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# THE DISPERSION OF ADHERED MARINE BACTERIA BY PYROPHOSPHATE AND ULTRASOUND PRIOR TO DIRECT COUNTING

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ABSTRACT - A technique has been investigated for dispersing adhered marine bacteria from various surfaces prior to direct counting. The bacterial cells were initially preserved and strengthened by use of 3.7 % formaldehyde (v/v final concentration). Seawater samples were then treated with 0.001 M of tetrasodium pyrophosphate (final concentration), a sequestering and deflocculating agent. Surface sediment and kelp disc samples were treated with 0.01 M tetrasodium pyrophosphate (final concentration) after having been suspended in 0.44 M sodium chloride solution. These samples were incubated for 15-30 min. Subsequently, the samples were treated with ultrasound (100 W) for 30 to 45 s. This technique resulted in an even dispersion of most bacterial cells in the samples. Bacterial cells dispersed in this manner were then stained with 4', 6 - diamino - 2 -phenylindole (DAPI) and enumerated by standard epifluorescent microscopy. Bacterial populations of surface sediment and older kelp blades which were previously difficult or impossible to enumerate were now found to be easily determined. A mechanism is proposed to explain the increase in dispersion of the bacterial cells by this technique.

RÉSUMÉ - Une technique pour disperser les bactéries marines des différentes surfaces avant les numérations directes a été testée. Les cellules bactériennes sont fixées et rendues plus fermes par addition de formaldéhyde à 3.7 % (concentration finale v/v). Les échantillons d'eau de mer sont ensuite traités par une solution de tétrasodium pyrophosphate 0.001 M (concentration finale), utilisée comme agent défloculant et séquestrant. Les échantillons de la surface du sédiment et des grandes algues sont traités par une solution 0.01 M de tétrasodium pyrophosphate (concentration finale) dans une solution de chlorure de sodium 0.44 M. Ils sont alors incubés 15 à 30 mn avant d'être traités aux ultrasons (100 W) pendant 30 à 45 s. Cette technique permet la dispersion de la majorité des bactéries dans l'échantillon. Les cellules bactériennes, dispersées par cette technique, sont ensuite colorées par du 4',6 diamino -2-phénylindole (DAPI) et dénombrées en microscopie à épifluorescence. Les populations bactériennes du sédiment de surface et des lames âgées d'algues, qu'il était jusque-là difficile ou même impossible de dénombrer, sont maintenant aisément quantifiées. Un mécanisme est proposé pour expliquer l'augmentation de la dispersion des cellules par cette méthode.

## 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 large proportion of bacterial population within the aquatic environment is adhered ; most of these cells are attached to sediment particles, plants, animals and detritus.

The direct microscopic count method (Porter and Feig, 1980 ; Francisco *et al.*, 1973 ; Hobbie *et al.*, 1977 ; Daley, 1979) is a very useful way of determining bacterial numbers within an aquatic sample. 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) fluochrome, e.g. acridine orange,

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 many 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, 1979), thus requiring special statistical treatments or homogenization (Daley, 1979).

The adherence of marine bacteria depends on the bacterial surfaces, the substrata, the media separating the surfaces and environmental factors (Fletcher, 1980). Bacterial adhesion is believed due, in part, to the extra-cellular polymers made up of acidic polysaccharide (Marshall, 1973 ; Fletcher and Floodgate, 1973 ; Costerton *et al.*, 1978) and/or protein (Danielsson *et al.*, 1977). The various factors effecting temporary and permanent adhesion of bacteria to smooth surfaces in laboratory studies has been discussed by Fletcher (1980).

Several investigators have attempted to disperse bacteria prior to counting by using blender homogenization of water, sediment and plants (Daley, 1979 ; Montagna, 1982 ; Scotten, 1971 ; Laycock, 1974 ; Mazure and Field, 1980) ; by sonication of sludge, soil, sand grains and epiphytic contaminants (Pike *et al.*, 1972 ; Banks and Walker, 1977 ; Zvyagintsev and Galkina, 1967 ; Bingle, 1980 ; Weise and Rhineheimer, 1978, Polne *et al.*, 1980) ; and by a combination of chemical treatments and ultrasound in sludge and soil (Gayford and Richards, 1970 ; Pike *et al.*, 1972 ; Banks and Walker, 1977 ; Bingle, 1980). These attempts met with varying success. In addition, prolonged homogenization and sonication lyse bacterial cells (Pike *et al.*, 1972 ; Coakley *et al.*, 1977 ; Paul and Myers, 1982) and therefore susceptibility of the cells is an important consideration.

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.

We report upon the use of sodium pyrophosphate and ultrasound for dispersing marine bacteria from surfaces to which they are attached into a suspension medium prior to epifluorescence microscopic counting. This technique was used to examine marine subsurface and epibenthic waters, and sediment. Different aged blades of the lesser giant kelp, *Macrocystis integrifolia* which had densities of bacteria, ranging from low on the young blades to very high on the other blades, were also examined.

## MATERIALS AND METHODS

### *Sample Collection*

Water, sediment and *Macrocystis integrifolia* samples were collected by SCUBA in January, 1983 from a kelp bed in Bamfield Inlet, Bamfield, Vancouver, Island, British Columbia. The site was approximatively 10 m from shore and 4.5 m in depth.

Subsurface (0.1 m depth) and epibenthic (4m) water samples were collected with sterile 1 L polypropylene bottles. Water salinities were 22‰ and 26‰, respectively. A surface sediment sample (4.5 m depth) was taken with a hand held sterile 50 ml syringe corer, the end of which was immediately capped.

A frond of *M. integrifolia* 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. All samples were preserved with 3.7 % formaldehyde (v/v). The final pH of the sample after fixation was ca.7.8.

### *Bacterial Enumeration*

The direct count method (Hobbie *et al.*, 1977), as modified for 4', 6 - diamino -2-phenylindole (DAPI) by Porter and Feig (1980), was used for staining the bacteria, except that the final DAPI concentration used was 1.0 $\mu$g/ml. After the specified treatment, the samples were incubated with DAPI for 10 min and mixed vigorously for 15 s before being filtered on to 0.2 $\mu$m pore size, 25 mm diameter, pre-dyed (Irgalan Black) Nuclepore filters. An epifluorescence light microscope was used to examine and enumerate the bacterial cells.

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 $\mu$l of the sediment sample was suspended in 5 ml of filter sterilized 26‰ sodium chloride solution (w/v) (ASW). 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 I.D. cork borer. The discs were stained with DAPI, rinsed with ASW 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 resulted in a dispersed mixture of microorganisms (including bacteria), kelp cells and mucillage. This mixture was stained and filtered in a similar manner as the sediment 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.

### *Effect of Ultrasound and Chemical Reagents*

A biosonik II generator with a high power level ultrasonic probe (Model BP-II) and a standard 4 mm diameter titanium tip (Bronwill Scientific, N.Y.) was used for the ultrasound experiments. The volumes of all samples or samples plus suspending media were 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 O°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. Two 500 ml cultures of the isolate were grown in 2 l Erylenmeyer flasks at 15°C using a reciproacting incubator with a shake rate of 50 strokes/mn.

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 mn. 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 $\mu$l subsamples from each of the two samples for each of the sonication periods.

The tolerance of bacterial cells in water and sediment samples, with and without 3.7 % formaldehyde was investigated. For each treament, two-5 ml epibenthic water samples and two-100 $\mu$l 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) and enumerated.

Several chemical reagents (viz, hydrochloric acid, sodium carbonate, sodium hydroxide, sodium periodate and tetrasodium pyrophosphate at concentrations ranging from 0.0001 M to 0.1 M dissolved in ASW) were tested for dispersal properties upon addition to sediment and kelp discs from blade 10. Each sample was incubated for 15 min after chemical addition and then treated with ultrasound. The samples were then examined under epifluorescence microscopy and qualitatively assessed for gross 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 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 $\mu$l sediment samples were each treated with 0.01 M pyrophosphate and sonicated at 75 to 125 W power level for up to 60 s. The samples were then examined for changes in bacterial numbers.

## RESULTS AND DISCUSSION

### *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 the 125 W power level, the bacterial count declined by 46 % and after 90 s treatment, only approximatively 1 % of the original number remained (Fig.1). Light absorbance measurement of the unfixed samples after treatment with sonication showed a similar trend (Fig.1).

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 in increase in tolerance of bacterial cells to sonication. After 5 s sonication at 125 W, there was a small increase in bacterial number (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.1). 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 treatment (Fig.1) confirming the increased tolerance of formaldehyde fixed cells to sonication.

Aldehyde fixatives form inter- and intra-molecular crosslinks with protein (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). Adherence of non-viable cells has been shown to decrease with increases in formaldehyde concentration and/or temperature (Stanley, 1983). Accordingly all water, sediment, and kelp blade samples were fixed with formaldehyde (3.7 %) for at least 30 min prior to sonication.

Microscopic observations of the marine surface 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. A small number of bacteria were single and unattached.

Discs from middle aged blade 10 of *M. integrifolia* had relatively more epiphytes than the young blades 1 and 4 but less than the old blade 22. 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. It was impossible to assess the tolerance of bacteria in 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 samples. 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. Treatment of fixed epibenthic water samples with sonication, at 100 W power level for up to 90 S, did not cause a decline in bacterial counts when compared to untreated samples (0 s, Fig. 2). However, sonication at 125 W power level for longer than 15 s caused a decline in bacterial numbers (Fig. 2).

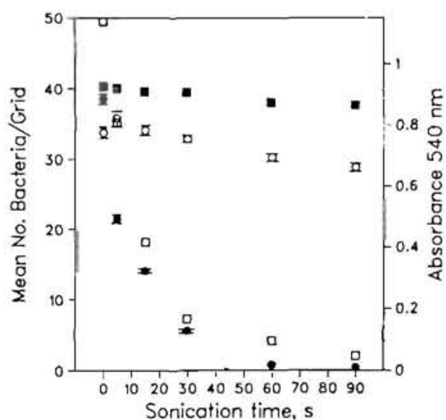


Figure 1 : Tolerance of Gram negative bacterial isolate cells to sonication at 125 W before and after fixation with formaldehyde (3.7 % for 30 mn). Bacterial means (± s.e.) and absorbance at 540 nm for m = 2 and number of microscopic grids (g) = 20 (s.e. smaller than the symbols are not represented). Symbols : unfixed cells sonicated and absorbance assayed (●), fixed cells sonicated and absorbance assayed (O), unfixed cells sonicated and cell numbers determined (□), fixed cells sonicated and numbers determined (■).

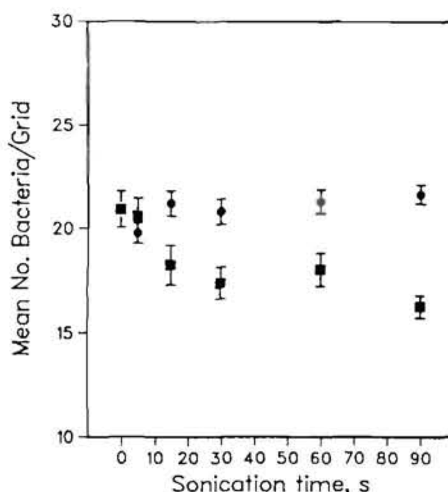


Figure 2 : The effect of power level (100 W, (●) and 125 W, (■), and duration of sonication on mean bacterial numbers (± s.e.) per microscopic grid (m = 2, g = 20) of an epibenthic water sample previously treated with 3.7% formaldehyde (final concentration).

Sediment particles 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. 3). 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. 3). 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. Sediment samples have been previously shown to require extensive treatment before interference from detrital particles and background fluorescence was eliminated (Montagna, 1982). Bacterial lysis may occur upon prolonged homogenization (Montagna, 1982).

It is likely that sonication severs some of the bridging polymers, causing large aggregate disruption (Marshall, 1980 ; Bingle, 1980). The bacteria and sediment material also accumulated at the edges of the filter adjacent to the glass barrel rather than spreading evenly over the entire filter. This may be due to the negative charge on the surface of bacterial cells and sediment particles at pH 7.9 resulting in attraction to the glass surface of the filter assembly. This may be mediated by cationic bridging mechanisms (Costerton *et al.*, 1978). Adherence of bacteria to glass can be reduced by coating the surface with dimethyldichlorosilane (Garvey *et al.*, 1977).

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 caused precipitation of cations from the sediment which interfered with the observation of bacteria.

Chemical reagents have not been widely used to disperse bacteria from aquatic samples. Various studies have demonstrated that bacterial isolates are repelled from surfaces such as soil particles, chitin and plankton, by high pH levels (Santoro and Stotsky, 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 rather than di- and tri-valent cations are used (Peele, 1936 cited in Marshall, 1980, Santoro and Stotsky, 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, a sequestering agent, followed by sodium periodate (Marshall, 1973). Fletcher (1980) examined the use of sodium periodate, sodium borate, ethylenediamine tetraacetic acid (EDTA), tetrasodium pyrophosphate, antibodies and bovine serum albumin in influencing the attachment of a *Pseudomonas* sp. to glass. Pyrophosphate was found to be the most effective in preventing attachment and removal of adhered bacterial cells. However, the results from these in vitro experiments may not apply to the natural environment. 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 the marine environment. Attachment of pure bacterial isolates to glass surfaces in laboratory experiments has been examined over relatively shorter time periods, usually less than 24 h (Marshall and Cruikshank, 1973 ; Corpe, 1974 ; Fletcher, 1980). One of the effects of the longer time period in the natural environment may be the production of a greater quantity and/or different quality of bridging polymers by the microorganisms and macrophytes than that which will occur in 24 h on glass surfaces.

### *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, showed a greater disaggregation of sediment associated bacteria and detachment of epiphytic bacteria than those samples treated with chemicals only. A change of pH or oxidation of polysaccharides by these reagents was insufficient to release the bacterial cells.

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 of the filters and a reduction in colloïdal 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. The use of pyrophosphate (or sodium tripolyphosphate) followed by sonication has proven to be more successful than the use of sonication alone, for dispersing bacterial flocs in activated sludge (Pike *et al.*, 1972 ; Gayford and Richards, 1970 ; Banks and Walker, 1977).

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. The formaldehyde fixed 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. 4).

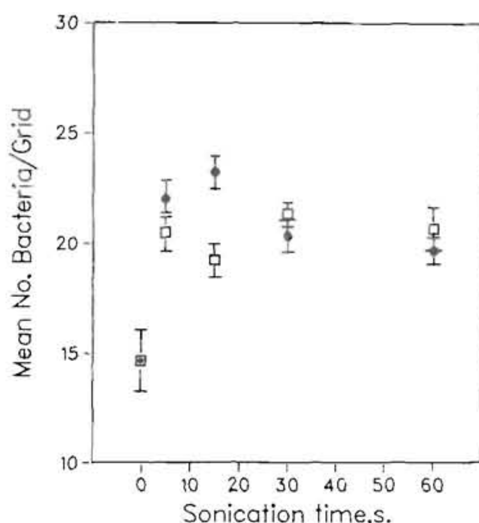


Figure 3 : The effect of power level (100 W, (□) and 125 W (●)) and duration of sonication on mean bacterial numbers (± s.e.) per microscopic grid (n = 2, g = 20) from sediment samples.

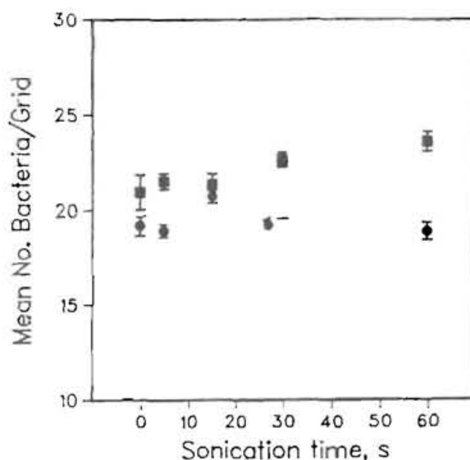


Figure 4 : The effect of duration of sonication (100 W) on mean bacterial counts (± s.e.) per grid of subsurface (●) and epibenthic (□) water samples containing 0.001 M tetrasodium pyrophosphate (m = 2, g = 20).

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. 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. 5). Maximum numbers were observed between 15 and 60 s of treatment at 100 W. Five s of treatment at 125 W power level caused the numbers of observed bacteria to decline.

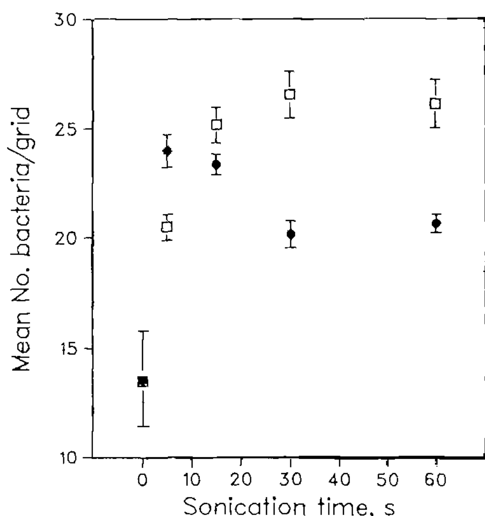


Figure 5 : The effect of power level (100 W, (□) and 125 W, (●)) and duration of sonication on pyrophosphate (0.01 M) treated sediment bacterial count means (± s.e.) per grid (n = 2, g = 20).

A sequestring (chelating) agent has more than one atom which may be bonded to a central metal ion at one time for form a ring structure (Manahan, 1975) and can therefore sequester ions such as 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 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. intergrifolia* 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 polysaccarides 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. Other investigators have alluded to a similar mechanism with use of sequestering agents such as

pyrophosphate, EDTA (Fletcher, 1980) and ethylene glycol-bis (B-aminoethyl ether)-N, N-tetraacetic acid (EGTA) (Turakhia et*al*., 1983) used in removal of adhered bacterial cells and biofilms. The use of sonication after treatment with pyrophosphate may simply have quickened the dispersal process by exposing 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. In addition, sonication may sever attached polymers. When the suspension is filtered, the water soluble complex ions formed by pyrophosphate and polyvalent cations remain in the filtrate. Since the surface charges have been neutralized, the bacteria and the organic and inorganic particulate matter will be evenly distributed on the filter.

The results of these experiments indicate that most bacteria in seawater, surface sediment and kelp blade samples can be dispersed by the following technique:

1. Preserve and fix the bacterial cells in seawater with formaldehyde (2-4 %, final concentration). Samples such as sediment and kelp blades should be suspended in filter sterilized seawater containing 2-4 % formaldehyde.

2. Add tetrasodium pyrophosphate (0.001 M, final concentration) to the seawater samples. Sediment and kelp samples are treated with 0.01 pyrophosphate. Incubate for 15-30 min.

3. Cool all samples to O°C. Sonicate for up to 90 s at 100 W, using a ca. 4 mm diameter probe tip immersed until 1 cm from bottom of tube.

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