INVESTIGATION OF THE RELATIONSHIP BETWEEN MARINE BACTERIA AND *PSEUDO-NITZSCHIA AUSTRALIS* (BACILLARIOPHYCEAE)

by

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Title: INVESTIGATION OF THE RELATIONSHIP BETWEEN MARINE BACTERIA AND PSEUDO-NITZSCHIA AUSTRALIS (BACILLARIOPHYCEAE)

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Lynda P. Shapiro, Ph.D

This thesis examines the relationship observed between marine bacteria and the diatom Pseudo-nitzschia australis. Bacteria counts were transformed to cells ml^{-1} in order to compare concentrations around the diatom cell (epialgal) with those in the media. A volume was calculated for P. australis and an assumed phycosphere using geometric approximations.

The results showed a distinct population of epialgal bacteria that interact throughout growth phase of the diatom cell and at higher abundances than that seen free in the media. Therefore, a population of bacteria exists epiphytically on P. australis during exponential phase and

does not seem to be supported by bacteria populations in the media. P. australis is a known domoic acid producer. It is not known whether this interaction with marine bacteria may have some role in growth mechanisms as well as toxin production by the diatom.

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CHAPTER I

INTRODUCTION

In this thesis I examine the relationship between marine bacteria and the diatom Pseudo-nitzschia australis. Free-living marine bacteria populations commonly increase at the end of algal blooms, utilizing decaying algae. Whereas, epialgal populations are present throughout algal growth and exist in a mutualistic association with algal assemblages. Epialgal bacteria may have quite different characteristics than those of free-bacteria because of their close association with phytoplankton. Algae-bacteria associations may also have an important impact on the type and amount of organic material that is channeled into the marine food web. In the following section, I will explain roles phytoplankton and bacteria play in associations, as well as discuss some specific algal-bacteria relationships that have been studied in depth.

Rhizosphere Comparison

Some understanding of the relationship between

phytoplankton and bacteria might be deduced by analogy from the better-studied association between bacteria and the roots of higher plants. The rhizosphere is the contact zone of roots and soil in which the microbial population is influenced physiologically by the plant. Production of root exudates by plants enriches the zone around the roots and helps to cultivate microorganisms in this zone (Werner 1992). The effect of bacterial stimulation is usually expressed as an R(root microorganisms): S(soil microorganisms) ratio. The R:S value is a ratio of abundance of micro-organisms in the soil adhering or in physical contact with the roots versus the density found in adjacent bulk soil. Typical R:S ratios range from 2 to 100 (Fitter et al. 1987).

Many of the bacteria stimulated in the rhizosphere are ammonifying and denitrifying bacteria. Both symbiotic and free-living bacterial genera can fix nitrogen in the rhizosphere utilizing energy derived from excreted plant photosynthate products (Fitter et al. 1987). A symbiotic association develops in Leguminosae in which the nitrogen fixing bacteria Rhizobium invade the plant and form nodules. Nodules developing in the host plant's root system are supplied directly by the plant vascular system. In nitrogen-limited soils nodules allow plants to utilize nitrogen fixed by bacteria and the plant in turn provide

carbon for the bacteria (Fitter et al. 1987).

Bacteria within the rhizosphere can often have important effects on factors governing plant growth, nutrient availability, respiration and root morphology. The phosphate content of barley shoots are increased under phosphate-limiting conditions in the presence of bacteria in the rhizosphere (Werner 1992). Bacteria growth in the rhizosphere can result in increased respiration rates in regions near roots (Bannister 1976). Silver beet plants inoculated with the bacteria Azospirillium brasilense increased the number of root hairs per millimeter of root by 25%. Azospirillium excretes a phytohormone in the rhizosphere which positively influences root growth (Werner 1992).

Plants can provide various materials as energy sources for microorganisms in the rhizosphere: sloughing of root tap cells, production of mucigel for passage of roots through soil, exudation by intact cells, and death of root cells. Plant exudation products include sugars, amino acids, carbohydrates, as well as some inorganic ions. Rhizosphere products often support populations of root-inhabiting, symbiotic (nitrogen fixing) as well as amino-acid requiring bacteria (Fitter et al. 1987). At 2mm from the root surface, concentrations of sugar and amino acids are too low to be effective in chemotactic response by

microorganisms. Chemotaxis responses only seem to be most important in the center of the rhizosphere, within 2mm from the root surface (Werner 1992).

Phycosphere

The phycosphere, the pelagic analog to the rhizosphere, is not as well studied. Bell et al. (1972) first defined the phycosphere as a zone extending outward from an algal cell or colony for an undefined distance in which bacterial growth is stimulated by the extracellular products released from the algae. The theory for development of a phycosphere is dependent upon two criteria; (1). there must be a source of enrichment for the microbial population in proximity to the algae, (2). the microflora must respond to the algal products by being attracted and/or growing in this region. The phycosphere then is a volume of water around photosynthetically active algae which is physiologically changed by the algae (Werner 1992). In this zone the associated algae and bacteria could have great influence over one another's existence.

Bacteria in association with algae (phycosphere) are believed to exhibit roles similar to those bacteria play in the rhizosphere, e.g., heterotrophic re-mineralization of limiting nutrients, providing useable nitrogen products for

algal assimilation, vitamin production, and inorganic ion mobilization. The physical dimensions of the phycosphere are dynamic depending upon concentration and rate of extracellular products, physiological and nutritional state of the algae as well as the movement of water. From literature values of excretion rates and primary productivity, Mitchell et al. (1985) calculated the radius of a microzone (phycosphere) surrounding phytoplankton to be approximately 1mm. The radius of the microzone was defined as the distance at which the nutrient gradient emanating from the cell is enhanced 10% above the background concentration. Bacteria colonization within the phycosphere does not appear to be random but occurs around areas of exudate release such as that seen in roots of the rhizosphere. Rosowski (1992) found that bacteria attached to specific sites near slits and pores on the mantle of the diatom Navicula confervacea. This supports the hypothesis that openings in these walls might be areas of extracellular material release.

There often exists a broad positive correlation between primary production of phytoplankton and bacterial abundances. Peaks in microbial activity or biomass tend to occur during the death phase of an algal bloom, furthering the theory that bacteria may respond to senescent algae or detritus rather than living algae (Cole 1982). Opinions in

the literature vary widely as to whether fixed carbon from photosynthesis (primary production) supports bacterial production. A population dominated by Asterionella sp. shuttled 16% of carbon it fixed into bacterial production. Data from an estuary in Australia exhibited a tight coupling of algal excretion rates 0.1 - .13 mg $Cm^{-3}h^{-1}$ to microheterotroph assimilation 0.1 - .12 mg $Cm^{-3}h^{-1}$, and respiration of algal products by heterotrophs .001 - .003mg $Cm^{-3}h^{-1}$ hypothesizing little accumulation of exudate products in open water (Jones et al. 1986). Rate of release will therefore be dependent on algal population density, as well as growth rate of phytoplankton.

There also exists a positive correlation between extracellular release and bacterial abundances. Heterotrophic bacteria may represent a sink for metabolites-organics that will tend to increase the rate of phytoplankton extracellular release (Bell et al. 1972). Phytoplankton have nutrient transport systems across membranes which function in transport of sugars, amino acids, and organic acids. Because of their affinity characteristics these transport proteins are hypothesized to transfer metabolites from intracellular pools to the outside by facilitated diffusion or active transport (Jones et al. 1986). A facilitated diffusion of organics would require that the extracellular pool of nutrients would be in

equilibrium with an internal pool of metabolites. The presence of heterotrophic bacteria would tend to increase the percentage of exudate released by the algae. Tight coupling of production and uptake of metabolizable organics would prevent accumulation. Therefore measurements made on the dissolved organic carbon in water reflect both bacterial utilization rates as well as rates of release in algae.

Role of Bacteria

The following are examples of cyanobacterium-bacteria and cyanobacteria-diatom associations; discussion of the mutual benefits lends credence to our discussion of the types of roles bacteria and algae can play in association. Trichodesmium is a non-heterocystous marine cyanobacterium that forms large colonies of filaments and is a major source of nitrogen fixation in tropical and sub-tropical waters. Tufts or aggregates are often found colonized extensively by diverse bacteria during bloom conditions. Analysis of zones within aggregates demonstrated enhanced cellular respiration or localized oxygen depletion 3-5 times as high as those cells located exteriorly. This demonstrates a mutualistic association between bacteria and a cyanobacterium in which bacteria actively reduce oxygen, facilitating nitrogen fixation by the cyanobacterium (Paerl et al. 1989).

The cyanobacterium Richelia intracellularis exists as an endophyte within the diatom Rhizosolenia sp. and is often found in oligotrophic waters (Weare 1974). R. intracellularis may function in concentrating nitrate, phosphate or other inorganic nutrients which may be limiting. 91-100% of three Hemiaulus sp. in the southwest North Atlantic Ocean contained cyanobacterial symbionts morphologically similar to R. intracellularis found in Rhizosolenia sp. The cyanobacteria seem to function in Hemiaulus as a nitrogen source similar to the association in Rhizosolenia. The relationship appears to be non-obligative, but diatoms without symbionts may be out-competed in nutrient-limiting conditions.

B_{12} is the most common vitamin requirement among marine phytoplankton. A number of marine bacteria test positive for Vitamin B_{12} production. In two-thirds of bacterial isolates that tested positive for B_{12} production the vitamin was detectable in culture medium (Swift 1980). Haines (1974) found that several marine diatoms that require Vitamin B_{12} can acquire it in culture from B_{12} producing bacteria. A "B_{12} cycle" might exist in which a B_{12} requirer and B_{12} producer would associate in order to utilize each others metabolic excretions.

Bacteria may also function in algal associations by reworking organic material throughout blooms, therefore

reducing the amount of material sinking out. Putt et al. (1994) describes a situation in McMurdo Sound, Antarctica in which Phaeocystis-bacteria interactions during a bloom are prominent enough to enhance remineralization or cycling of minerals within the mixed layer, further facilitating entry of these organics into the microbial food web. Epialgal bacteria concentrations at midbloom were often 8 times greater than abundances of free-bacteria. Microbial activity appeared to be high throughout all phases of the bloom, including the exponential growth phase of the algae. Phaeocystis-bacteria interactions in this case may have a direct influence on the amounts of organic material that sinks out to the benthos.

Role of Phytoplankton

Phytoplankton may select their own populations of epialgal bacteria by controlling exudate materials. Substances released by phytoplankton can include lipids, peptides, organic phosphates, volatile substances, sex factors, growth inhibitors, vitamins, toxins, metabolic regulators such as cyclic adenosine 3′,5′-monophosphate (cAMP) that can serve as cues to the nutrient status of the phycosphere as well as organic carbon in the form of carbohydrate (Jones et al., 1986). Certain populations of

chemotactic bacteria seem to prefer algal exudates when given a choice of organic substrates. Bacteria isolates across taxa responded better to 30 day old culture filtrates versus a 0.5% peptone enriched media (Bell et al,1972). Bacteria also had an increased chemotactic response to old culture filtrates versus filtrates of young cultures. It would be plausible to hypothesize that certain strains of bacteria are preferentially chosen for epialgal association. Wang et al. (1994) found that bacterial species in a freshwater lake that lived in close association with cyanobacteria had an increased response to cyanobacterial exudates than free-living bacteria. Heterotrophic bacteria responses to phytoplankton assemblages can be very specific.

Phytoplankton may control succession of bacteria populations by providing two types of organics products; exudate and algal decay. Studies suggest that two different bacterial flora exist in relation with phytoplankton; 1. living on products from healthy cells and 2. a population utilizing products of algal decay. Smaller amounts of low-molecular weight compounds are released during exponential growth versus more of high-molecular weight substances after stationary growth (Jones et al. 1986). Vaque et al. (1990) found that high within-species variability of epialgal bacteria may indicate that physiological state of the algal

population may be the most important factor governing diatom-bacteria interactions and the colonization process.

Phytoplankton may also offer a microhabitat or niche that provides escape from environmental stress and where excess nutrients may collect. Sand grains and large detrital particles often act as nutritional foundations for bacteria populations (Hoppe, 1984). In some cases an epialgal association may even offer escape from predation by those ciliates, or other protists which can graze quite effectively on free-bacteria populations.

Associations between phytoplankton and bacteria may so dominate their existence that composition of species may be determined by that interaction. Oscillatoria sp. growth was enhanced when grown with its satellite bacteria (Pseudomonas, Xanthomonas, Flavobacterium). This bacterial consortia, however, had differential effects on other groups of algae, Chlorella, Chlamydomonas, Anabaena, and Euglena. The deterrence suggests that interactions between bacteria and phytoplankton may be species-specific. Satellite bacteria found with certain species of algae may interact strongly enough to regulate interactions between groups of algae and determine species composition (Delucca et al. 1977).

Bacteria-algal associations may benefit both parties in their ability to exist in oligotrophic areas where nutrients

(nitrogen) are limiting to growth and where neither organism could exist alone. A tight coupling seems to exist between bacteria and Trichodesmium in which remineralization, growth and exchange of compounds may occur. The obvious advantage to both parties is that in providing for each other organic compounds and enhanced conditions for nitrogen fixation this consortium can exist in oligotrophic conditions where either might not live otherwise (Paerl et al. 1989).
Cyanobacterial symbionts in several species of diatoms seem to occur in oligotrophic waters where nutrients are limiting. Symbionts probably provide fixed nitrogen and concentrate other nutrients that might limit diatom growth. Diatom populations containing symbionts would then be favored in nutrient depleted conditions allowing both to co-exist.

Physical Interactions

Physical conditions around the phycosphere seem to dictate bacteria-algal associations much more strongly than those of the rhizosphere. Clustering of epialgal bacteria maybe possible only at the thermocline where algal sinking speeds are slower due to lower turbulence, and where sheets of water may form. Average sinking rates of pelagic diatoms are approximately 10μm sec^{-1}. Bacterial net chemotaxis

behavior is around 3μm sec^{-1}. Thus conditions in the mixed layer would not allow bacteria utilizing chemotaxis to catch sinking phytoplankton. Nutrient enrichment extends about 1mm from phytoplankton and zones are less than 10 times concentrated above the background. Microzones could be established in the thermocline where less mixing would allow nutrient gradients to form and bacteria could utilize chemotaxis to stay within this nutrient zone (Mitchell et al. 1985). Colonization by bacteria also may depend upon the densities of both algae and bacteria in the oceanic environment. Colonization of algae would then depend upon the rate of encounter with bacteria. If colonization is dependent upon encounter then bacteria maximize survivability by maintaining dense populations in dormant stages which can react quickly to changes in nutrient availability.

Movement of water at the surface of algal cells should have an effect on colonization by bacteria. The velocity of a fluid is zero at the interface between a stationary solid and a moving fluid. A speed gradient zone, called the boundary layer, develops from the surface of a solid to the free-stream velocity some distance away. Flow in the boundary layer does not follow the speed of the bulk fluid but is relatively stationary. Eddy diffusion would work to maintain bacteria and exudate nutrients within this boundary

layer. An accumulation of nutrients could then occur within this layer and would cause a phycosphere effect. The motion of the bulk fluid would place microbial cells near the substratum and because the cells are travelling faster than the surrounding fluid a force acts upon the cell towards the surface (Hoppe, 1984). Bacterial cells would maintain themselves in the phycosphere by becoming attached or via behavioral chemotaxis. Physical movement of water around an algal cell as described would function in creating a boundary layer and because of the physical dynamics would continually move bacteria in and near the algal surface.

Bacteria can attach to solid surfaces in three consecutive ways: reversible, irreversible (eg. adhering fibers), and biological (eg. clustering or division) (Kogure et al. 1982). Low adhesion is typical in early log phase growth; highest adhesion is seen during stationary growth phase. Rosowski (1992) described bacterial strands, termed locomotory trail fibers, singly and posteriorly attached to bacteria, on diatom valve surfaces. Locomotory and anchoring bacterial fibers were found over the full extent of diatom surfaces attesting to wide microbial activity. Apical
attachment was the most common orientation observed. All attachment did not involve anchoring fibers. There are no known examples of bacteria attached directly to diatom

mucilage strands, although locomotory fibers have been found in these areas. Bacteria appear to be able to interact in a loose-motile association or a tight-fitting immobile association with diatoms.

Paradox

Phytoplankton and bacterial interactions have been described as paradoxical. Extracellular release can account for 1-20% of photoassimilated carbon. This release stimulates the microbial community, which in turn can out-compete phytoplankton for limiting nutrients. Bacteria are more efficient competitors for mineral nutrients than algae because of their larger surface to volume ratio. Bratbak et al. (1985) found that in a phosphorous limited chemostat culture, bacteria out-competed algae. A fine line may exist between commensalism and competition in the relationships between algae and bacteria. Competition would result in bacteria dominance, while commensalism may allow both populations to co-exist.

It is believed that marine bacterial growth is limited by organic carbon while phytoplankton growth is limited by levels of inorganic phosphate and nitrogen sources. Products released by phytoplankton can provide organic carbon and stimulate bacteria growth. Wang et al. (1994)

found that phytoplankton products control bacterial growth only when phosphorous and nitrogen sources were not limiting to growth. A commensalistic association could only develop when phosphate and nitrogen sources were not limiting to growth. A competitive situation may develop when phytoplankton exudates indirectly stimulate bacterial growth, thus leading to P and N uptake by bacteria.

Evolutionary Advantages

The release of extracellular materials by phytoplankton would not appear adaptive in evolutionary terms. Wood et al. (1990) hypothesized that algae benefit from excretion when high light conditions are coupled with low nutrient availability. Excretion would protect the photosynthetic apparatus as well as prepare phytoplankton for rapid uptake when nutrient conditions become beneficial. The bacterial community living in close association with the phytoplankton would benefit from utilizing excreted organic compounds. Wood et al. (1990) explained this interaction as a type of patch selection in which interspecific assemblages are chosen as a group. This type of interaction would favor a mixotrophic lifestyle. Patch selection could also be argued for nonmixotrophic phytoplankton such as diatoms. A community of diatoms and associated/epialgal bacteria could

be favored and selected for because of the benefits they afford to one another. By providing exudate to epialgal bacteria in low nutrient conditions phytoplankton may be stimulating vitamin production, or nitrogen fixation by bacteria and are able to utilize bacteria as a nutrient mobilizer to enhance its own growth.

Specific Examples

Observations on diatom populations found that colonization by bacteria can vary widely across a bloom. Vaque et al. (1990) looked at the importance of between-species and within-species variability of two diatoms Leptocylindrus danicus and Skeletonema costatum upon colonization by bacteria. Within-species variability contributed (80%) much more to epialgal bacteria than that explained by between-species variability (11%). The time course of colonization was characterized by 1. slow (lag) algal growth allowing rapid colonization by bacteria (average 3 bacteria per cell), 2. exponential algal growth consisting of a majority of bacteria-free algae, followed by 3. algal bloom decline with heavily colonized bacteria dominating thereafter. High within-species variability of epialgal bacteria indicates that physiological state of the algal population may be the most important factor governing

diatom-bacteria interactions and the colonization process.

Kogure et al. (1982) found that the numerical abundance of epibacterial populations living on the diatom Skeletonema costatum do not numerically reflect the same abundances in free-media. Kogure et al. followed bacterial flora populations attached to algae as well as free bacteria in the media to see what proportion of the population each maintained over time. There were large differences in the make-up of flora populations living epiphytically on the diatom versus those in the media. Acinetobacter sp., for example, was rarely found attached to algae but often dominated the media flora. Initial attachment of bacteria to algae was not dependent on the abundance in the media. With addition of glucose to the media free-living bacterial concentrations increased but did not effect bacterial attachment. Success of bacterial attachment was dependent upon algal growth phase or physiological state but seemed independent of surrounding microbiological state. A succession of attached bacterial flora also seemed to occur over time and was often not reflective of bacteria in the media.

In summary I began my discussion of bacteria-phytoplankton associations by comparing the well-studied rhizosphere associations. Many of the same analogous functions are provided by bacteria in the phycosphere, the

pelagic analogy to the rhizosphere, such as: fixing nitrogen, concentrating inorganic ions, vitamin production, and re-cycling of limiting nutrients. In return phytoplankton can provide a percentage of fixed carbon as well as a microhabitat for bacteria. This type of community may be selected evolutionarily in cases where both algal cell and bacterium are in turn enhancing each others growth. Finally, I have tried to discuss a couple of diatom-bacteria association examples in order to facilitate discussion of my own results.

What seems to be unknown about algal-bacterial associations is whether or not epialgal bacteria are present throughout growth of the algae. What determines epialgal abundances, and why can it be so variable within a population of algae? Little is known about epialgal bacteria populations as well. Is living epiphytically on algal cells a transient lifestyle capable by all bacteria or is this type of association very specific and singular?

The purposes of my thesis therefore are twofold:

1. Is there an significant association between marine bacterium and the diatom Pseudo-nitzschia australis?
2. What are the growth dynamics of free bacteria, epialgal bacteria and P. australis, and how do they relate to one another?

CHAPTER II

MATERIALS AND METHODS

Field Collection

Pseudo-nitzschia clones were isolated in waters from September 1991 through March 1992. Plankton tows were taken off of the Oregon Institute of Marine Biology (OIMB) dock at high tide. A 63μm mesh size net was used for isolation of diatoms. Water was also collected by boat at the mouth and outside the jetties of Coos Bay at high tide (Figure 1). Concentrated tows were immediately returned to the lab and isolations were made into F/20 within an hour of collection. Studies were performed on a Pseudo-nitzschia australis clone that was isolated November 10, 1992 immediately outside the bar of Coos Bay.

Growth Media

Clones were grown in incubators at 12-14 °C, with illumination on a 14:10 hour light:dark cycle, by cool-white

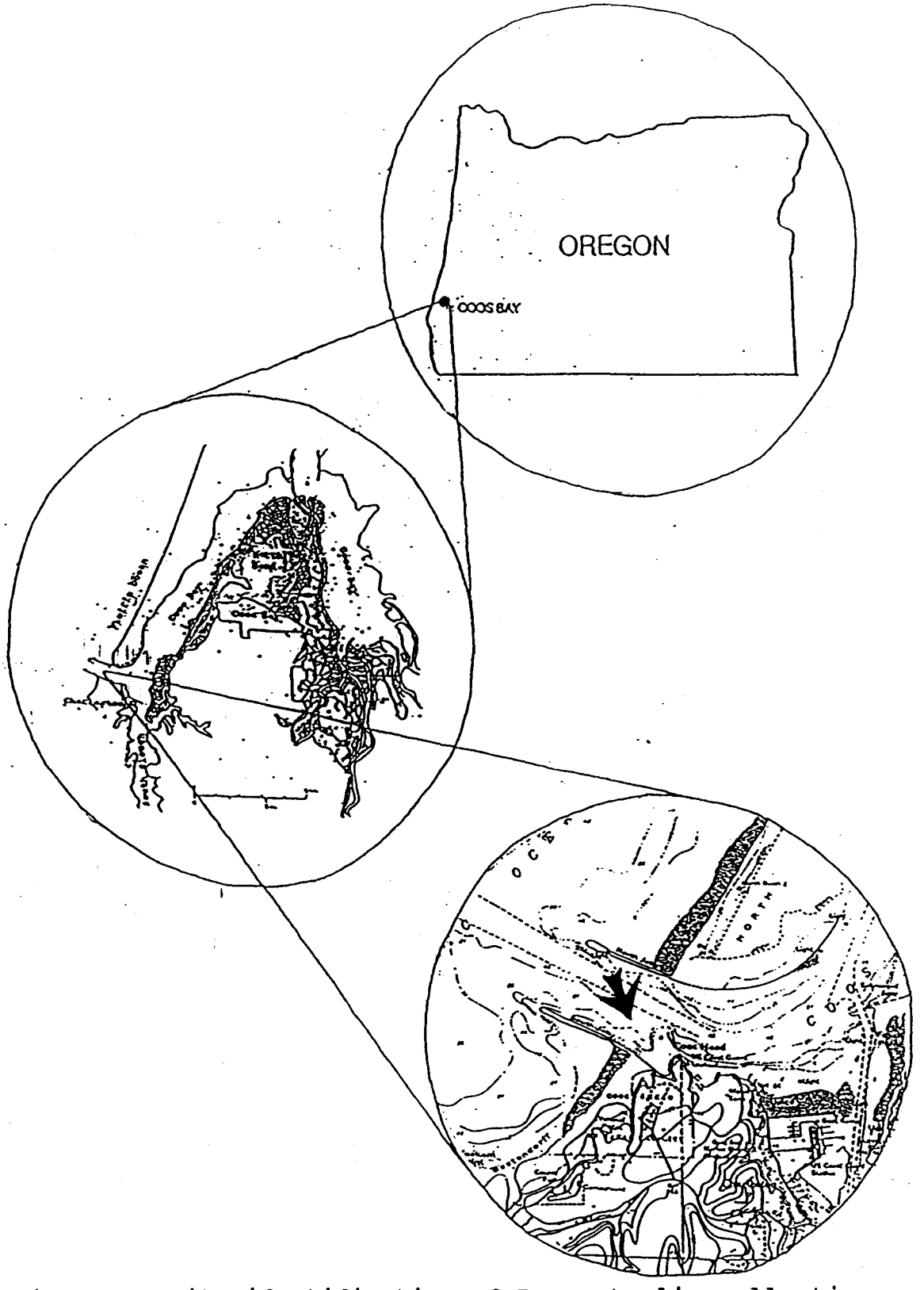


Figure 1. Site identification of P. australis collection.

fluorescent bulbs at ~90 $\mu Em^{-2}sec^{-1}$. Isolates were grown in batch culture in f/2 (Guillard 1975) with added soil extract in 250 millimeter flasks with cotton stoppers. Media was made with local seawater filtered through a 0.2μm filter and microwave sterilized (Keller et al. 1988). Soil extract was made by combining 1 cup of rich organic potting soil with 1L of filtered sterile seawater. To the potting soil and seawater 2.5g of NaOH was added and autoclaved for 2 hr. The mixture was filtered down through a 0.2μm filter when cool and diluted 1:50 for the final working stock (Provasoli et al. 1957).

SEM Preparation

An acid digestion procedure for SEM was adapted from a protocol obtained from Peter Walz (personal communication) for Pseudo-nitzschia sp. in the Monterey Bay area. A cloudy culture of P. australis was centrifuged at 4000 x g for 5 min. to pellet diatoms. Supernatant was removed and the moist pellet was added to a saturated solution of $KMnO_4$. After 48 hrs, concentrated HCl was added, a drop at a time, until the solution turned brown. The solution was then heated on a heating block at 95°C until the solution became clear or yellow. The sample was then diluted with distilled water into Eppendorff tubes and centrifuged at 2500 x g for

10 min. or until a clear pellet formed. Supernatant was removed and the pellet was resuspended again with distilled water. Centrifugation was repeated until the solution neared a neutral pH (6-7). The cleaned diatoms were filtered down onto a 3μm Nuclepore filter and repeatedly washed (3-4 times) with distilled water. Once the diatoms had been rinsed and collected onto the filter they were rinsed off into an Eppendorff tube with distilled water. Samples were then centrifuged again and stored until EM could be performed. EM was done by Eric Schabtach at the electron microscope facilities at the University of Oregon in Eugene.

Staining

All marine bacteria were enumerated with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain. Samples were first fixed at a 0.25% final concentration with a biological grade glutaraldehyde solution at 5°C for 1 hr. A 1 mg ml^{-1} stock solution of DAPI was thawed immediately before usage. Samples were stained with DAPI at 5°C for 10 minutes, and filtered down on to polycarbonate membrane filters (Poretics). A gentle vacuum was applied to the samples at 5 p.s.i. Filters were placed on slides, a drop of oil (Cargille Type DF) was placed on top with a coverslip

and slides were frozen in a light resistant slide box until analysis. All samples were analyzed with epifluorescence a Leitz Laborlux S microscope equipped with a 100-W Mercury light source. A BP 340-380 excitation filter, RKP 400 dichromatic beam splitter and LP 430 barrier filter were used for analysis of DAPI stained cells. All bacteria were enumerated at 1000x magnification under oil immersion objectives.

Geometric Approximation

A geometric approximation was done on P. australis in order to determine a volume of water for the phycosphere. The shape of P. australis was approximated to be a rectangular solid topped by two pyramids. Length of cell, width of valves and width of valve in girdle view were taken for length, width and height measurements respectively. Measurements were done on fixed samples (0.25% glutaraldehyde) with an ocular micrometer at 1000x. At least 50 cells were measured and the means were used for volume estimations. To estimate the volume of the phycosphere 2μm were added to all measurements and the volume was recalculated.

$$eq1.\ (h \cdot w \cdot l) + 2\left(\tfrac{1}{3}(h \cdot w \cdot l)\right) = CellVolume$$

$$eq2.\ [(h+2)(w+2)(l)] + 2[\tfrac{1}{3}(w+2)(h+2)(l)] = clvlm + physphere$$

$$eq2. - eq1. = VolumeofPhycosphere$$

Cell Enumeration

Bacteria and P. australis were enumerated utilizing two comprable computations for calculating cells per milliliter. All enumerations were done by filtering samples onto polycarbonate filters (Poretics). Field of view analysis was calculated by counting cells in a number of fields of view up to a total of 500 cells. This type of analysis was often used for enumerating bacteria.

$$FieldofViewAnalysis = \left(\frac{avgcells}{FV}\right) \times \left(\frac{FV}{filter}\right) \times \left(\frac{filter}{ml}\right) = \left(\frac{cells}{ml}\right)$$

A strip area count was used for P. australis enumeration. At total of at least 200 cells were counted and cells per area were averaged.

$$StripAreaAnalysis = \left(\frac{avgcells}{areaofstrip}\right) \times \left(\frac{areaoffilter}{ml}\right) = \frac{cells}{ml}$$

Fixative Stickiness

Putt et al. (1994) suggested that fixatives might have an effect of sticking bacteria to algal cells. Therefore, live and fixed samples of P. australis cultures were compared in order to test for an effect of fixative on epialgal bacterial concentrations. Live samples were stained with Hoechst 33342, and fixed samples with DAPI as previously described. Two 1 ml samples from the same culture were pipetted into sterile plastic vials. One sample was fixed and stained with DAPI as previously described. The second unfixed sample was incubated for 90 minutes at culture temperature with a 1mg ml^{-1} stock solution of Hoechst dye 33342. Epialgal bacteria were enumerated as previously described.

Statistical Analysis

Data was compiled and organized using spreadsheets in Microsoft Excel v.4.0a. Data was analyzed and graphed in Systat for Dos v.1.0. All cell counts were square root transformed for statistical analysis as recommended by Sokal and Rohlf (1981). All experiments assuming a random association of free bacteria with the diatom cell were analyzed using Analysis of Variance (ANOVA) tests. Linear

regressions were performed on all growth data for P. australis and bacteria populations, for a growth rate.

Association Experiment

The first experiment performed was done to determine if a numerically significant number of epialgal bacteria were associated with P. australis cells. Three flasks were inoculated with an exponentially growing culture and kept at culture conditions for 5 days. Each flask was sampled daily for two 1 ml samples. Both samples were fixed and stained with DAPI as previously described. One sample was filtered down onto a 3μm filter (Poretics) and washed 3 times with sterile filtered seawater. This procedure should ensure that remaining bacteria were closely associated with the diatom cells. The second sample was filtered onto a 0.2μm filter (Poretics) for free bacteria, and P. australis enumeration. Cell counts were done as previously described.

Growth Rate

The method used for determining growth rate of P. australis was described by Brand et al. (1981). Clones were grown as previously described. Cultures were kept in

25x100mm tubes at 14°C on a 14:10 light dark cycle at 89.5 $\mu Em^{-2}sec^{-1}$. Clones were measured once a day at the same time of day in a Model 10-AU-000 Turner Designs Fluorometer. The fluorometer was equipped with a 10-045 daylight white lamp source, 10-050R excitation filter, 5-60 color specification filter, and 10-051R emission filter. Cultures were taken out 10 minutes prior to fluorometric readings and vortexed immediately right before reading. Replicate tubes were transfered 3 days after inoculation in order to ensure cultures never lagged in growth. Fluorometric readings were log transformed (base of 2) and plotted versus time. Clones were deemed in continuous culture when slopes of replicates were approximately similar and no lag phase ocurred. Slopes were determined by the least squares method of linear regression. A mean rate and standard deviation were calculated to determine a 95% confidence limit.

Sampling for growth dynamics of free and epialgal bacteria populations was done on cultures being monitored for growth rate of P. australis. Every third day two 500 µl samples were taken from growth rate replicates. Both samples were fixed and stained with DAPI as previously described. One sample was filtered onto a 0.2µm polycarbonate membrane filter for free-media bacterial counts. The other sample was filtered onto a 3µm filter and cells were washed 3 times with filtered sterile seawater for

tightly-associated epialgal bacteria counts. Clones were monitored every 3 days for the duration of the growth phase (20-25 days).

Dilution Experiment

A dilution of bacteria in culture media was performed in order to test the hypothesis that there is a random association between bacteria and P. australis. A 5 day old culture (exponential phase) was used for manipulations. An initial count was performed of P. australis, free-bacteria, and loosely associated epialgal numbers. Samples were fixed with glutaraldehyde and stained with DAPI as previously described. The culture was diluted with sterile filtered seawater at ratios of 1:10, 1:100, and 1:1000. Dilutions were performed in Erlenmeyer flasks, lightly vortexed and sampled immediately. Samples were then taken from each dilution, fixed and stained with DAPI. Samples were filtered down and frozen for later analysis. P. australis and bacteria enumeration was performed as previously described.

Concentrate Experiment

Free-bacteria were concentrated in culture media conditions in order to test the hypothesis that there is a random association between bacteria and P. australis. An exponentialy growing culture (5-7 days old) was grown up in one liter of f/2 media. Three treatments were performed with five replicates in each treatment.

1. Approximately 250 mls of culture media was centrifuged twice at 1000 g for 10 min., which separated free bacteria in supernatant from diatom and associated bacteria in pellet. The supernatant was sampled, fixed, and stained with DAPI for free bacteria counts and to ensure no P. australis was retained in the supernatant. Collected supernatant was then reverse filtered through a 0.2μm polycarbonate filter. The filter was attached to one end of a tube which was open on the other end and was placed in the supernatant in order to filter and remove bacteria-free media. The concentrate was sampled for bacterial counts until a suitable concentration could be reached for experimentation and was added back to the original culture to produce the high free-bacteria:epialgal bacterial experimental condition.

2. A 100 ml aliquot of culture media was again centrifuged as previously described. The supernatant was

collected, fixed, and stained with DAPI for a free bacteria count and to ensure no P. australis remained. This supernatant was again added back to the original culture to produce the low free-bacteria:epialgal bacterial experimental condition.

3. 25 mls of the original culture media was monitored as a control for normal epialgal growth. The three experimental conditions were set up in 25x100mm culture tubes and monitored for 3 days. Cultures were sampled daily for P. australis, epialgal bacteria, and free-bacteria fixing and staining with DAPI as previously described.

CHAPTER III

RESULTS

Taxonomic Identification

The pennate diatom clone isolated at the mouth of Coos Bay, Oregon was identified as Pseudo-nitzschia australis also known as Nitzschia australis, Pseudo-nitzshia seriata, and Nitzschia pseudoseriata (Hasle and Fryxell 1995). Taxonomic descriptions from Hasle (1965, 1993) were used for positive identification of the clone: length of cell 75-144μ, width of cell 6.5-8μm, valves linear to lanceolate and cell ends slightly rostrate and rounded at tip (Figure 2). Keel puncta and transapical ribs are present in equal numbers 12-18 in 10μm (Figure 3). No pseudonodules are present and two rows of large poroids, 4-5 per 1μm are present at each side of costae (Figure 4). Cells formed stepped colonies by overlapping of cell ends. The clone at isolation measured approximately 100μm in length and 8μm in width. It has diminished in culture and now measures 90.9±.94μm in length and 6.4±2.6μm in width (Table 1).

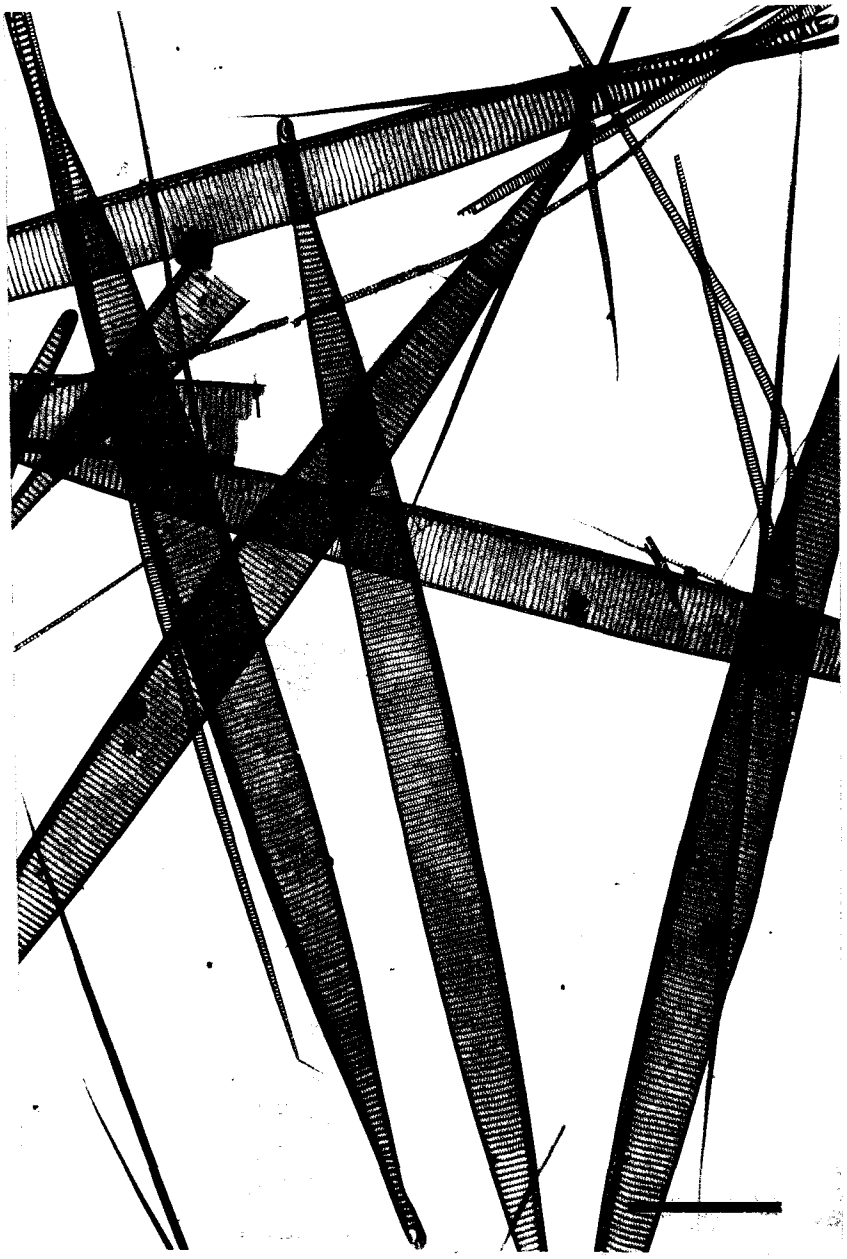


Figure 2. TEM of acid cleaned frustules of P. australis (mag 2000x, scale bar equals 5μm).

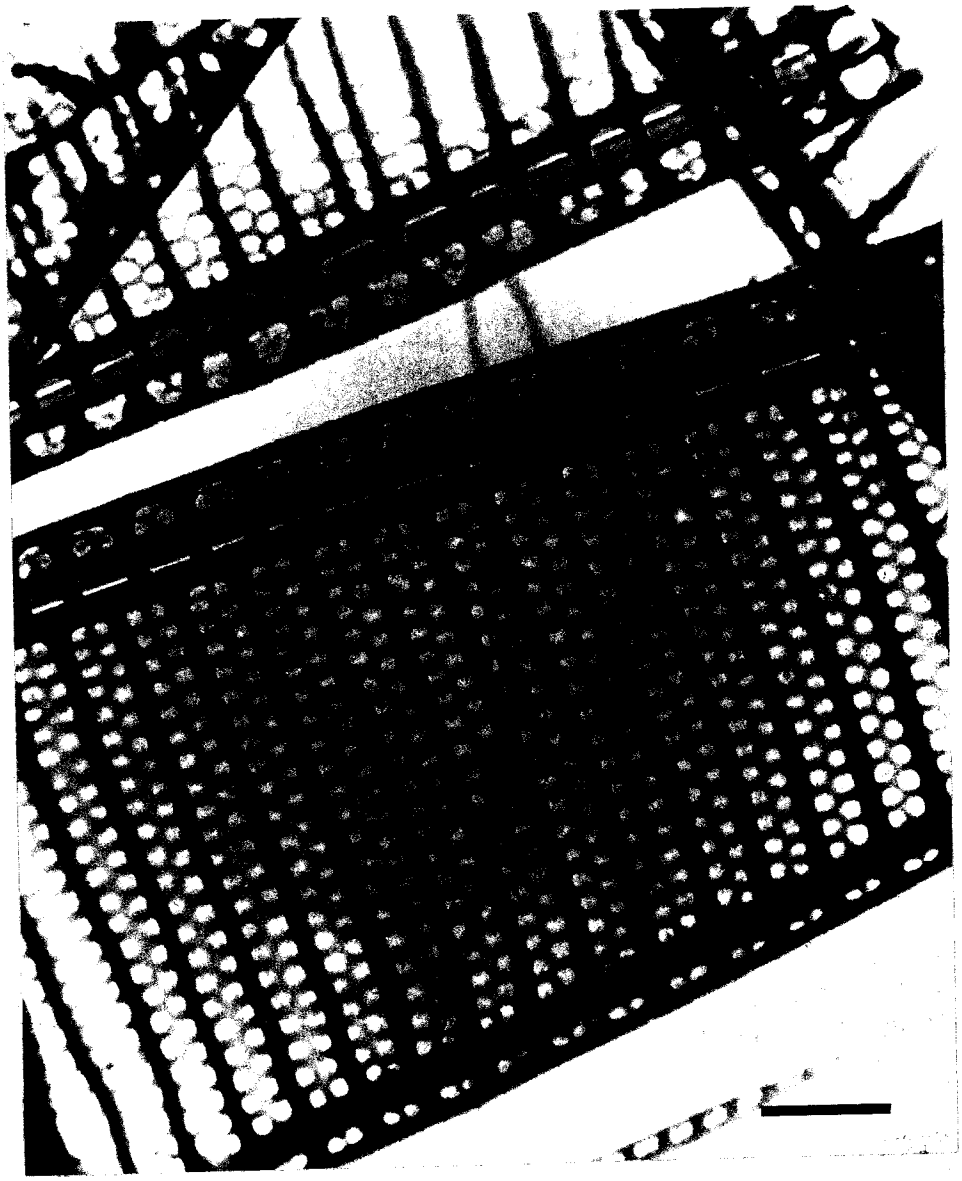


Figure 3. TEM of P. australis frustules, 2-3 rows of poroids between costae (mag 12500x, scale bar equals 1μm).

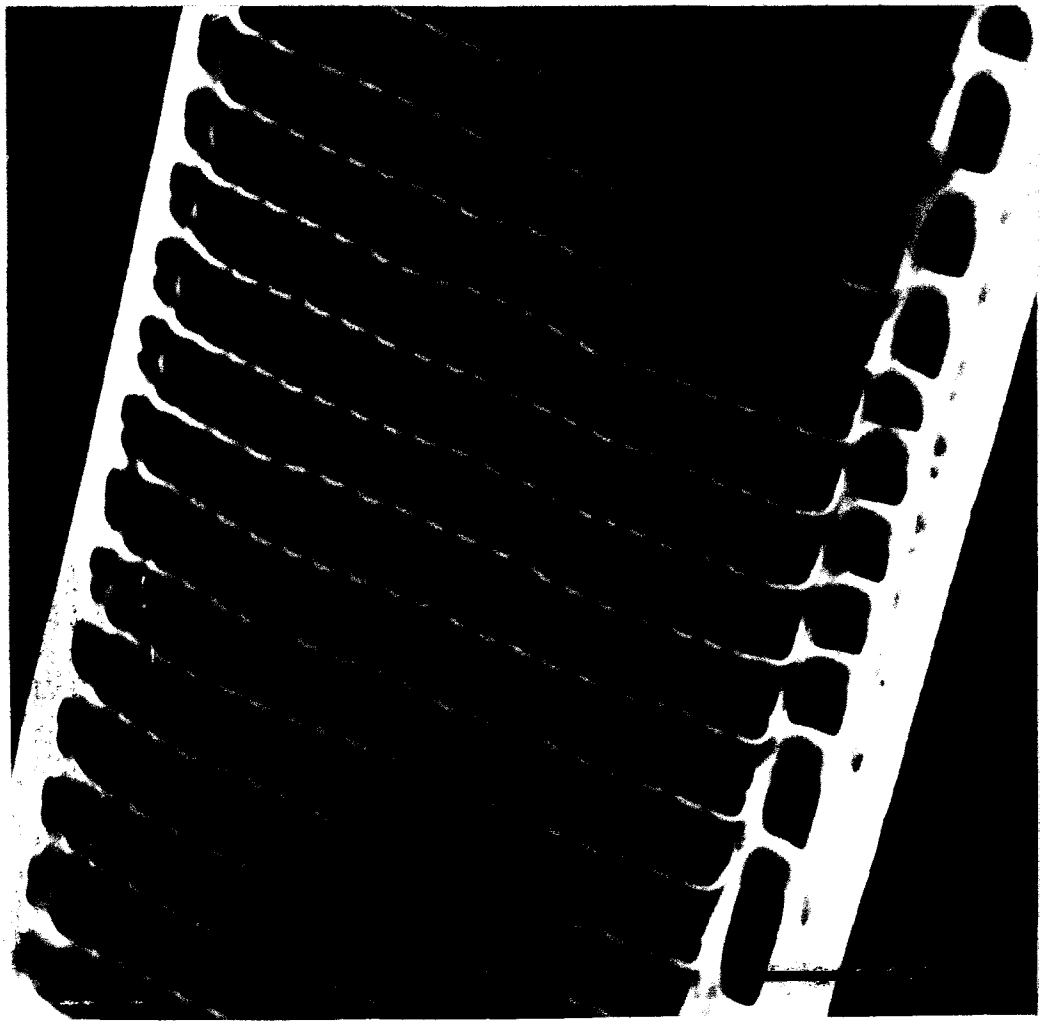


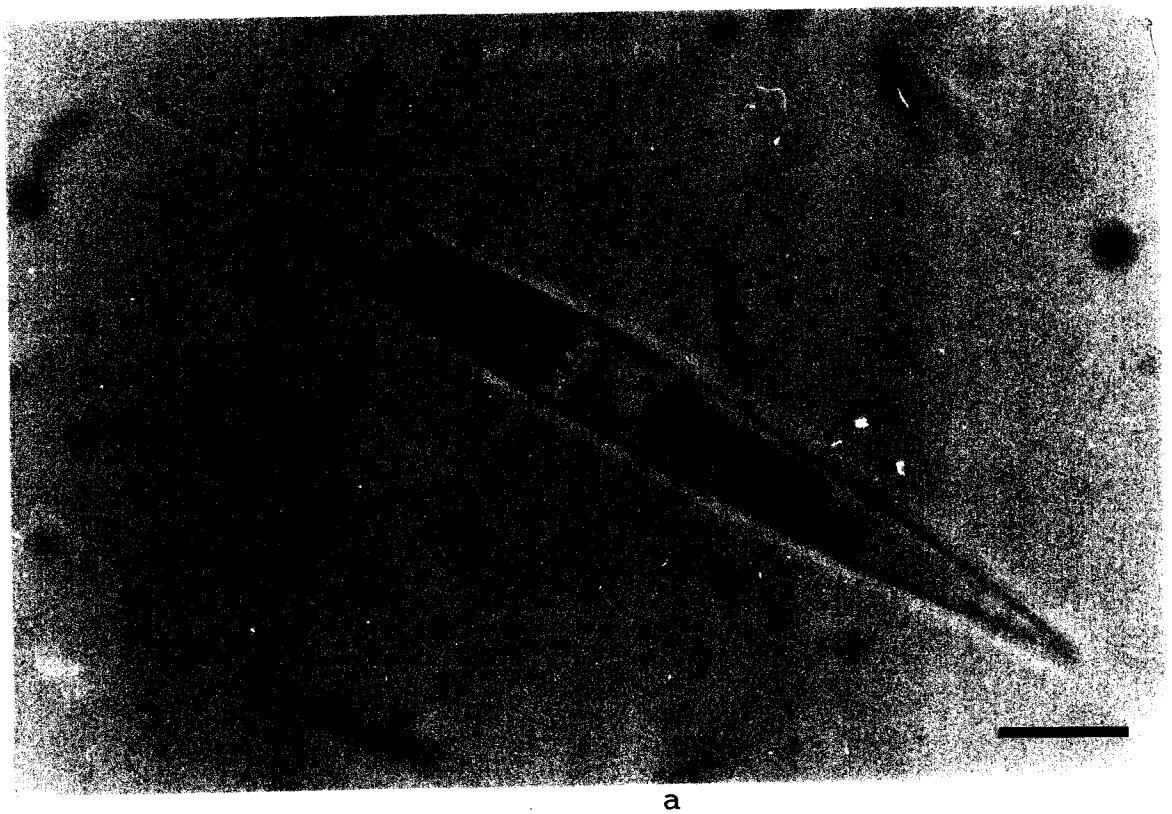
Figure 4. SEM of P. australis frustule (mag 28600x, scale bar equals .5μm).

The calculated volume using a geometric approximation (Figure 5) of P. australis is 3067μm^3 while the calculated volume of an assumed 2μm phycosphere is 2324μm^3 = 2.3248x$10^{-9}mm^3$.

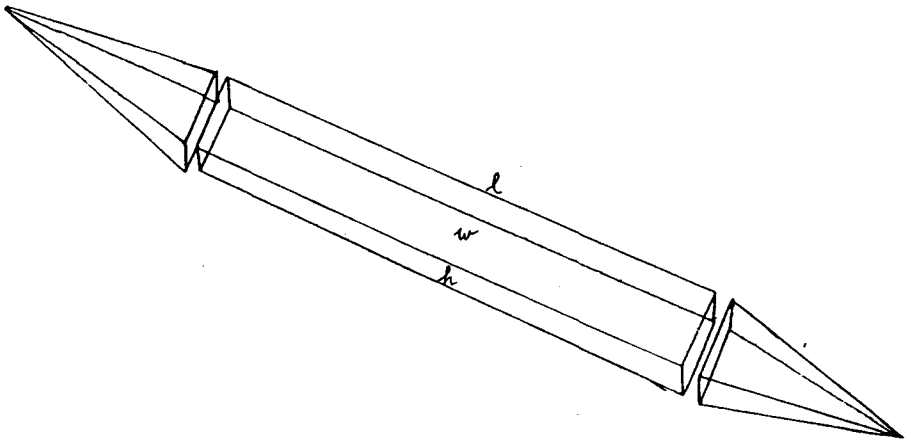
Table 1. Biometric measurements and geometric approximation of the volume in the phycosphere surrounding P. australis. Sensitivity analysis volumes were calculated by adding and subtracting 2μm to each measurement in order to assess epialgal abundances if sizes were over or underestimated.

Measurements	N	Mean(μm)	Std.Err	C.V.
Height	50	6.40	0.1309	0.145
Length	50	90.92	0.4676	0.036
Width Ends	50	3.60	0.1143	0.224
Width Middle	50	6.42	0.1281	0.141

Volume of Cell = 3067.5μm^3
Volume of Cell + Phycosphere = 5392.3μm^3
Volume of Phycosphere = 2324.79μm^3 = 2.3248x10^{-9}mls
Sensitivity Analysis (+2μm to size) = 3.0308x10^{-9}mls
Sensitivity Analysis (-2μm to size) = 1.6508x10^{-9}mls



a



b

Figure 5. Picture of a typical P. australis cell (mag 5500x, scale bar equals 10μm) (a), and diagram of shapes used for geometric approximation of cell volume (b).

Fixative Experiment

P. australis cultures were sampled and stained with DAPI (fixed) and Hoechst (live) stains for epialgal enumeration in order to test for a sticky effect on bacteria from glutaraldehyde fixative. Replicate counts of epialgal and free bacteria, both fixed and living, were compared in 5 and 11 day old P. australis cultures. Fixed cells were stained with DAPI as previously described, and live with Hoechst 33342. Initial results were not conclusive.

Growth Rate of P. australis

Replicates of P. australis (replicates 1,2,3) were grown in semi-continuous batch culture to determine the cellular division rate of the diatom , which was calculated as the slope of the semi-log plot of fluorescence units versus time. The replicates were deemed in semi-continuous culture at that time because no lag phase occurred and a semi-logarithmic plot of fluorescence versus time demonstrated similar slopes between replicates (Brand et al., 1981) (Figure 6). Calculated slopes for linear regressions on all three replicates were #1, 0.352; #2, 0.331; and #3, 0.364 (Table 2). All slopes of replicates were significantly different from a slope of zero ($t>2$, and $P<0.05$). The 95% confidence intervals for each replicate's

slope overlapped. The mean slope of all replicates (1-3) was 0.349 doublings day^{-1}.

Table 2. Summary table of linear regressions on growth curves for replicates of P. australis.

Rplct	Cnst	Time	Std. Error	R^2	P - value	t - value
1	-0.00	0.352	0.011	0.998	0.001	32.61
2	0.077	0.331	0.013	0.997	0.002	24.60
3	-0.04	0.364	0.023	0.992	0.004	15.51

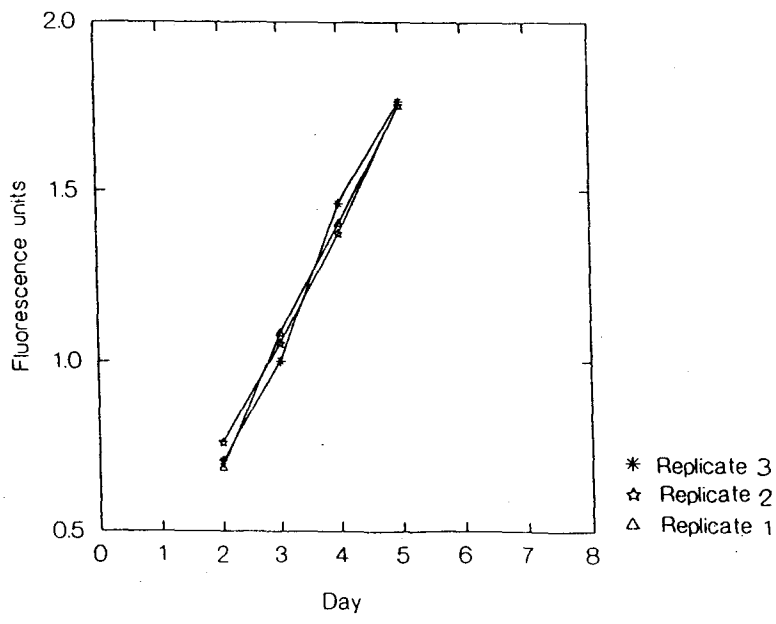


Figure 6. A plot of fluorescence units (log_2 transformed) versus time, used for the linear regression analysis of replicates 1, 2, and 3 of P. australis.

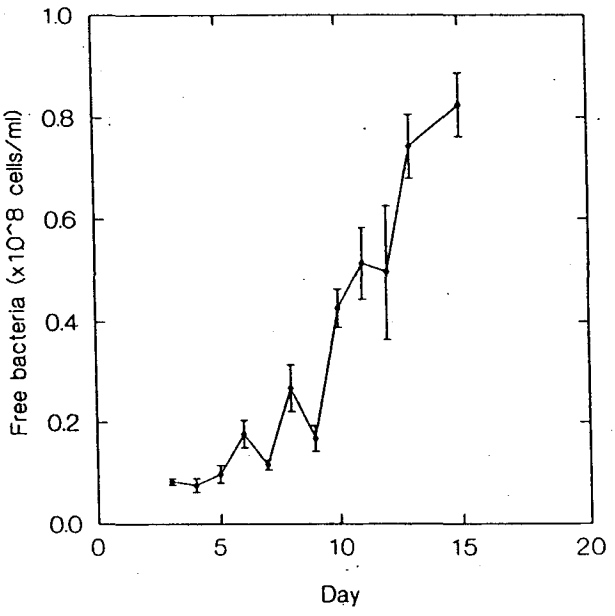
Growth Dynamics of Bacteria

Epialgal and free bacteria concentrations were monitored throughout P. australis growth rate analysis in order to assess growth dynamics for bacteria in culture with the diatom. All epialgal and free bacteria counts taken from replicates 1-3 were averaged for graphing and statistical analysis. Epialgal abundances were 2-3 orders of magnitude higher than free bacteria throughout all stages of growth of P. australis (Figure 7a,b). Linear regressions were performed on replicates of bacteria growth counts ($\log_2$ transformed). Average division rates were 0.381 doublings day^{-1} for free and 0.226 doublings day^{-1} for epialgal bacteria.

The concentration factor is a ratio:

$$CF = \frac{\text{epialgal-free}}{\text{free}}$$

Concentration factors calculated using the growth dynamics data represent epialgal bacteria in the tightest-association with P. australis, because a filtered seawater wash was performed on epialgal bacteria in order to rid counts of loose-associating bacteria. Fitting a line to CF calculations shows a negative trend during P. australis growth (Figure 8). Therefore, during batch culture growth



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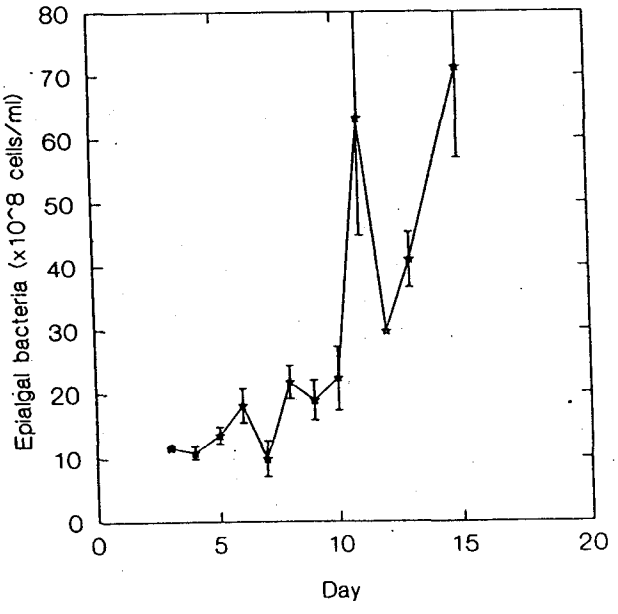

b

Figure 7. Mean abundances of free (a) and epialgal (b) bacteria throughout growth of P. australis.

of P. australis, free bacteria increased more rapidly than epialgal bacteria.

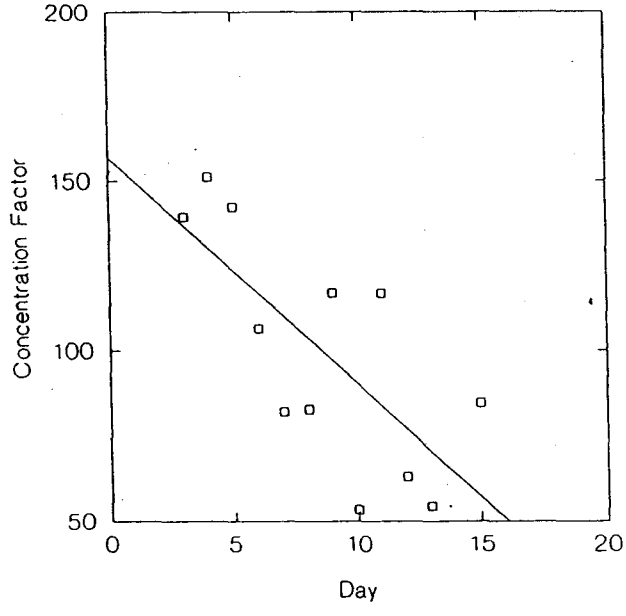

Figure 8. Concentration Factor calculated for bacteria populations throughout growth of P. australis.

Association Experiment

Epialgal and free bacteria abundances were monitored throughout exponential growth of P. australis in order to assess quantitatively if more bacteria were in association (living epiphytically) with the diatom cell as compared with the abundance of cells present free in the media. The null hypothesis was that in assuming a random association of bacteria epialgal and free abundances would be the same. Throughout all 5 days of P. australis growth, epialgal

bacteria abundance was at least 2-3 orders of magnitude higher than free bacteria abundance (Figure 9). ANOVA results showed a signficant interaction with the replicate (Table 3). Analysis of the interaction plot revealed that replicate number 1 was abnormal (Figure 10). Another ANOVA was performed by elimination of replicate 1 based on the significant p-value for location. Without replicate number 1 the interaction with replicate was not significant ($P=0.556$) (Table 4). Similiar p-values were obtained for volume adjustment measurements (+/-2μm) ($P<0.001$). Because the analysis using original values for the volume of phycosphere showed significantly higher epialgal bacterial concentrations compared with free bacteria the underestimated volume adjustments (-2μm) were not tested. Therefore, our volume calculation for the phycosphere appears to be elastic enough for mismeasurements of up to 2μm. The null hypothesis that bacteria were randomly associating with the diatom was rejected.

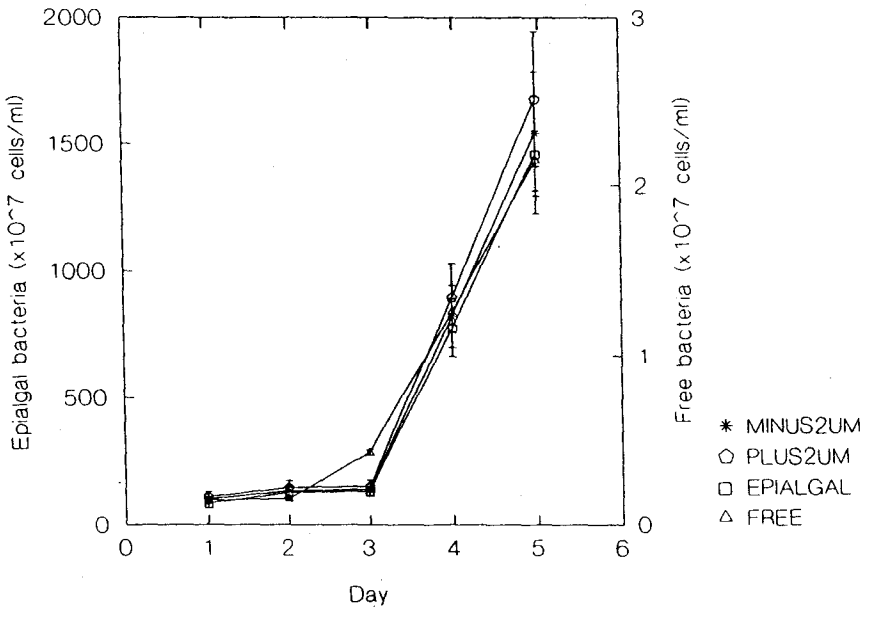

Figure 9. Mean bacteria abundances, minus2μm and plus2μm refers to sensitivity analysis phycosphere volume.

Table 3. ANOVA table for association experiment all replicates included, R^2=0.388.

Source	Sm-of-Sqrs	DF	Mn-Square	F-Ratio	P
Lction	8.41034×10^{10}	1	8.4103×10^{10}	71.689	0.000
Rplct	1.07736×10^{10}	2	5.3868×10^{9}	4.592	0.011
Lct*Rplt	1.02602×10^{10}	2	5.1301×10^{9}	4.373	0.013
Day	1.68173×10^{11}	1	1.6817×10^{11}	143.35	0.000
Error	5.20886×10^{11}	444	1.1732×10^{9}		

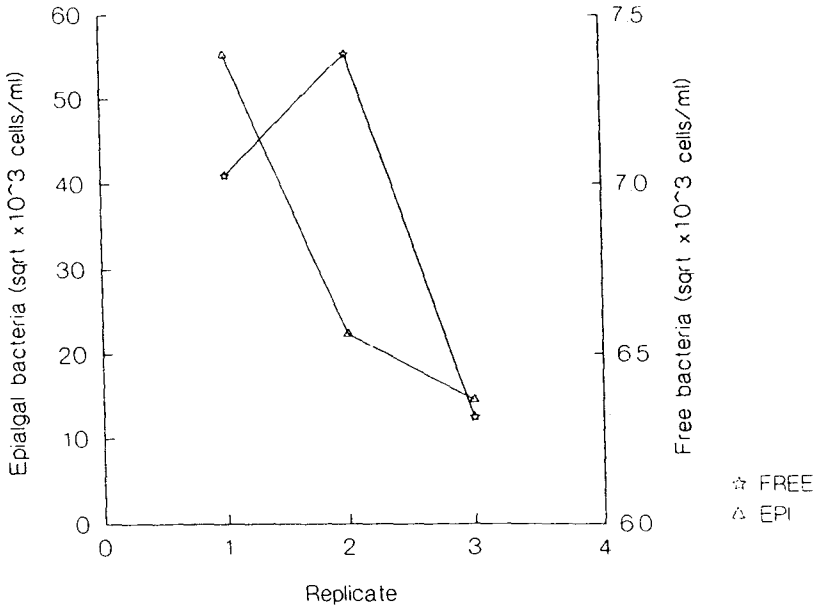

Figure 10. Interaction plot for ANOVA in association experiment.

Table 4. ANOVA for association experiment replicate 1 removed, R^2=0.327.

Source	Sm-of-Sqrs	DF	Mn-Square	F-ratio	P
Lction$	3.68651×10^{10}	1	3.68651×10^{10}	50.598	0.000
Rplct	4.32884×10^{8}	1	4.32884×10^{8}	0.594	0.441
Lct*Rplt	2.53795×10^{8}	1	2.53795×10^{8}	0.348	0.556
Day	5.34129×10^{10}	1	5.34129×10^{10}	73.311	0.000
Error	2.17118×10^{11}	298	7.28584×10^{8}		

Dilution Experiment

A dilution of free bacteria in the media was performed on an exponentially growing culture of *P. australis* in order to determine if epialgal bacteria were randomly associated with the diatom cell. Again the null hypothesis: epialgal bacteria should decrease with a decrease in free bacteria concentrations. Analysis of variance results showed there was a significant interaction of dilution and location (epi vs.free) effect ($P<0.001$) (Table 5). Epialgal bacteria did not exhibit similar patterns to that of the dilution of free bacteria (Figure 11). Epialgal replicates were not significantly different from one another while the free bacterial concentrations declined steadily with dilution ($P=0.221$) (Table 5). Epialgal bacteria essentially did not dissociate further after the 1:5.5 dilution (Figure 12). Therefore, the null hypothesis can be rejected; epialgal bacteria abundances do not decrease with a decrease of free bacteria in the media and therefore the association of bacteria with the diatom cell is not random.

Table 5. ANOVA for dilution experiment, $R^2 = 0.986$.

Source	Sm-of-Sqrs	DF	Mn-Squares	F-Ratio	P
Diltn$	4.01418×10^9	3	1.33806×10^9	19.657	0.000
Loctn$	1.10563×10^{11}	1	1.10563×10^{11}	1624.25	0.000
Replct	4.20841×10^8	4	1.05210×10^8	1.546	0.221
Dlt*Lct	3.09753×10^9	3	1.03251×10^9	15.168	0.000
Error	1.63368×10^9	24	6.80700×10^7		

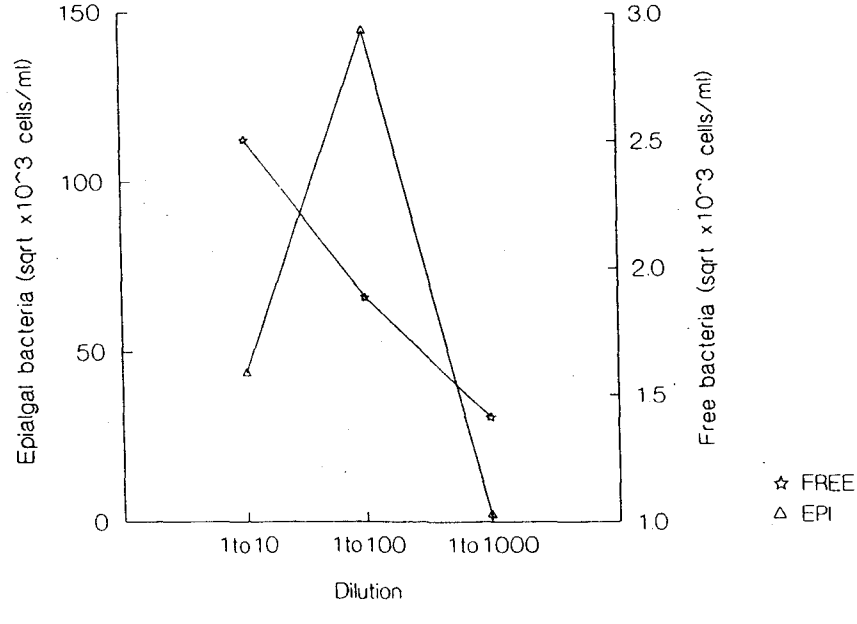

Figure 11. Interaction plot of dilution (diltn$) with location (loctn$) of bacteria.

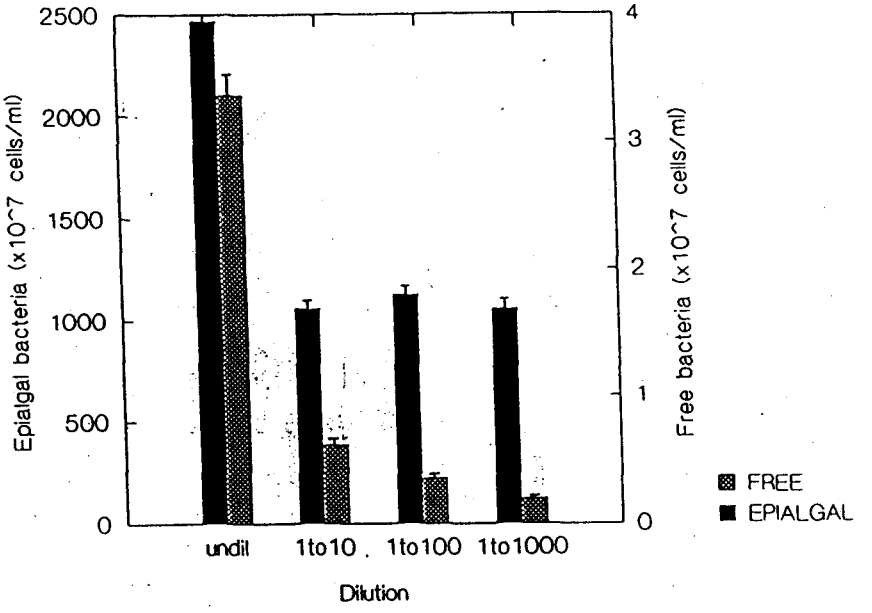

Figure 12. Mean epialgal and free bacteria concentrations in dilution experiment.

Concentration Experiment

A concentration of free bacteria from a P. australis culture was performed by reverse filtration of a centrifuged supernatant, and the concentrate (free bacteria) was added back to an exponentially growing culture of P. australis as another test of whether bacteria are randomly associated with the diatom cell. Therefore the null hypothesis; an increase in free bacteria concentration will increase epialgal bacteria abundances proportionately.

Analysis of variance results showed a significant interaction between treatment (free bacteria concentration)

and location (epialgal and free) of bacteria ($P<0.001$) (Figure 13). The deviation from the pattern occurs in the concentrate treatment where the epialgal bacteria are less abundant than the experimentally concentrated free bacteria (Figure 13). Therefore epialgal bacteria concentrations are not significantly increasing due to an increase of free bacteria. Replicates within this analysis were not significantly different ($P=0.282$) (Table 6). Therefore the the null hypothesis was rejected: again bacteria are not randomly associating with the diatom cell.

Table 6. ANOVA for concentrate experiment, $R^2=0.985$.

Source	Sm-of-Sqrs	DF	Mn-Squares	F-Ratio	P
Replicat	6.47441×10^{8}	4	1.61860×10^{8}	1.289	0.282
Treat$	3.33700×10^{9}	2	1.66850×10^{9}	13.291	0.000
Locatin$	6.17620×10^{11}	1	6.17620×10^{11}	4919.72	0.000
Trt*Lctn	2.43988×10^{9}	2	1.21994×10^{9}	9.718	0.000
Error	9.28994×10^{9}	74	1.25540×10^{8}		

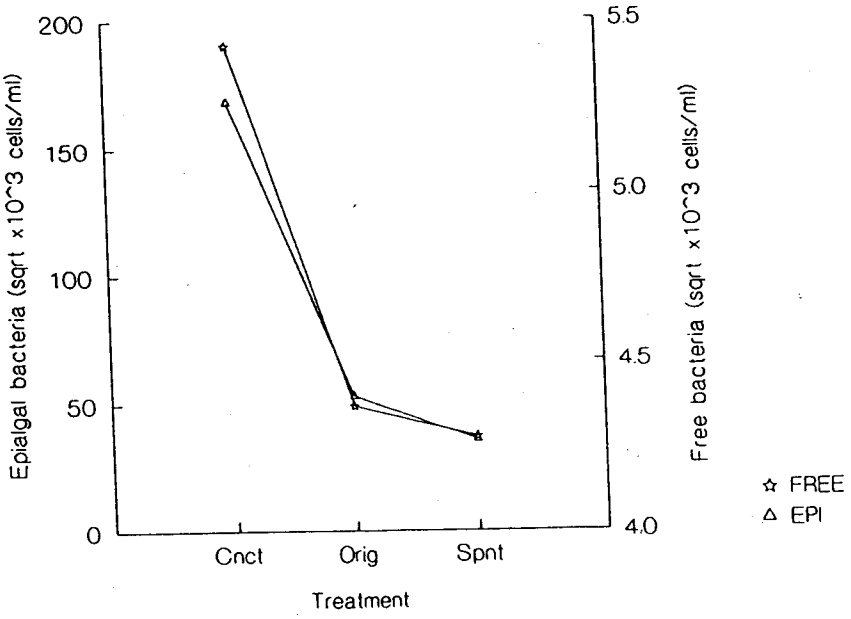

Figure 13. Interaction plot of treatment (free bacteria concentrations) with location of bacteria.

CHAPTER IV

DISCUSSION

Epialgal Bacteria

The epialgal bacteria enumerated by the methods used in this study represent those bacteria that live in the tightest association with the diatom. Since a series of washes were performed on these samples, any extraneous bacteria that might have filtered down on the diatom were removed. The results showed a population of epialgal bacteria that interact throughout growth phase of the diatom cell and at higher concentrations than that seen in the free media. Bacteria have been observed living in the valve pores of this clone of P. australis (pers.obsv.), suggesting these pores might be sites of extracellular material release, as previously suggested for the diatom Navicula confervaceae (Rosowski, 1992). Thus, these data do not support the null hypothesis that bacteria associated with phytoplankton are solely utilizing algal decay products at the end of algal growth. However, at the onset of

stationary phase epialgal bacteria populations peak and drop off suddenly with quite a wide variation in abundances seen between cells. Jones et al. (1986) suggested that in fact two different populations of bacteria may interact with algae, one living on exudate products and one utilizing algal decay products. During exponential growth, P. australis may provide exudate products that support a certain, select population of bacteria. At the onset of stationary phase however, algal products may become more abundant and less selective and a shift or a recruitment of free bacteria from the media may occur. Epialgal abundances at this point then would vary widely as both epialgal and free bacteria populations interact with the diatom. Therefore, epialgal populations enumerated at stationary phase and exponential phase may represent two widely varying types of bacteria capable of different interactions with P. australis.

Concentration Factor

CF values greater than 0 indicate that densities of bacteria living epiphytically are greater than those in the free media (Putt et al., 1994). Since all of the CF values calculated in our experiments were greater than 50, it is obvious that significant concentration is occuring at the

cell surface. Furthermore, CF values calculated for bacteria abundances during P. australis growth demonstrated a negative trend. CF values were near 150 at the beginning of P. australis exponential growth and decrease to 50 during stationary growth. This negative trend supports the hypotheses that; (1) epialgal bacteria during exponential growth of the diatom are independent of free bacteria concentrations, and (2) perhaps two different populations of bacteria are associating with the P. australis cell.

A decreasing CF value during P. australis growth could suggest a couple of different possibilities. High CF values at the beginning of diatom growth reflect higher epialgal abundances than those of free bacteria. Throughout P. australis growth epialgal abundances may not be decreasing but rather, dividing more slowly than that of free bacteria. Free bacteria divide at 0.381 divisions day^{-1}, compared with 0.226 divisions day^{-1} for epialgal bacteria. A negative slope again rejects the null hypotheses that bacteria were randomly associated with the diatom cell. If epialgal bacteria abundances were dependent on free bacteria concentrations, then both abundances would increase equally and no trend would be demonstrated. Therefore, a negative slope of CF values rejects the null hypothesis that bacteria associations are random with this diatom.

During stationary phase, CF values stray from the

expected (regression line) much more than those in exponential phase and are extremely variable (large SE bars). This variability may be due to a shift in bacterial populations that may occur between exponential and stationary phase. I discussed earlier that two bacteria populations may be living epiphytically on the diatom surface during growth. When the diatom reaches stationary phase exudate products may become more abundant and less selective. CF values vary widely because free bacteria, who before were just inhabiting the media, may be colonizing the surface of the diatom cell. Variable CF values then support an hypothesis of free bacteria contributing to epialgal populations during stationary phase of P. australis.

Fixative Experiment

Putt et al. (1994) suggested that there might be an effect of fixative on the "stickiness" of bacteria cells to algal cells and therefore use of fixatives may result in overestimating epialgal concentrations. I tried to test for an effect of fixative on epialgal abundances using DAPI stain with fixed and Hoechst stain with live samples. However, the stains do not result in comparable enumerations of bacteria. Hoechst fades more rapidly under ultraviolet excitation then DAPI, and Hoechst counts may have been underestimates. Because of the confounding factor of stain

responses, a statistical analysis was not peformed on these data and results are inconclusive.

Sensitivity Analysis

In order to compare epialgal and free bacteria, abundances were converted to cells ml^{-1}. Therefore, the volume of the 2μm phycosphere in which epialgal bacteria were enumerated was estimated in order to get both counts to cells per milliliters. Because all epialgal abundance measurements were based on this volume measurement a sensitivity analysis was performed in order to establish how much elasticity was in the estimations. A 2μm envelope was added and subtracted to each of our size measurements and the phycosphere volume was recalculated. Phycosphere volumes for plus 2μm (3.0308×10^{-9}mls) and minus 2μm (1.6508×10^{-9}mls) are relatively close. Epialgal abundances using adjusted volume estimations are still 2-3 orders of magnitude greater than those of free bacteria abundances. Therefore if size measurements were as grossly over or under estimated as 2μm, volume estimations would still give significantly more epialgal bacteria associated with *P. australis* than free bacteria in the media.

Nonrandom Association

Two experiments were performed in order to provide evidence that a distinct population of bacteria was associating with P. australis separately and was not just a random association with free bacteria. In the dilution experiment the epialgal population did not dilute out to concentrations that would be expected if free bacteria were determining epialgal abundances. Epialgal bacteria on average diluted down 1.4×10^{10} cells ml^{-1} or 32.5 bacteria cells per diatom cell with the first 1:5.5 dilution, and did not dilute further despite an additional hundred-fold dilution. This means bacteria living epiphytically on the diatom did not dilute out with a dilution of free bacteria in the media. Therefore a population of bacteria exists epiphytically on P. australis during exponential growth and does not seem to be supplied by bacteria populations in the media.

The inability to dilute out free bacteria may be explained by the tendency for this P. australis culture to grow in sturdy clumps at the bottom of flasks, leading to initial counts that may have underestimated actual bacterial concentrations. When diluting cultures with filtered sea water these clumps of bacteria and P. australis may have been broken up with mixing and thus contributed more

bacteria to dilutions than expected. It is also possible that bacteria populations may not be dilutable. Free bacteria populations within this P. australis culture divide at 0.381 doublings day^{-1} under normal conditions. Dilution of bacteria concentrations may trigger a cell density cue that may begin exponential growth to maintain a threshold density.

In the concentrate experiment cultures were centrifuged to separate out epialgal bacteria and P. australis cells from free bacteria. Therefore a concentrated population of free bacteria was added back to the original culture. Epialgal bacteria did not increase with an increase in free bacteria concentrations. Both the dilution and concentrate experiment permitted rejection of the null hypothesis that bacteria association with P. australis is random, and demonstrated a population of bacteria living in close association with the diatom.

Bacteria and Toxin Production

P. australis is known to be a domoic acid producer on the west coast of the United States. This project was born out of an interest in the possible influence marine bacteria might have on the effect of toxin production in this diatom. These questions could not be followed up because this clone

of P. australis only produced domoic acid in trace amounts and regular testing methods for domoic acid production were not available. Also, I was unable to render this clone axenic, and therefore the effects of bacteria directly on P. australis growth and domoic acid production, could not be assessed. Recent work with clones of axenic domoic acid producing Psuedo-nitzschia sp. has shown that clones decrease production of domoic acid and length of production compared with xenic clones, (Douglas et al., 1993). This indicates that marine bacteria may have some influence on Pseudonitzschia sp. growth and subsequent domoic acid production.

Summary and Conclusions

Growth dynamics of this P. australis culture can be characterized by gradual epialgal and free bacteria growth during exponential growth of the diatom. At the onset of stationary phase of the diatom epialgal and free bacteria peak in abundances. It is evident from these intial observations that epialgal bacteria are present throughout growth of P. australis and in larger concentrations than that seen in the free bacteria. This supports the theory that there is a distinct population of bacteria independent of that of the free bacteria that lives epiphytically on P. australis. The increased variability within epialgal

abundances during stationary growth of the diatom may indicate that a shift in bacteria populations is occuring between exponential and stationary growth. Manipulations with free bacteria concentrations in the dilution and concentrate experiment demonstrated again that there is a distinct population of bacteria living epiphytically in the phycosphere of P. australis that is independent of that of free bacteria concentrations. Epialgal abundances were not a factor of the concentration of free bacteria in the media.

I have demonstrated that a distinct population of marine bacteria interact closely with a clone of P. australis throughout all stages of growth. This interaction may have some role in growth mechanisms of the diatom as well as toxin production. Future studies on domoic acid producing clones must ensure that cultures are axenic and that these tightly interacting bacteria have been eliminated. Future work should also include research into characterizing the nature of the interaction between marine bacteria and toxin producing Psuedo-nitzschia clones.

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