Fig. 5).

Sediment and their associated bacteria did not disperse within 60 s of sonication until a threshold value of 75 W was applied. At the 100 W power level bacterial and sediment dispersion began within 5 s of sonication (Fig. 6). Maximum bacterial numbers were observed after 30 s treatment. At 125 W maximal numbers of bacterial cells were observed after 15 s of sonication, thereafter the bacterial counts declined as the time of sonication increased (Fig. 6). In all cases small aggregates of bacterial cells and detrital material remained after sonication. There still appeared to be colloidal material surrounding the bacterial and sediment aggregates after treatment with sonication. It is likely that sonication severs

Fig. 4. Photomicrograph of bacteria from untreated epibenthic water sample : (a) free bacterial cell, (b) bacterial cells surrounded by colloidal material. Magnification, x1200.

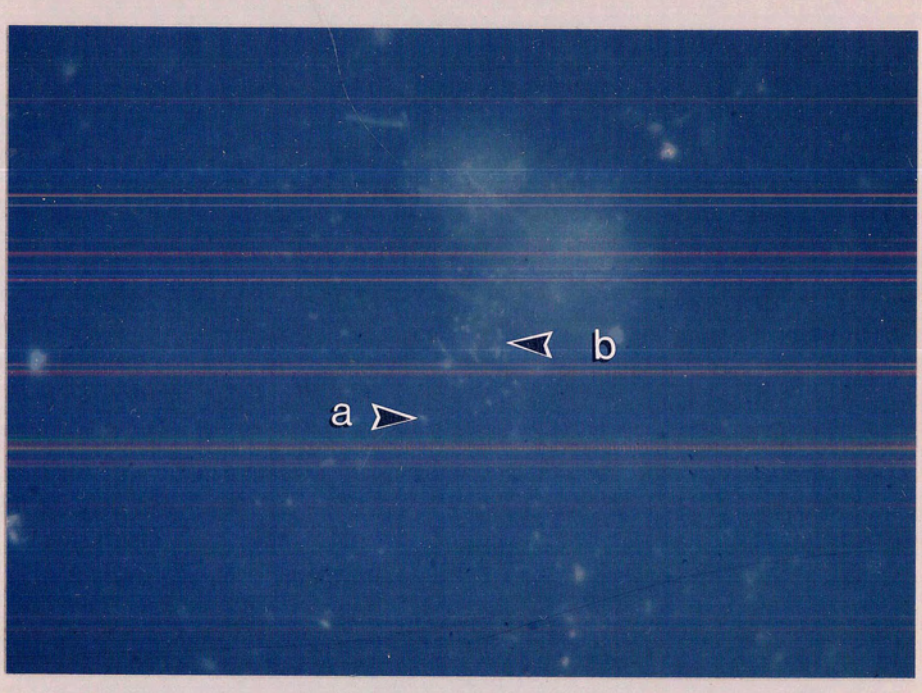

b
a

Fig.5. The effect of power level and duration of sonication on mean bacterial numbers ((±SE) per microscopic grid of epibenthic water samples. (n=2 and g=20).

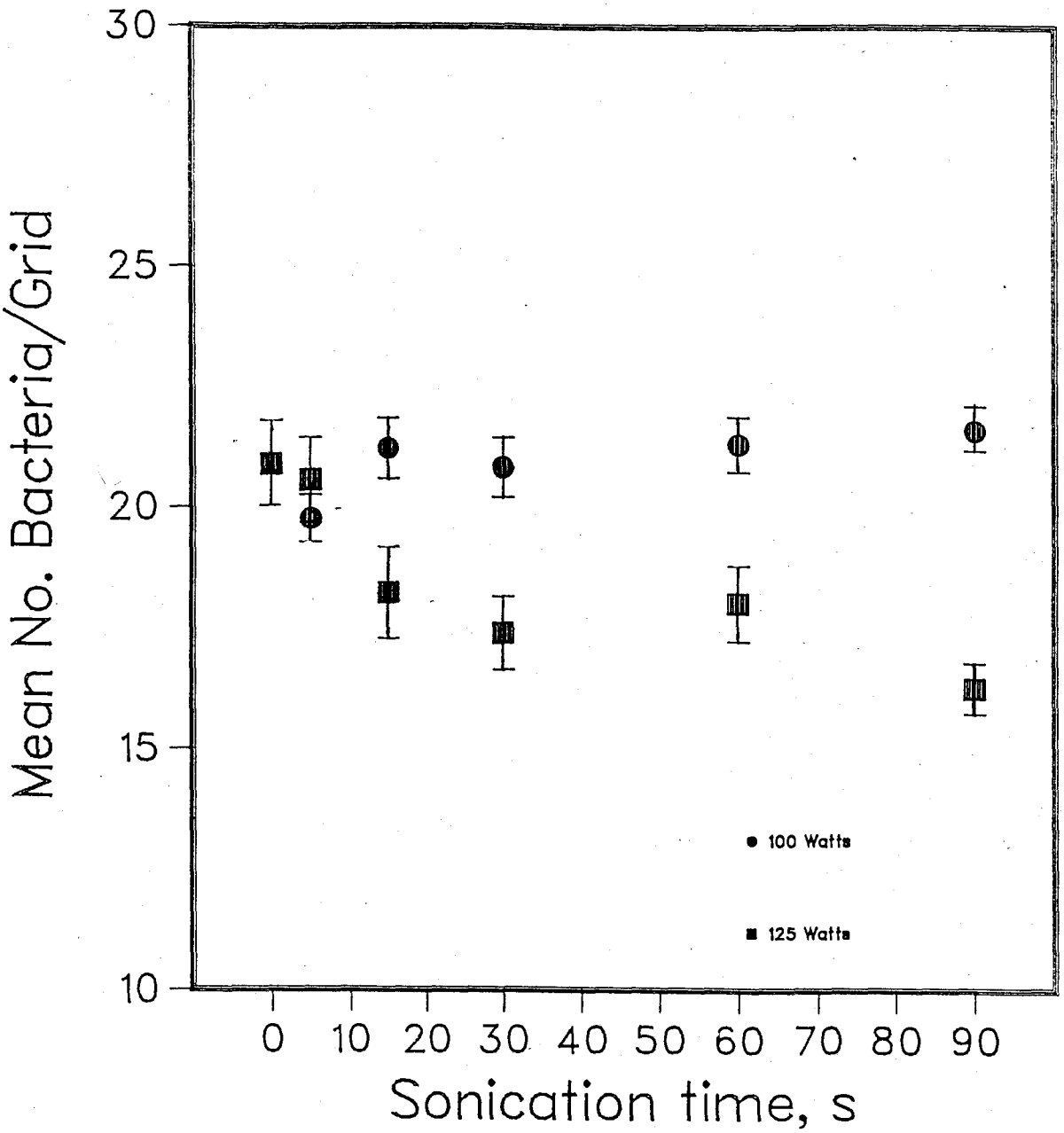
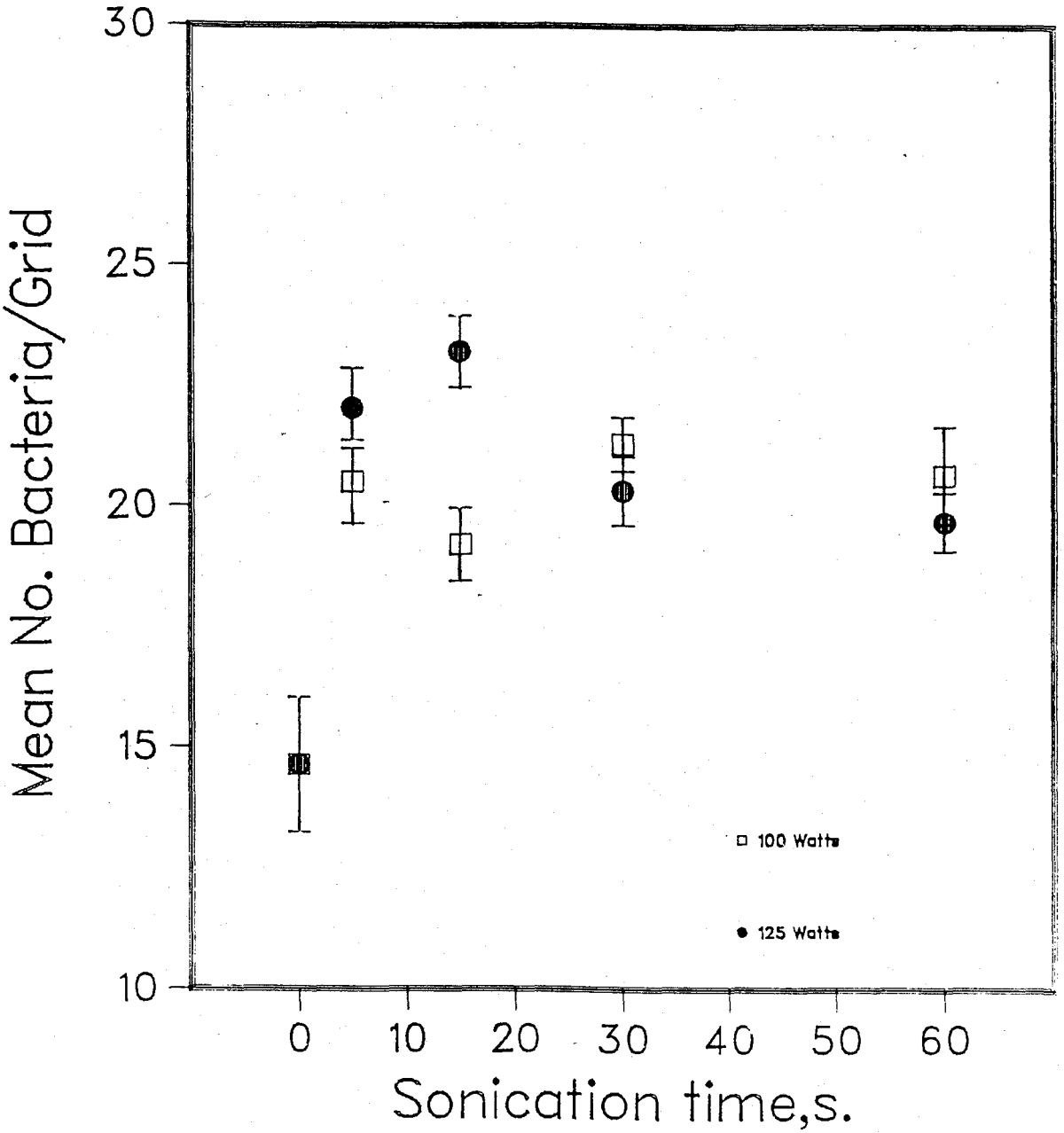

Mean No. Bacteria/Grid
30
25
20
15
10
0
10
20
30
40
50
60
70
80
90
Sonication time, s
100 Watts
125 Watts

Fig.6. The effect of power level and duration of sonication on mean bacterial numbers ((± SE) per microscopic grid (n=2, g=20) from sediment samples.


30
25
20
15
10
Mean No. Bacteria/Grid
0
10
20
30
40
50
60
Sonication time,s.
100 Watts
125 Watts

some of the bridging polymers, causing large aggregate disruption (Marshall, 1980a; Bingle, 1980). The bacteria and sediment material also tended to accumulate at the edges of the Nuclepore filter rather than spread evenly over the entire filter surface. This may be due to the negative charge on the surface bacterial cells and sediment particles at pH 7.9 resulting in an attraction to the glass surface of the millipore filter assembly, which may be mediated by a cationic bridging mechanism (Costerton et al. 1978) particularly since divalent cations were still present.

1.3 Effect of chemical reagents followed by ultrasound

Microscopic examination of sediment and kelp disc samples treated with hydrochloric acid, sodium hydroxide, sodium carbonate, sodium periodate and tetrasodium pyrophosphate followed by sonication, at 100 W power level for 15 s, showed a greater disaggregation of sediment associated bacteria and detachment of epiphytic bacteria than those samples treated with the chemicals only (sec. 1.1). Incubation of the samples suspended under acidic, mild basic and basic conditions followed by sonication did not appear to cause any greater dispersion of bacteria than occurred in those suspended in ASW and sonicated (sec. 1.2). The accessibility of the reagents to bacterial cells was increased by breaking of the large sediment aggregates with sonication but this was insufficent to cause total dispersion of

the cells. A large number of small aggregates of bacteria and amorphous organic matter were still present in the sediment sample. Treatment of the kelp discs from blade 10 with sonication resulted in only partial removal of epiphytic bacteria into the suspending medium. The small bacterial aggregates in the sediment and the epiphytic bacteria on the blade surface were observed to be enmeshed by colloidal material that may have been polysaccharide in nature (Corpe, 1972; Marshall and Cruickshank, 1973; Costerton et al., 1978).

Cleavage of the polysaccharides in kelp requires strong hydrolytic conditions, e. g., 0.5 M sulphuric acid at 100° C for 20 h (K. Rosell, per. comm.). Therefore simple altering of pH may change the surface charge of the polysaccharides and the medium but does not cause dispersion of the individual cells that may have been enmeshed in the colloidal matrix. In hard water washing where presence of soil interferes with the cleaning process, Cahn and Lynn (1983) reported that sodium hydroxide and sodium carbonate do not sequester cations, do not have a soil suspending effect and, in addition, sodium carbonate forms insoluble calcium carbonate with calcium. Treatment of the sediment and kelp samples with pyrophosphate (0.01 M) or periodate (0.005 and 0.01 M) followed by sonication increased bacterial dispersion when compared to samples treated with only sonication or change in pH followed by sonication.

Bacterial cells treated with periodate and then stained with DAPI (and AO) lost fluorescence within 15 s when examined

by epifluorescence microscopy. Periodic acid oxidizes compounds which contain two or more adjacent hydroxyl ,carbonyl (Morrison and Boyd, 1974) or alpha-amino groups (Hayat, 1981) with cleavage of carbon-carbon bonds. Therefore periodate may have oxidized the nucleic acids or the fluorochromes resulting in the rapid quenching of the fluorescence.

Sediment suspensions treated with pyrophosphate at concentrations of 0.05 M or greater formed a precipitate, which greatly interfered with bacterial observations on the filter. Samples treated with 0.01 M pyrophosphate followed by sonication resulted in an even distribution of bacteria and other sediment components on the filters (Fig.7) and a reduction in colloidal material surrounding the bacterial cells. A concentration of ≤0.001 M pyrophosphate did not enhance sonication-induced dispersion. Kelp discs treated with 0.01 M pyrophosphate for 15 to 30 min followed by sonication at 100 W for 30 to 60 s dispersed into unattached single cells or packets of 2 to 5 kelp cells. Observation of the residue after filtration revealed an even dispersion of bacterial and kelp cells (Fig.8).

A sequestering (chelating) agent has more than one atom which may be bonded to a central metal ion at one time to form a ring structure (Manahan, 1975). Pyrophosphate can bind to 2 sites on a calcium ion (Manahan, 1975). Polyphosphates can sequester cations like calcium in both soluble or suspended forms. This property has been used for reducing the equilibrium concentration of calcium ions in water pipes and boilers to

Fig. 7. Photomicrograph of DAPI stained bacteria from sediment sample treated with tetrasodium pyrophosphate (0.01 M) and sonicated (100 watts, 15 s) treatment, (c) dispersed bacterial cells, and (d) dispersed sediment aggregate. Magnification, x1200.

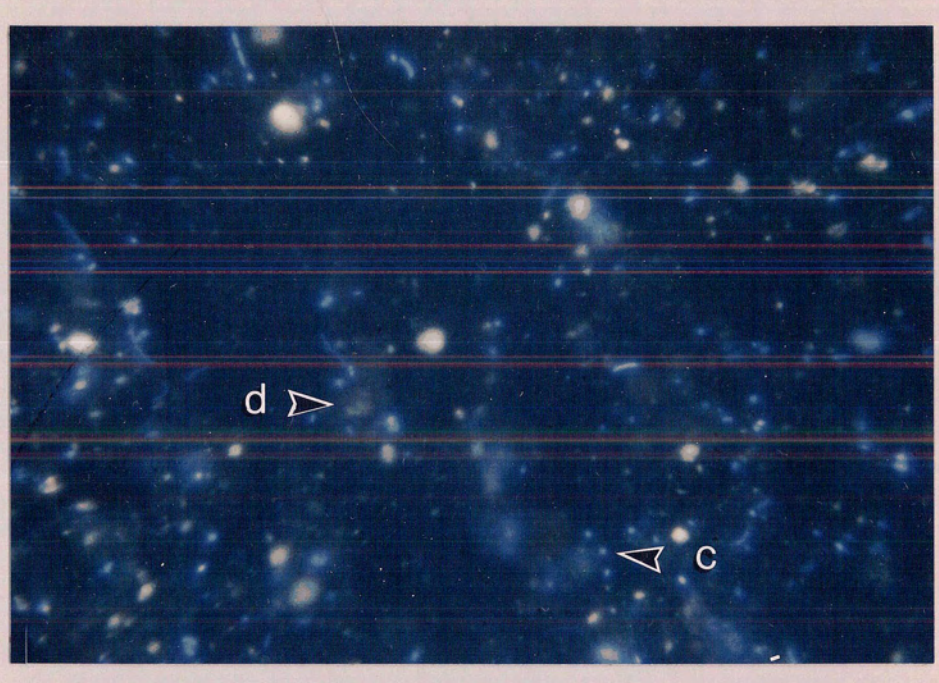
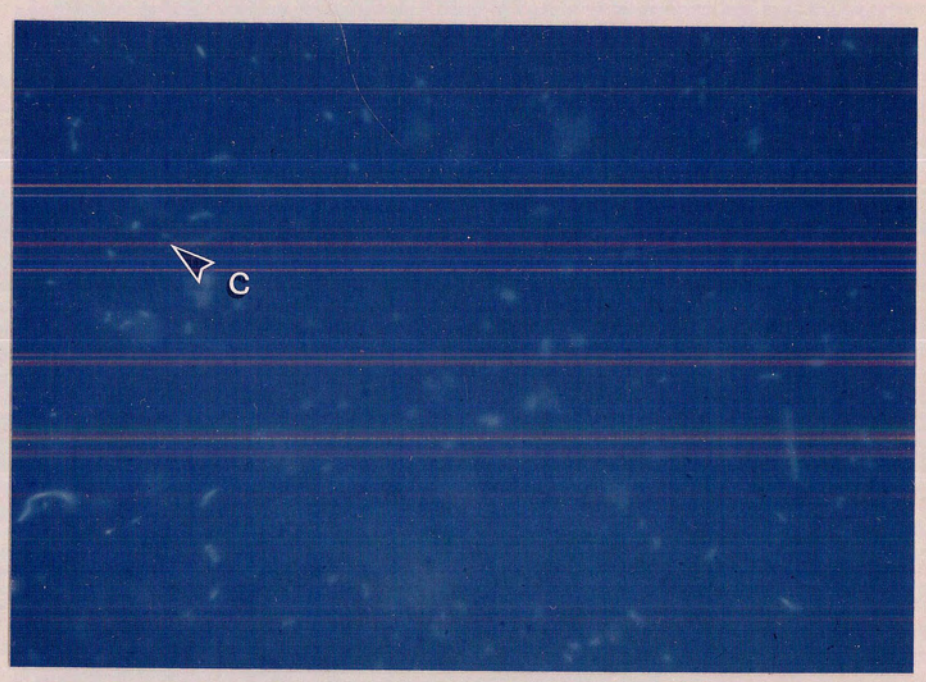

d
c

Fig. 8. Photomicrograph of DAPI stained epiphytic bacteria from blade 22 of Macrocystis integrifolia after treatment with tetrasodium pyrophosphate (0.01 M) and sonication (100 watts, 45 s), (c) dispersed bacteria. Magnification, x1200.


C

prevent precipitation of calcium carbonate (Manahan, 1975). Sodium pyrophosphate, sodium tripolyphosphate and pentasodium tripolyphosphate have been used to keep soil in suspension and to sequester calcium and magnesium cations in heavy duty fabric washing (Cahn and Lynn, 1983; Considine and Considine, 1983). These cations form water-soluble complexes with the polyphosphates and therefore cannot react with cleaning agents to form precipitates (Considine and Considine, 1983).

The increase in dispersion of bacteria and other sediment components as well as the dispersion of the kelp cells and their associated bacteria may be due to pyrophosphate sequestering of polyvalent cations present in these samples. The majority of the monovalent and divalent inorganic elements in M. integrifolia appear to be ionically bound. Most of the divalent cations have been found to be associated with the alginate and fucoidin components (Rosell and Srivastava, 1984). The binding mechanism between the acidic polysaccharides and cations in the sediment and kelp samples may be more complicated than ion exchange and salt formation (Dudman, 1977). The affinity of polyglucuronides and sulphated fucans for calcium and magnesium cations has been proposed to be a result of chelation and coordination complexes (DeLucas et al., 1975, and, de Lestang and Quillet, 1974 quoted in Dudman, 1977). Therefore pyrophosphate must be a relatively stronger sequestering agent than the compounds binding the cations so that it results in sediment disaggregation as well as in disruption of kelp tissue into cells. Sonication simply

quickened this process.

When the subsurface and epibenthic water samples were incubated in 0.001 M pyrophosphate and then sonicated, there was a noticeable decline in the occurrence of bacterial cells enmeshed in colloidal material (Fig. 9). The bacterial cells appear to be able to tolerate sonication at a power level of 100 W since the numbers of bacteria in these waters did not decrease following treatment with 0.001 M pyrophosphate and sonication for up to 60 s (Fig. 10).

The trend in tolerance of bacterial cells treated with 0.01 M pyrophosphate followed by sonication in the sediment samples was similar to samples treated with sonication only (sec. 1.2). Treatment for 5 s or longer at 75 and 100 W power levels caused an increase in bacterial counts due to increase in dispersion (Fig. 11). Maximum numbers were observed between 15 and 60 s treatment at 100 W and after 5 s treatment at 125 W. Treatment for longer than 5 s at 125 W power level caused the numbers of bacteria observed to decline.

There appeared to be no decline of bacterial numbers in seawater samples treated with 100 W power level for up to 60 s (with and without pyrophosphate) and the maximal numbers in sediment samples were observed after 15 s of sonication treatment (with and without pyrophosphate). Therefore the power level of 100 W for 30 to 45 s in the presence and absence of pyrophosphate was used for detailed examination of the dispersal effect of bacterial cells in subsurface and epibenthic water,

Fig. 9. Photomicrograph of bacteria from epibenthic water sample after treatment with tetrasodium pyrophosphate (0.001 M) and sonication (100 watts, 15 s) treatment : (c) free bacterial cell. Magnification, x1200.

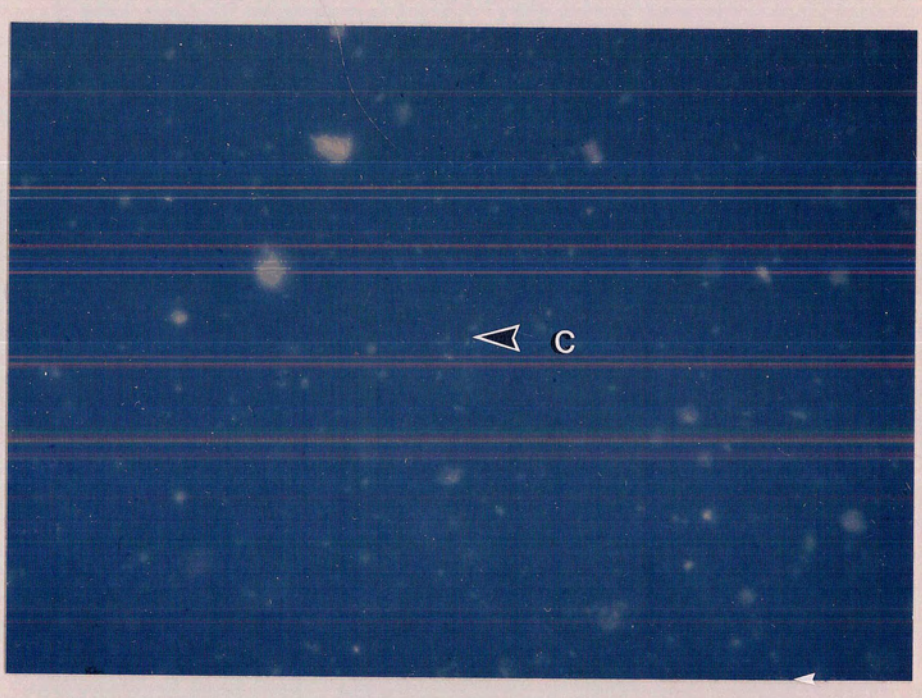

C

Fig. 10. The effect of duration of sonication (100 W) on mean bacterial counts ((± SE) per grid of subsurface and epibenthic water samples containing 0.001 M tetrasodium pyrophosphate (n=2, g=20).

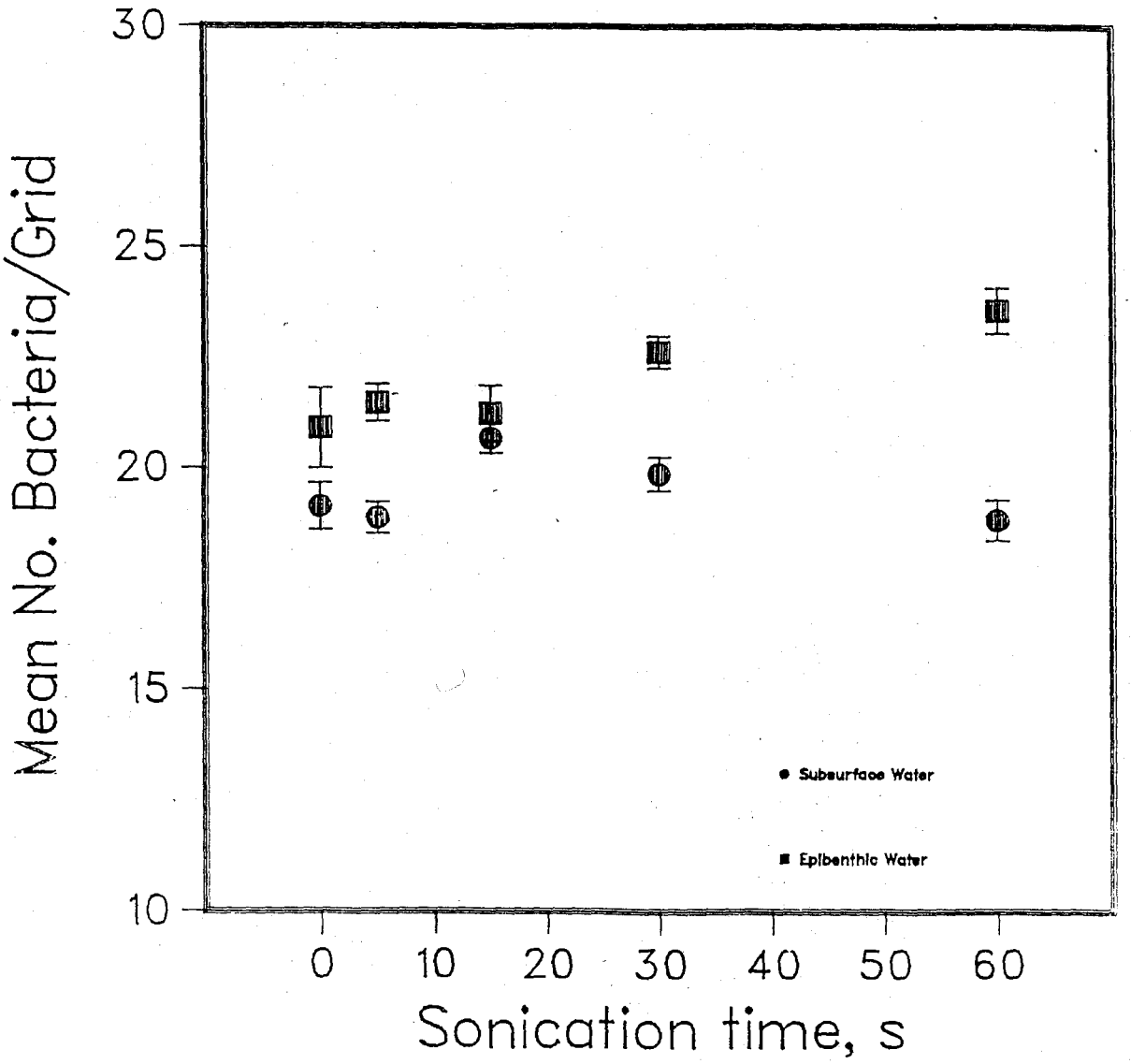
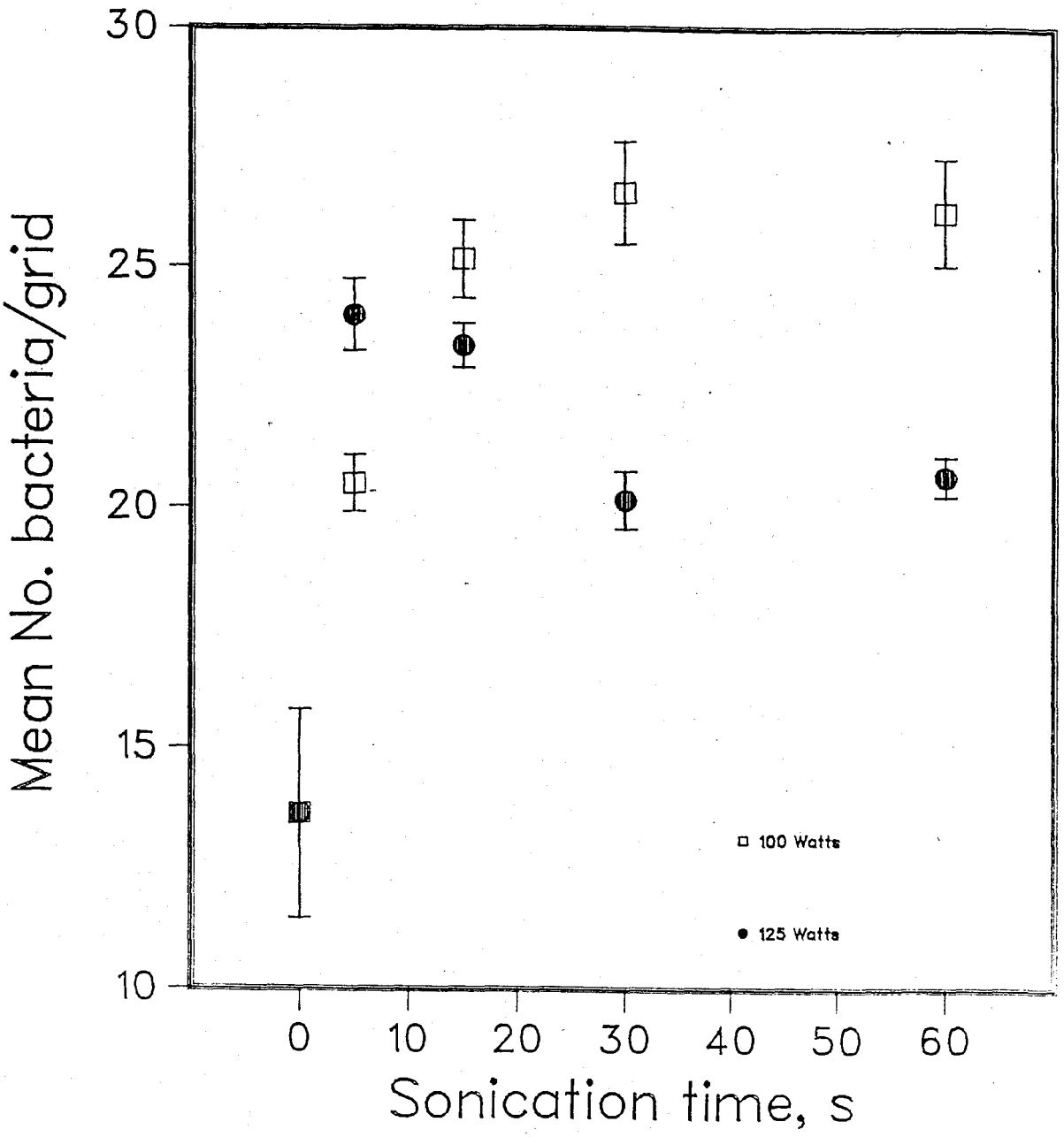

Mean No. Bacteria/Grid
30
25
20
15
10
0
10
20
30
40
50
60
Sonication time, s
Subsurface Water
Epibenthic Water

Fig.11. The effect of power level and duration of sonication on pyrophosphate (0.01 M) treated sediment bacterial count means ((± SE) per grid (n=2, g=20).


30
25
20
15
10
Mean No. bacteria/grid
0
10
20
30
40
50
60
Sonication time, s
100 Watts
125 Watts

sediment, fecal material and different aged kelp blades.

2. Statistical analyses of bacterial samples from different habitats treated with pyrophosphate and ultrasound

Individuals of a population are found in three basic spatial distributions : random; regular (uniform, even or repulsed); and contagious (clumped or aggregated) (Elliot, 1977; Sokal and Rohlf, 1969). The relationship between the variance and the arithmetic mean of the population is determined by the dispersion of the population. In a random distribution, the variance equals the mean; in a regular distribution, the variance is less than the mean while in a contagious distribution, the variance is greater than the mean (Elliot, 1977). Calculation of the coefficient of dispersion (CD=variance/mean) is a rapid method for determining if the bacterial cells are randomly distributed (Sokal and Rohlf, 1969). Therefore, in a randomly distributed sample the CD value will be near 1, while the value will be <1 for regular distribution and >1 for contagious distribution (Sokal and Rohlf, 1969). Many environmental factors are unevenly distributed and this often leads to a contagious distribution in some populations. In others there is a a tendency to aggregate, thereby producing a contagious distribution (Elliot, 1977).

In several aquatic habitats bacterial distributions have been found to be non-random and this has been attributed to

clumping and attachment of bacteria (El-Shaarawi et al., 1981; Daley, 1979; Hossel and Baker, 1979a; Montagna, 1982; Kirchman et al., 1982). This leads to significant variations in bacterial counts at the subsample and microscopic field levels (Kirchman et al., 1982; Montagna, 1982). If the bacterial samples can be manipulated so that the eventual spatial distribution is random, the simple methods of Poisson series can be used to calculate confidence limits and compare samples from different areas. This would be simpler than using the more complex methods for regular or contagious distribution (Elliot, 1977).

It is generally difficult to compare variances of two populations if their means differ greatly. For instance, in the untreated bacterial sediment samples the means of the bacterial counts were found to be considerabley lower than those samples that had been dispersed by sonication or treated with a combination of pyrophosphate and sonication. The coefficient of variation has been found to be useful for comparing two populations independent of the magnitude of their means (Sokal and Rohlf, 1969). Coefficient of variation (CV) is the standard deviation (SD) expressed as a percentage of the mean. This statistical technique was used to compare the variations within and between the various microbial samples before and after treatments.

2.1 Subsurface and epibenthic water

The spatial distribution of the bacterial cells in the subsurface and epibenthic water samples ranged from random to regular (n=4) for each treatment (control, sonicated and pyrophosphate followed by sonication). All samples had CD $\leq$ 1. Most of the cell counts per sample fit the uniform distribution ($P>0.05$), all cell counts fit the Poisson distribution ($P>0.05$) and the normal distribution ($P>0.05$). This meant that the cell frequency distribution overlapped the three types of distributions and there was insufficent information to distinguish between the three.

The variances of the subsurface and epibenthic water samples were unequal over the three treatments (Bartlett's test, $P<0.001$). Since the various transformations of the data did not enhance normality and homogeneity of variances simultaneously, the assumptions of analysis of variance (ANOVA) could not be satisfied. The means of the three treatments were found to be significantly different in the subsurface water (K-W test, $X^2=19.386, P<0.001$) and epibenthic water ($X^2=42.113$, $P<0.001$). The cell means were also significantly different between each of the treatments (Table 2).

The variances from each treatment were examined separately. The untreated and the combination treated samples from both the subsurface and epibenthic water had equal variances, while samples that were sonicated only did not have equal variances

Table 2. Kruskal-Wallis test for significace of the the means[1] between the treatments in the subsurface and epibenthic water samples.

Water Samples		TREATMENTS[2] C with S	C with PS	S with PS
Subsurface	X^2[3]	17.633	5.441	6.062
	P[4]	0.001	0.020	0.014
Epibenthic	X^2	10.80	43.021	9.235
	P	0.001	0.001	0.002

[1] The means of each treatment in the subsurface and epibenthic waters are given in Table 6.
[2] Treatments : C untreated samples
S sonicated samples (100 W for 30 s)
PS samples treated with pyrophosphate (0.001 M) followed by sonication (100 W for 30 s).
[3] Chi-square value.
[4] Two tailed probability.

(Table 3).

The estimates of the mean number of bacterial cells per sample were relatively more precise in the pyrophosphate followed by sonication (combination) treatment as compared to untreated or treated samples with sonication. Both the standard error (SE) of the mean (Fig. 12 and 13) and the coefficeient of variation (CV) were the lowest. The CV for the combination treatment samples was ca. 10 % (range 9-12%), while the CV's for control samples were ca. 15% (range 12-21 %) and for sonicated samples ca. 15 % (range 11-25%). The untreated samples' CV was in a similar range as reported by Kirchman et al. (1982) for Great Sippewisset Marsh samples (12-25%). When the CV of the bacterial count was examined for 5, 10, 20 and 30 grids per sample in the different treatments and in both subsurface and epibenthic water, the combination treatment had the lowest and constant CV for grids between 10 and 30 (Table 4 and 5). The CV of the control and sonicated samples fluctuated or declined with the increase in number of grids counted (Table 4 and 5). This suggests that the combination treated water samples had a homogeneous distribution of bacteria on the filter. Therefore counting of at least 10 grids would give as good an estimate of the mean and variance of the bacterial numbers in the combination treated samples as would counting 20 or 30 grids. The improved precision in the combination treated samples was also reflected in the lower SE for the overall mean (g=80) when compared to the untreated or sonicated treated samples in both

Table 3. Test of homogeneity of variances of bacterial numbers in samples within each treatment in subsurface and epibenthic water.

Treatment	Subsurface Water	Epibenthic Water
Control	0.616[1]	0.146
Sonication	0.017	0.014
Combination[2]	0.908	0.635

[1] Bartlett's test for homogeneity of variances, probability.
[2] Pyrophosphate with sonication treatment.

Fig. 12. The mean number of bacteria mL^{-1} ($\pm$SE) in the subsurface water before and after treatments. Treatments : Control (C), sonicated (S) and pyrophosphate and sonication (PS). (n=1-4 and g=20).

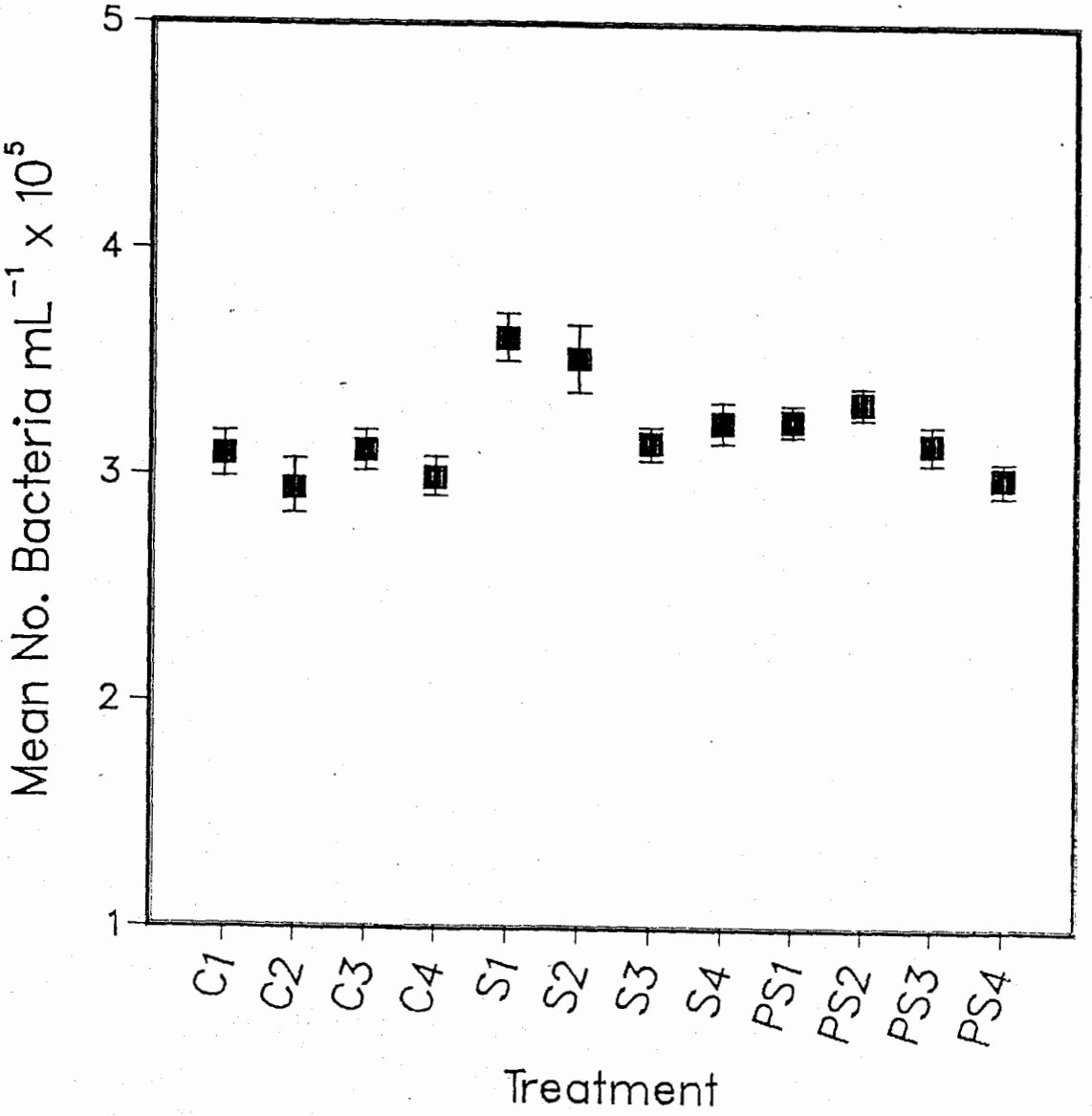

Mean No. Bacteria mL^{-1} x 10^5
5
4
3
2
1
C1
C2
C3
C4
S1
S2
S3
S4
PS1
PS2
PS3
PS4
Treatment

Fig. 13. The mean number of bacteria mL^{-1} ($\pm$SE) in the epibenthic water before and after treatments. Treatments : Control (C), sonicated (S) and pyrophosphate and sonication (PS). (n=1-4 and g=20).

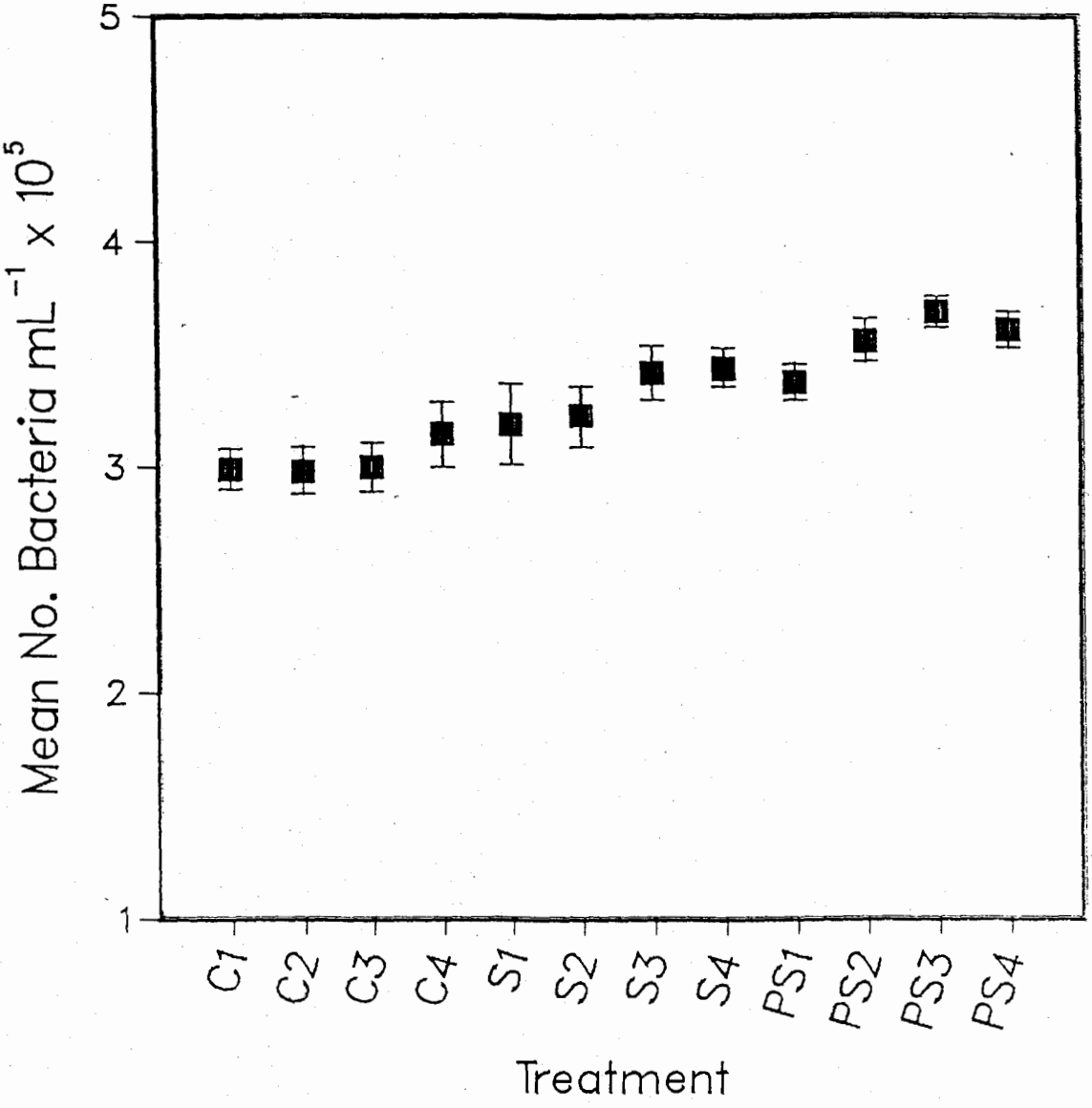

Mean No. Bacteria mL^{-1} x 10^5
5
4
3
2
1
C1
C2
C3
C4
S1
S2
S3
S4
PS1
PS2
PS3
PS4
Treatment

Table 4. Coefficient of variation of bacterial numbers using different numbers of microscopic grids for two subsurface water samples . CV=(Standard deviation/mean)x100.

# grids	TREATMENTS Control 1	Control 2	Sonication 1	Sonication 2	Combination 1	Combination 2
5	5.56	18.84	8.86	19.28	14.02	12.89
10	11.46	14.23	10.31	14.37	10.87	9.63
20	12.92	12.63	11.05	11.96	11.30	10.62
30	11.15	11.22	10.75	11.18	10.37	10.79

Table 5. Coefficient of variation of bacterial numbers using different numbers of microscopic grids for two epibenthic water samples. CV = (Standard deviation/mean)/100.

# grids	TREATMENTS Control 1	Control 2	Sonication 1	Sonication 2	Combination 1	Combination 2
5	21.06	14.23	9.94	9.06	7.14	8.07
10	18.84	26.08	11.27	12.54	6.43	9.87
20	17.05	21.02	17.09	11.35	8.41	10.16
30	15.84	18.96	15.24	14.47	8.66	10.09

the subsurface and epibenthic water (Table 6).

The improvement in the precision of bacterial sample means after the combination treatment as compared to the untreated samples has important implications. Kirchman et al. (1982) found that bacterial abundances varied spatially on a very small scale, i.e. <10 cm. The variation at the lowest level of replication in their sampling scheme was attributed to the non random spatial distribution of the bacteria. This could have been caused by particle associated bacteria, aggregation of unattached bacteria or the non random distribution of the unattached bacteria. Treatment of water samples with pyrophosphate followed by sonication removes these sources of variation and allows for detection of small and large scale variation in the water column with greater certainty.

Table 6. Estimate of bacterial densities in the subsurface and epibenthic water samples by different treatments (g=80).

Treatment	Bacteria mL^{-1} ($\pm$SE x)$x10^5$ Subsurface Water	Epibenthic Water
Control	3.03($\pm$0.05	3.03($\pm$0.06
Sonication	3.38($\pm$0.06	3.37($\pm$0.07
Combination	3.06($\pm$0.04	3.34($\pm$0.04

2.2 Sediment samples

Bacteria were enumerated in four sediment samples (n=4). The sediment samples were untreated, sonicated and treated with pyrophosphate followed by sonication (combination). Two subsamples (ss=2) were analyzed per sample after treatment and 20 grids (g=20) were enumerated for bacterial cells per subsample.

All 8 subsamples from the untreated samples had cell counts with CD>1, indicating a contagious type of spatial distribution. When samples were treated with sonication there was a change in the spatial distribution. Half the subsamples had CD>1 while the other half had CD≤1. There was a complete shift in spatial distribution from the the contagious to random or regular in the combination treated samples (CD≤1). There was insufficient information to distinguish the frequency distribution of the bacterial cell count since there was an overlap of the uniform, the Poisson and the normal distributions. Montagna (1982) found that the bacterial cell counts from mud and sand sediments that had been homogenized by a blender fit a log-normal distribution rather than normal, Poisson or negative binomial distributions. This may have been due to a difference in sediment type since his sediment samples had to be homogenized for at least 4 min before he was able to eliminate particle interference, and he used 8 min treatment for routine analysis.

The variances of the samples over the three treatments were unequal (Bartlett's test, $P<0.001$). No single transformation examined enhanced the homogeneity of variances simultaneously in all the sample in the three treatments. Examination of individual subsamples per treatment indicated that the variances were homogeneous for the combination treatment ($P=0.698$) but not for the control and the sonicated treatments ($P<0.001$). The means of the samples from the three treatments were significantly different (K-W test, $X^2=187.699, P<0.01$). The bacterial means were significantly different between the control and sonicated treatment ($X^2=143.035$, $P<0.001$) and control and combination treatment ($X^2=138.121$, $P<0.001$).The sonication and combination treatment means were similar ($X^2=0.842$, $P=0.359$). The estimate of the bacterial density per g ($\pm$SE) of sediment was $5.20(\pm 0.36x)x10^{10}$ in the untreated samples. There was an increase in the number of bacteria that could be observed after treatment with sonication. The bacterial density was $11.46(\pm 0.37x)x10^{10}$ in the sonicated samples and $11.30(\pm 0.24x)x10^{10}$ in the combination treated samples.

Though the bacterial densities were similar in the sonicated and the combination treated samples, the SE of the mean was lower for the combination treated samples. This becomes obvious when the subsamples' means are compared within each treatment(Fig. 14). The means in the sonication treatment were significantly different ($X^2=20.471$, $P=0.001$) while the means were similar in the subsamples from the combination treatment

(X^2=2.445, P=0.485). In addition the estimates of the mean counts per subsample were more precise in the combination treatment due to the lower SE of mean (Fig. 14) and the lower CV. The CV of the combination treated subsamples was 28.1 % (range 22.8-34.5%) as compared to the CV of untreated subsamples of 77.5 % (range 60.10-108.3 %) and sonicated treated subsamples of 34.6 % (range 24.3-55.9). Montagna (1982) using 8 min of homogenization for his sediment samples and 5 fields per sample, had less precise estimates. They were 50% of the mean ((confidence interval/mean)x 100) compared to 11-16% of the mean, calculated similarly for the combination treatment with 20 grid counts in the present work.

The samples that had been subjected to the combination treatment gave stable CV's after counting 10 grids compared to untreated and sonicated treated samples for which CV's did not stablize when up to 30 grids had been counted (Table 7). In addition the CV between the samples and subsamples was lower in the combination treatment compared to control or sonication treatment. This point is further illustrated when the CV of the combined 8 subsamples within each treatment is compared. The combination treatment had CV of 6.2%, the control had 27.5% and the sonicated treatment had 15.95% .This indicated that the combination treatment not only reduced variation in bacterial cell counts within a subsample, but also between subsamples and between the samples. Montagna (1982) attributed most of the variance in his samples to the subsampling and counting

Fig. 14. The number of bacteria g^{-1} ($\pm$SE) in the sediment samples before and after treatments. Treatments : Control (C), sonicated (S) and pyrophosphate followed by sonication (PS).(n=1-4, ss=1-2 and g=20).

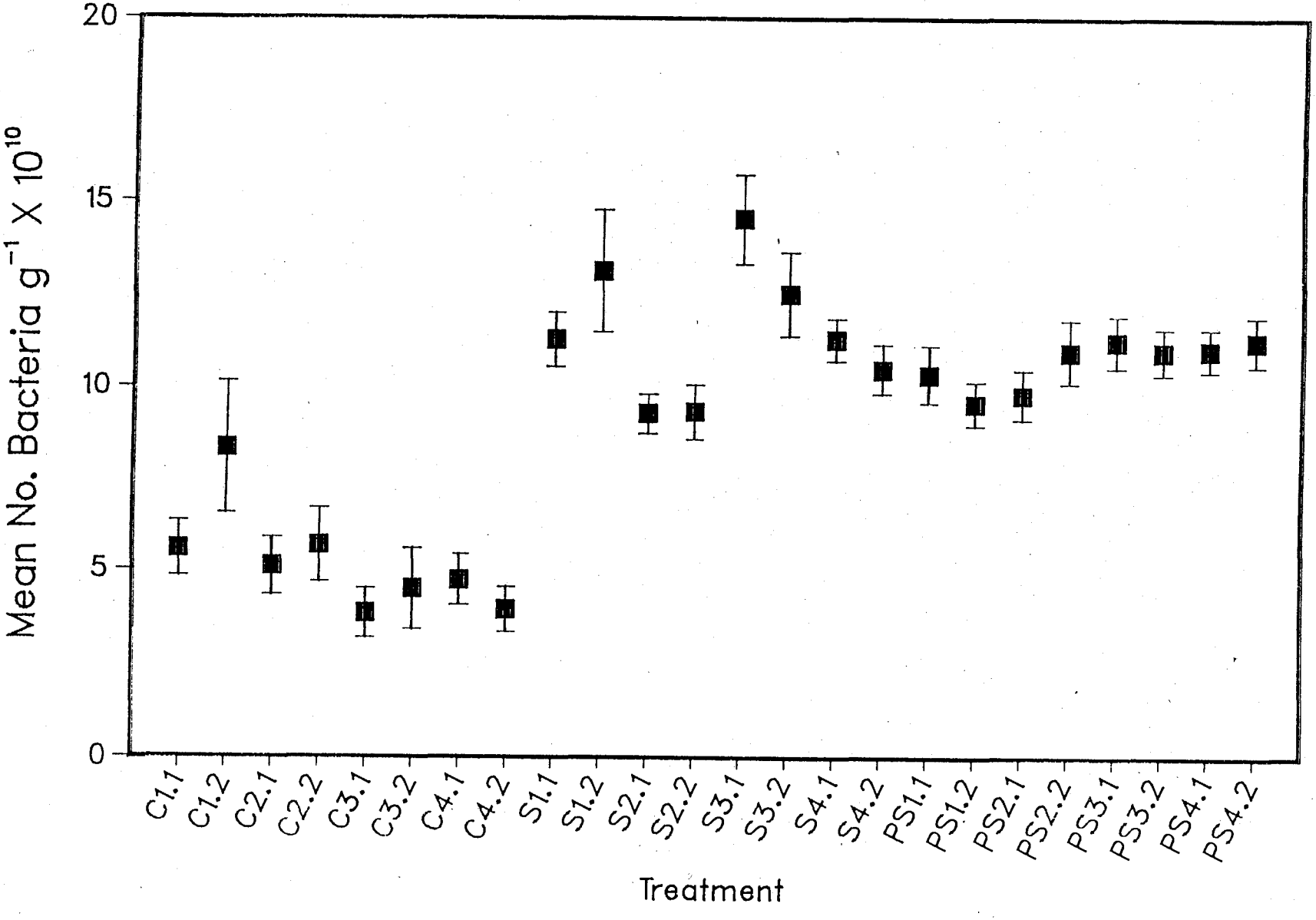

Mean No. Bacteria g^{-1} X 10^{10}
20
15
10
5
0
C1.1
C1.2
C2.1
C2.2
C3.1
C3.2
C4.1
C4.2
S1.1
S1.2
S2.1
S2.2
S3.1
S3.2
S4.1
S4.2
PS1.1
PS1.2
PS2.1
PS2.2
PS3.1
PS3.2
PS4.1
PS4.2
Treatment

Table 7. Coefficient of variation of bacterial numbers using different numbers of microscopic grids for two sediment subsamples per treatment.

# grids	TREATMENTS[1] Control 1	Control 2	Sonication 1	Sonication 2	Combination 1	Combination 2
5	47.32	97.91	31.43	28.40	36.30	15.59
10	63.74	74.95	35.01	25.28	34.79	28.43
20	60.10	97.06	28.68	55.96	30.58	28.56
30	68.46	89.03	26.26	49.76	31.54	29.08

[1] CV=(SD/Mean)x100.

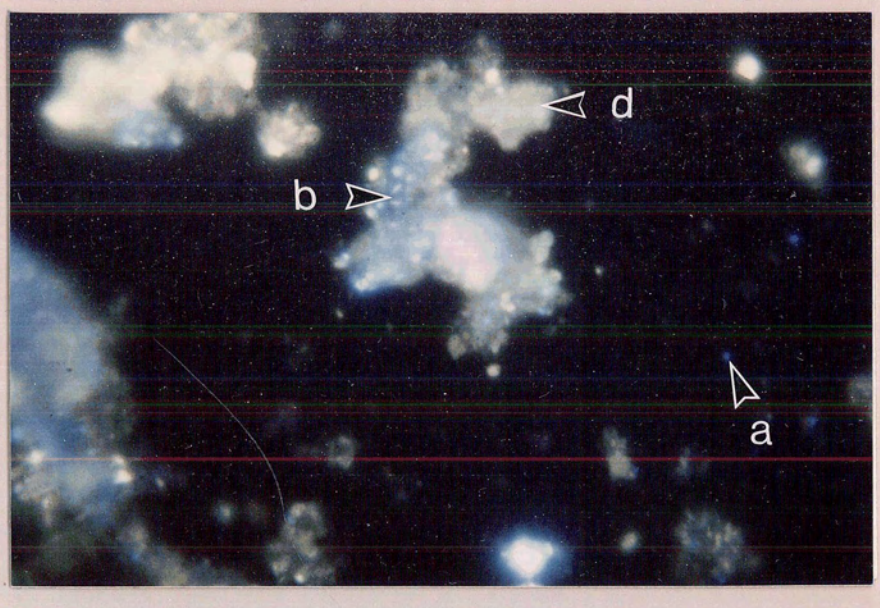
procedures. Since the variation in the counting procedure within each subsample and the variation between the subsamples and samples has been minimized by the use of combination treatment in the present work, spatial variation between sediments from different areas should be easier to detect.

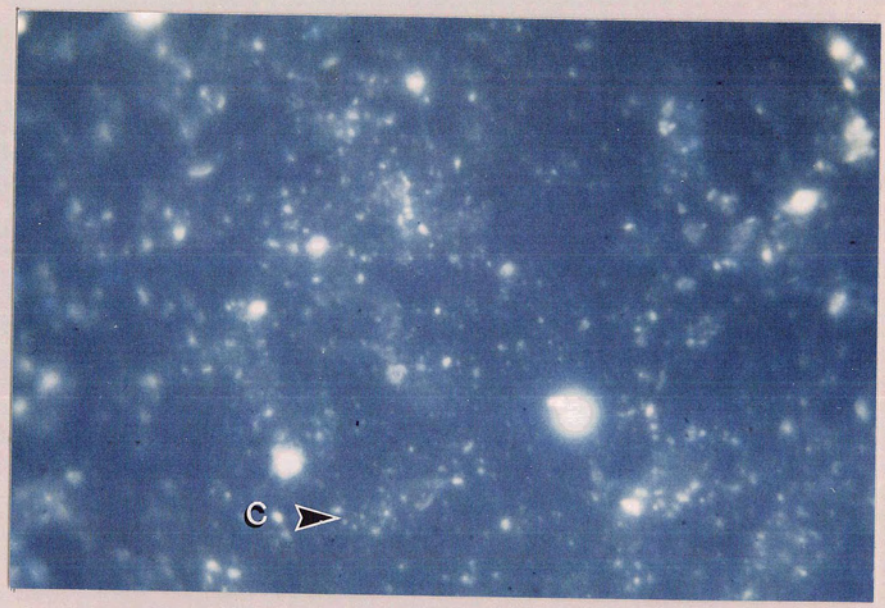
2.3 Parastichopus californicus fecal material

The fecal material's consistency and appearance was similar to that of the sediment sample. There were organic aggregates but with far fewer sand grains. There were also a large number of coelomocytes (Binyon, 1972) from the sea cucumber's gut and a few diatom frustules were also observed. Bacteria were easier to observe in fecal material than in the unsonicated sediment sample, and were generally attached to organic debris (Fig. 15). Sonication partially disrupted the fecal debris-bacterial matrix. Samples treated with pyrophosphate and sonication combination showed very few to no bacterial aggregates and a more even distribution of the fecal material on the filter (Fig.15) than occurred without sonication.

The spatial distribution of the bacteria in the untreated fecal material was contagious since most of the cell counts in in the subsamples had CD>1. Treatment with sonication resulted in contagious to random type of distribution (CD≥1). When the samples were treated with the combination treatment, the spatial distribution was changed from contagious to random or regular

Fig. 15. Photomicrographs of bacteria from fecal material sample: Untreated (top) and after tetrasodium pyrophosphate (0.01 M) and sonication (100 watts, 30 s) treatment (bottom). (a) bacteria cell, (b) bacterial aggregates, (c) dispersed bacterial cells, and (d) detrital aggregate. Magnification, x1200.


d
b
a


c

type (CD≤1) in all the subsamples.

The frequency distributions from the three treatments were in agreement with Poisson and normal distributions and about half the subsamples were in agreement with uniform distribution ($P>0.05$). Examining the frequency distributions of the samples from the different treatments was of limited use since the test lacked sensitivity for detecting changes in distribution. The variances of the samples in the three treatments were not similar (Bartlett's test, $P<0.001$). Transformation of the data did not improve homogeneity of variances and normality simultaneously. This indicated that the data tested in the three treatments were heterogenous in nature.

Examination of the subsamples within each treatment for equality in variances indicated a similar trend as in the sediment treatments. Variances were homogeneous for the subsamples counts in the combination treatment ($P=0.129$) but not in the untreated or treated with sonication treatments ($P<0.001$). The means of the samples in the three treatments were significantly different (K-W test, $X^2= 90.514, P<0.01$). Differences were also significant in the comparisons of (a) untreated and sonicated ($X^2=6.997$, $P=0.008$), (b) untreated and combination ($X^2=45.078$, $P<0.001$) and (c) sonicated and combination ($X^2 = 83.571$, $P <0.001$) treated samples.

The estimates of mean bacterial count per g ((±SE) of the combined subsamples per treatment were, for the untreated samples $7.06(\pm 0.24x)x10^{10}$, for the sonicated treated $4.12(\pm 0.41x)x10^{10}$ and for the combination treated samples $6.48(\pm 0.17x)x10^{10}$. There were no large increases in number of bacterial cells observed after treatment with sonication due to reduction in clumping as was observed in the sediment samples. The means of the bacterial cell counts of the subsamples (Fig. 16) were similar in the untreated (X^2=2.164, P=0.455) and the combination treated samples (X^2=8.334, P=0.400) but were significantly different in the sonicated samples (X^2=11.703, P=0.008).

The trend in the precision of the subsamples' cell counts in the three treatments was similar to that of the sediment treatments. The SE of the mean per subsample (Fig. 16) and the CV were the least for the combination treated samples. The CV was 30.4 % (range 20.4-43.9) for the combination treated, 56.9 % (range 33.4-103.9%) for the untreated and 46.8 % (range 25.9-68.5%) for the sonicated treated subsamples. The improvement in the precision after combination treatment of the samples was also reflected in the relatively low and stable CV after counting 10 microscopic grids of the subsamples. The CV was higher and unstable for samples that had been untreated or treated with sonication only (Table 8). Further, the CV of the subsample means was also lower in the combination treated samples (9.3 %) compared to the untreated (14.8 %) and sonicated

Fig. 16. The mean number of bacteria g^{-1} ($\pm$SE) in the fecal samples before and after treatments. Treatments : Control (C), sonicated (S) and pyrophosphate and sonication (PS).(n=1-4, ss=1-2, g=20).

Mean No. Bacteria g^{-1} X 10^{10}
10
5
0
C1.1
C1.2
C2.1
C2.2
C3.1
C3.2
C4.1
C4.2
S1.1
S1.2
S2.1
S2.2
S3.1
S3.2
S4.1
S4.2
PS1.1
PS1.2
PS2.1
PS2.2
PS3.1
PS3.2
PS4.1
PS4.2
Treatment

Table 9. Coefficient of variation of bacterial numbers using different numbers of microscopic grids for two of the fecal material subsamples per treatment.

# grids	TREATMENTS Control 1	Control 2	Sonication 1	Sonication 2	Combination 1	Combination 2
5	66.57	104.18	44.61	30.29	20.79	34.49[1]
10	51.17	97.73	47.19	25.84	24.32	28.00
20	56.22	103.95	52.73	25.89	24.49	32.77
30	52.59	92.24	51.96	25.96	24.40	30.09

[1]CV=(SD/Mean)x100.

samples (35.7 %). Combination treated fecal material samples showed a decline in variation within subsamples, and in the variation between the subsamples. This trend was similar to the sediment samples treated with the combination treatment and compared to untreated or samples treated with sonication.

2.4. Macrocystis integrifolia blades

The bacterial counts in the majority of the untreated disc samples from blades 1, 4 and 10 had a CD≥1 which indicated a contagious type of distribution (Table 9). Roland (1980) also found greater variance to mean ratios in the bacterial samples from the different aged blades of M. integrifolia. The cell frequency of more than half the disc samples from each blade fit the normal distribution. Most of the samples from blades 1 and 10 did not fit Poisson distribution ($P<0.05$), the majority of blade 4 samples did ($P>0.05$). The variances of the samples per blade were not homogeneous ($P<0.001$) and none of the transformations examined enhanced the homogeneity except for $\log(x+1)$ and $\sinh^{-1}$ for blade 1 samples only ($P>0.1$) (Table 10). The reason for the failure of transformation could be due to the association of bacteria with the microtopography of the kelp surface, other epiphytes and the presence of bacteria ranging from single cells to microcolonies over small spatial scales.

In the combination treatment the CD was ≤2 for all the disc samples from all the 4 blades, indicating a change in bacterial spatial distribution from contagious to the random type. This was confirmed when the cell frequency from all the samples from the four blades fit the Poisson distribution ($P>0.05$). Most cell frequencies from blades 1 and 4 did not fit the normal distribution ($P<0.05$), while samples from blades 10 and 22 did

Table 9. Coefficient of dispersion and coefficent of variation of bacterial counts in discs of different aged blades treated with and without pyrophosphate (0.01 M) and sonication (100 watts).

Blade no.	Treatment	Range of CD[1]/blade	Range of CV within discs	CV[2] between discs
1	Control	2.0-23.0	78.9-148.7	85.3
	Combination	0.6-1.2	54.2-145.6	41.1
4	Control	0.8-6.6	42.2-65.7	30.1
	Combination	0.9-1.8	74.3-127.5	25.5
10	Control	9.1-40.0	41.1-77.5	54.3[3]
	Combination	1.0-2.0	30.2-57.3	26.5
22	Control	*	*	*[4]
	Combination	0.9-1.6	20.8-26.9	10.0

[1] CD=Variance/Mean.
[2] CV=(SD/Mean)x100.
[3] n=6 and g=30 except in untreated blade 10 where n=4.
[4] Bacteria could not be enumerated on disc samples from blade 22 due to clumping and other interference.

Table 10. Test of homogeneity of variances of bacterial numbers from disc samples of each blade in the untreated and pyrophosphate with sonication combination treatment, before and after transformation.

	Treatment						
	Control			Combination			
Blade No.	1	4	10	1	4	10	22
Transform							
x	<0.001	<0.001	<0.001	<0.001	<0.001	0.007	0.414[1]
$x^{0.5}$	<0.001	<0.001	0.190	0.049	0.656	0.016	0.694
$(x+0.5)^{0.5}$	<0.001	<0.001	0.018	<0.001	0.129	0.059	<0.001
Log(x+1)	0.102	<0.001	<0.001	0.086	0.650	0.006	0.758
$Sinh^{-1}$	0.257	<0.001	<0.001	0.128	0.707	0.001	0.752

[1] Probability, Bartlett's test for homogeneity.

($P>0.05$). The cell frequencies did not fit the uniform distribution ($P<0.05$). Microscopic observation of the combination treated blade discs revealed that the density of bacteria in the young blades was naturally low. There was a reduction in the association of the bacteria with kelp surface components as well as with other epiphytes. The variances were similar only in samples from blade 22 but not for the samples from blade 1, 4 and 10 (Table 10). Transformation enhanced the homogeneity of variances in most of the samples from each individual blade (Table 10).

The range of CV within the combination treated samples was lower than untreated samples in blades 10 and 22. In blade 1, the range of CV was high but similar to the untreated samples, while in blade 4 the CV of the combination treated samples was greater than the untreated samples (Table 9). A possible reason for the higher variation in the younger blades could be due to the low numbers of bacteria present and the high number of zero bactrial counts encountered in these samples. The variation between the samples was lower in the combination treated samples and the CV decreased with the blade age (Table 10). This may be due to the greater density of bacterial cells present in the older blades and therefore a lower chance of zero count per microscopic grid.

The means of bacterial counts per sample on each blade (Fig. 17 and 18) were shown to be significantly different for both the untreated and the combination treatment (K-S

Fig. 17. The number of epiphytic bacteria cm^{-2} ((±SE) on untreated different aged blades of Macrocystis integrifolia. Blade no.: K=1, 4, 10 or 22, n=1-6 and g=30.

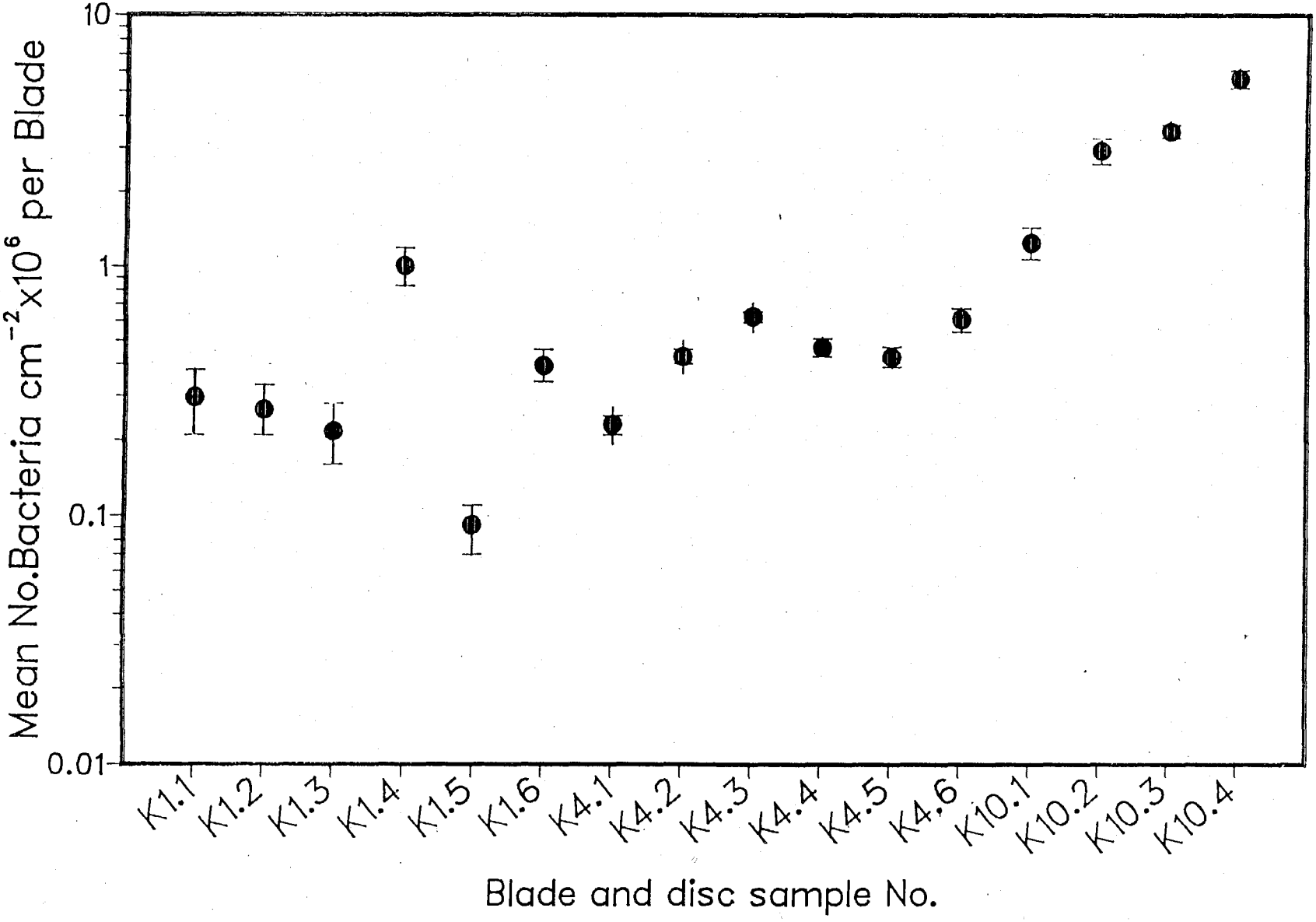

Mean No.Bacteria cm^{-2}x10^{6} per Blade
10
1
0.1
0.01
K1.1
K1.2
K1.3
K1.4
K1.5
K1.6
K4.1
K4.2
K4.3
K4.4
K4.5
K4.6
K10.1
K10.2
K10.3
K10.4
Blade and disc sample No.

Fig. 18. The number of epiphytic bacteria cm^{-2} ($\pm$SE) on different aged blades of *Macrocystis integrifolia* after pyrophosphate and sonication treatment. Blade no., K=1, 4, 10 or 22, n=1-6 and g=30.

Mean No.Bacteria cm^{-2}x10^{6} per Blade
100
10
1
0.1
0.01
K1.1
K1.2
K1.3
K1.4
K1.5
K1.6
K4.1
K4.2
K4.3
K4.4
K4.5
K4.6
K10.1
K10.2
K10.3
K10.4
K10.5
K10.6
K22.1
K22.2
K22.3
K22.4
K22.5
K22.6
Blade and disc sample No.

test,$P<0.001$) with the exception of blade 4 in the combination treatment ($P=0.143$) K-W test. This demonstrates that there was sample variation within each blade.

The densities of the bacteria on blades 1 and 4 for the untreated and combination treatment (Table 11) were lower than those reported by Roland (1980) for the young and mature blades. The bacterial densities for both the untreated and treated samples increased with the age of the blade (see also Roland, 1980). The means of the combination treated samples were greater than those of the untreated samples (Table 11). When the disc samples were treated with the combination treatment there was total disruption of the kelp tissue. This resulted in the release of epiphytic bacteria from both sides of the kelp discs and enumeration of the total number of bacteria (or an aliquot of the sample where the density of the bacterial cells was very high). In the untreated samples only one side of the sample could be enumerated and if the bacterial density was too high, or if there was interference from other epiphytes, then the bacterial cells could not be effectively enumerated at all.

Table 11. Mean number of bacteria of combined bacterial counts from disc samples of *Macrocystis integrifolia* blades before and after being treated with pyrophosphate and sonication.

Treatment	Control	Combination
	Mean no. bacteria ($\pm$SEx)x10^{6} cm^{-2}	
Blade no.		
1	0.38($\pm$0.04 g=180[1]	2.00($\pm$0.14 g=180
4	0.47($\pm$0.02 g=180	1.51($\pm$0.12 g=180
10	3.31($\pm$0.21 g=120	6.35($\pm$0.23 g=180
22	*[2] g=0	37.74($\pm$1.44 g=180

[1] total number of grids enumerated for bacteria from the kelp discs per blade (g=30/disc sample)

[2] samples which could not be enumerated due interference. from very high densities and other epiphytes.

2.5 Dispersal mechanism

The increase in dispersion of bacteria and other components in samples treated with pyrophosphate and sonication was probably due to several factors. These include sequestering of polyvalent cations by pyrophosphate that were present in the suspending medium or exposed on the different surfaces. Marshall (1980a) and Bingle (1980) suggest that sonication disrupts large aggregates. This in turn, exposes more cations for further sequestering by pyrophosphate. Exposed organic and inorganic anionic sites may then be neutralized by excess sodium cations present in the suspending medium. When the suspension is filtered onto Nuclepore membranes the water soluble complex ions formed by pyrophosphate and polyvalent cations remain in the filtrate and since the surface charges have been neutralized, the bacteria may be randomly distributed on the filter. An even distribution of the bacterial cells was observed microscopically.

Treatment of the samples with ultrasound alone had variable results on the dispersion of bacteria. There was a decrease in the size of aggregates of bacteria in sediment and fecal material with only partial removal of epiphytic bacteria from the kelp blades. There was an uneven distribution of bacteria and other components on the Nuclepore filters. Sonication may have only partially disrupted aggregates and bridging polymers.

Since colloidal material was still observed to be surrounding the bacterial cells after sonication, it may be that the presence of polyvalent cations may have contributed to aggregate reassociation. Neutralization of the surface charge by sodium cations may not occur under these conditions since polyvalent cations outcompete the monovalent cations for the anionic sites on the various surfaces. This may be the reason why the variances of bacterial means were greater in various samples as compared to those treated with pyrophosphate followed by sonication.

IV. Conclusions

The fluorochromes DAPI and Hoechst were found to be useful for staining bacteria from the different marine habitats due to their specificity and low background fluoresence. The majority of bacteria in sediment, fecal material and those epiphytic on the blades were found to be irreversibly attached to their substrates. Vortex mixing of sediment and kelp discs from blade 10 suspended in 26 % sodium chloride and chemical reagents did not cause disaggregation or detachment of bacteria under the specified conditions. Sonication of the samples was found to be of limited use. The tolerance of cells of a Gram negative rod shaped marine isolate to sonication was increased by fixation with 3.7 % formaldehyde (v/v) before treatment. The power level of 100 W and treatment time of up to 90 s did not cause a decline in bacterial numbers in formaldehyde fixed epibenthic water sample. In sediment samples a minimum of 15 s sonication treatment at 100 W was required before the the large sediment aggregates broke into smaller aggregates and the bacterial counts began to stablize with prolonged treatment. Sonication treatment of the samples from seawater, sediment, fecal material and older kelp blades was insufficient to cause dispersion of the smaller bacterial and organic matter aggregates and complete removal of attached bacteria.

With the exception of sodium periodate and tetrasodium pyrophosphate, treatment of sediment and kelp discs with various chemical reagents followed by sonication did not appear to cause any greater disaggregation or detachment of bacteria when compared to sonication only. Sodium periodate could not be assessed further since it quenched fluorescence from DAPI stained bacteria. Tetrasodium pyrophosphate treated samples followed by sonication resulted in further dispersion of the small bacterial, sediment, and fecal material aggregates and also disrupted the kelp tissue which resulted in detachment of the epiphytic bacteria. This dispersion was attributed to the initial break down of large aggregates by sonication and the subsequent sequestering of the exposed polyvalent cations by the pyrophosphate.

The method for disaggregating bacteria involves intial fixation of the samples with 3.7 % formaldehyde (v/v), followed by incubation in 0.01 M tetrasodium pyrophosphate suspended in 0.44 M sodium chloride solution (0.001 M pyrophosphate for water samples) for 15 min (30 min for kelp discs) and finally treatment with sonication at 100 W for 30 s (45 s for kelp discs). The samples were then stained with DAPI and prepared as in the standard epifluorescence direct count method (Hobbie et al., 1977; Porter and Feig, 1980).

This method allows for examination and estimation of bacteria in habitats which were previously difficult to examine, e. g., sediment, fecal material and kelp blades. In addition,

there was a reduction in interference from the particulate and colloidal material, and the increased dispersion of bacterial cells resulted in the cells being observed as separate entities. The dispersion of bacteria led to an improvement in accuracy of bacterial estimates in samples such as sediment and kelp blades, where the bacterial numbers were previously underestimated. Statistical analyses demonstrated that the combination treatment of pyrophosphate and sonication on samples from the different habitats, except water, resulted in random to regular spatial distribution of the bacteria. There was a reduction in variance within each sample from different habitats. This included the subsurface and epibenthic water, which had lower organic and colloidal material compared to other habitats, and an initial random spatial distribution before treatment. The improvement in precison of the count was illustrated by the decline in the CV in the combination treated samples compared to untreated samples. The CV decreased by 4 % in the subsurface water, 6 % in the epibenthic water, 49 % in the sediment, 26 % in the fecal material, 12 % in blade 1, and 35 % in blade 10. Since untreated blade 22 could not be enumerated, the change in CV could not be calculated. The time and tedious nature of counting bacteria can now be reduced by enumerating fewer randomly chosen microscopic grids of the sample on the filters since the CV stablized after counting 10 grids. This improvment in accuracy and precision of bacterial counts, and the reduction in time and tedium of counting will allow a greater number of samples to be processed

on a routine basis.

V. Literature Cited

Albert, A. 1966. The Acridines. (2nd ed.). St. Martin's Press, New York. 604 pp.

Alexander, M. 1977. Introduction to soil microbiology (2nd ed.). Wiley and Sons, N.Y. 467 pp.

Babiuk, L.A., and E.A. Paul. 1970. The use of fluorescein isothiocyanate in the determination of the bacterial biomass of a grassland soil. Can. J. Microbiol. 16:57-62.

Banks, W.J. and J.Walker. 1977. Sonication of activated sludge flocs and the recovery of their bacteria on solid media. J. Gen. Microbiol. 98:363-368.

Bingle, W.H., 1980. Methods for seperating microorganisms from soil. M.Sc. Thesis, University of Saskatchewan, Saskatchewan. 90 pp.

Binyon, J. 1972. Physiology of echinoderms. Pergamon Press. Oxford. 264 pp.

Burns, A.R., L. Oliveira and T. Bisalputra. 1982. A histochemical study of bud initiation in the brown alga Sphacelaria furcigera. New Phycol. 92:297-307.

Cahn, A. and J.L. Lynn. 1983. Surfactants and detersive system. In: Kirk-Othmer Encyclopedia of Chemical Technology. (3rd ed.). Vol. 22. Wiley, New York. pp 332-432.

Cassel, E.A. 1965. Rapid graphical method for estimating the precision of direct microscopic counting data. Appl. Microbiol. 13:293-296.

Coakley,W.T., A.J. Baker, and D. Lloyd. 1977. Disruption of micro-organisms. In: A.H. Rose and D.W. Tempest (eds.) Advances in Microbial Physiology. Academic Press, London. pp. 279-340.

Coleman, A.W. 1980. Enhanced detection of bacteria in natural environments by flurochrome staining of DNA. Limnol. Oceanogr. 25:948-951.

Considine, D.M. and G.D. Considine (eds.). 1983. Van Nostrand's Scientific Encyclopedia. 6 th edition. Van Nostrand Reinhold Co. New York. 3067 pp.

Corpe, W.A. 1972. Microfouling : The role of primary film forming marine bacteria. In : Proc. Third. Int. Congr. Marine Corrosion and Fouling. Dept. of Commerce, Gaithersburg, Md. pp. 598-609.

Corpe, W.A. 1974. Detachment of marine periphytic bacteria from surfaces of glass slides. Dev. Ind. Microbiol. 15:281-287.

Costerton, J.W., G.G. Geesey, K.J. Cheng. 1978. How bacteria stick. Sci. Am. 238:86-95.

Cowden. R.R. and S.K. Curtis. 1981. Microflurometric investigations of chromatin structure. Histochemistry 72:11-23.

Cundell, A.M., T.D. Sletter, and R. Mitchell. 1977. Microbial populations associated with the surface of the brown alga Ascophyllum nodosum. Microb. Ecol. 4:81-91.

Dale, N.G. 1974. Bacteria in interdial sediments: factors related to their distribution. Appl. Environ. Micobiol. 41:107-116.

Daley, R.J. 1979. Direct epiflouresecence enumeration of native aquatic bacteria: uses, limitation and comparative accuracy. In: J.W. Costerton and R.R Colwell (eds.) Native Aquatic Bacteria Enumeration, Activity, and Ecology, ASTM STP 695. American Society for Testing and Materials. pp. 29-45.

Daniels, S.L. 1980. Mechanisms involved in sorption of microorganisms to solid surfaces. In: G. Bitton and K.C. Marshall (eds.)Adsorption of microorganisms to surfaces. John Wiley & Sons New York. pp. 7-58.

Dudman, W.F. 1977. The role of surface polysaccharide in natural environments. In : I. Sutherland (ed.). Surface carbohydrates of the prokaryotic cell. Academic Press, Lon. pp. 357-414.

Elliot, J.M. 1977. Statistical analysis of samples of benthic invertebrates. Freshwater Biological Association, Scientific Publication No. 25. (2nd ed.). 156 pp.

El-Shaarawi, A.H., S.R. Esterby, and B.J. Dutkea. 1981. Bacterial density in water determined by Poisson or Negative binomial distributions. Appl. Environ. Microbial. 41:107-116.

Fenchel, T. and B.B. Jorgensen. 1977. Detritus food chains of aquatic ecosystem : The role of bacteria. Adv. Microbiol. Ecol. 1:1-57.

Fletcher, M. 1980. Adherence of marine micro-organisms to smooth surfaces. In: Bacterial adherence. E.H. Beachey (ed.) Chapman & Hall. London. pp. 345-398.

Fliermans, C.B. and E.L. Schimdt. 1975. Fluorescence microscopy: direct detection, enumeration and spatial distribution of bacteria in aquatic systems. Arch. Hydrobiol. 76:33-42.

Franscisco, D.E., R.A. Mah and A.C. Rabin. 1973. Acridine orange epiflourescence technique for counting bacteria in natural waters. Trans. Am. Mirosc. Soc. 92:416-421.

Gayford, C.G. and J.P. Richards, 1970. Isolation and enumeration of aerobic heterotrophic bacteria in activated sludge. J. Appl. Bact. 33:342-350.

Geesey, G.G., W.T.Richardson, H.G. Yeoman, R.T. Irvin and J.W. Costerton. 1977. Microscopic examination of natural sessile bacterial populations from an alpine stream. Can. J. Microbiol. 23:1733-1736.

Hattori, T. 1970. Adhesion between cells of E. coli and clay particles. J. Gen. Appl. Microbiol. 16:351-359.

Hayat, M.A. 1981. Principles and techniques of electron microscopy. Vol. 1. (2nd. ed.). University Park Press, Baltimore. 412 pp.

Hobbie, J.E. R. Daley and S. Jasper. 1977. Use of nucleopore filters for couting bacteria by flourescence microscopy. Appl. Environ. Microbiol. 33:1225-1228.

Hossel, J.C. and J.H.Baker. 1979a. Epiphytic bacteria of the freshwater plant Ranunculus penicillatus : enumeration, distribution and identification. Arch. Hydrobiol. 86:322-327.

Hossel, J.C. and J.H. Baker. 1979b. A note on the enumeration of epiphytic bacteria by microscope methods with particular reference to two freshwater plants. J. Appl. Environ. Microbiol. 46:87-92.

Hull, C.H. and N.H. Nie (eds.). 1981. SPSS update 7-9 : New procedures and facilities for releases 7-9. SPSS Inc. USA. 402 pp.

Kaneko, T and R.R. Colwell. 1975. Adsorption of Vibrio parahaemolyticus onto chitin and copepods. Appl. Microbiol. 29-269-274.

Kirchman, D. and R. Mitchell. 1982. Contribution of total microheterotrophic activity in five ponds and two marshes. Appl. Environ. Microbiol. 43:200-209.

Kirchman, D., J. Sigda, R. Kapuscinski, R. Mitchell. 1982. Statistical analysis of the direct count method for enumerating bacteria. Appl. Environ. Microbiol. 44(2):376-382.

Laycock, R.A. 1974. The detrital food chain based on seaweeds I. Bacteria associated with the surface of Laminaria fronds. Mar. Biol. 25:223-231.

Manahan, S.E. 1978. Environmental Chemistry. (2nd ed.). W.G. Press, Bos. Mass. 532 pp.

Marshall, K.C. 1973. Mechanism of adhesion of marine bacteria to surfaces. In: S.R.F. Acker, B.F. Brown, J.R. DePalma and W.P. Iverson (eds.) Proc. 3rd Intern. Congr. Mar. Corrosion Fouling. Northwestern Unversity Press, Evanston, USA. pp. 625-632.

Marshall, K.C. 1980a. Adsorption of microorganisms to soil and sediments. In: Adsorption of microorganisms to surfaces. G. Bitton and K.C. Marshall (eds.) Wiley. New York pp. 317-330.

Marshall, K. C.1980b. Reactions of microorganisms, ions and macromolecules at interfaces. In: Contemporary microbial ecology. D.C. Ellwood, J.N. Hedger, M.J. Latham, J.M. Lynch and J.H. Slater (eds.). Academic Press. London. pp. 92-106.

Marshall, K.C. and R.H. Cruickshank. 1973. Cell surface hydrophobicity and the orientation of certain bacteria at interfaces. Micbiol. 91:29-40.

Mazure, H.G.F. and J.G. Field. 1980. Density and ecological importance of bacteria on kelp fronds in an upwellilng region. J. Exp. Mar. Biol. Ecol. 43:173-182.

Montagna, P.A. 1982. Sampling design and enumeration statistics for bacteria from marine sediments. Appl. Environ. Microbiol. 43:1366-1372.

Morrison, R.T. and R.N. Boyd. 1974. Organic chemistry. 3rd ed. Allyn and Bacon, USA. 1258 pp.

Nie, N.H., C.H. Hull, J.G. Jenkins, K. Stembrenner and D.H. Bent. 1975. SPSS : Statistical package for the social sciences . 2nd ed. SPSS Inc., USA. 675 pp.

Paul, J.H. 1982. Use of Hoechst dyes 33258 and 33342 for enumeration of attached and planktonic bacteria. Appl. Environ. Microbiol. 43:939-944.

Paul, J.H. and B. Myers. 1982. Fluorometric determination of DNA

in aquatic microorganisms by use of Hoechst 33258. Appl. Environ. Microbiol. 43:1393-1399.

Pike, E.B., E.G. Carrington and P.A. Ashburner. 1972. An evaluation of procedure for enumerating bacteria in activated sludge. J. Appl. Bact. 35:309-321.

Polne, M., A. Gibor and M. Neushul. 1980. The use of ultrasound for removal of macro-algal epiphytes. Botanica Marina 23:731-734.

Porter, K.G. and Y.S. Feig. 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. 25:943-948.

Puelo, J. R., M. S. Favero and N. J. Petersen. 1967. Use of ultrasonic energy in assesing microbial contamination on surfaces. Appl. Microbiol. 15:1345-1351.

Roland, W. 1980. Epiphytism and endophytism of Macrocystis integrifolia and Nereocystis luetkeana: Seasonality, succesion and tactics on temporary living substrate. M.Sc. Thesis, Simon Fraser University. 56 pp.

Rosell, K.G. and L. Srivastava. 1984. Binding of inorganic elements to kelp residues. Proc. Int. Seaweed Symp., 11: (in press).

Santoro, T. and G. Stotzky. 1968. Sorption between microorganisms and clay minerals as determined by electrical sensing zone particle analyzer. Can. J. Microbiol. 14:299.

Scotten, H.L. 1971. Microbiological aspects of the kelp bed environment. In: The biology of giant kelp beds (Macrocystis) in California. W.J. North (ed.). Beileft Nr. 32 Zur. Nova Hedwiga, Heft 32. pp. 313-318.

Sieburth, J.McN. 1979. Sea Microbes. Oxford University Press. New York. 491 pp.

Sokal, R.R. and F.J. Rohlf. 1969. Biometry. W.H. Freeman and Co. San Francisco. 776 pp.

Weise, W., and G. Rheinheimer. 1978. Scanning electron microscopy and epiflourescence investigation of bacterial colonization of marine sand sediments. Microb. Ecol. 4:175-188.

Zvyagintsev, D.G. and G.M. Galkina. 1967. Ultrasonic treatment as a method for preparation of soils for microbiological analysis. Mikrobiologiya 36:1087-1095.