EFFECT OF THE INTERACTION BETWEEN TWO SPECIES OF MARINE DIATOMS ON THEIR INDIVIDUAL COPPER TOLERANCE

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ABSTRACT

The importance of species interactions in studies assessing the trace metal tolerance of individual species, has been largely overlooked. Most toxicity assessments involve single-species tests. Although relevant in some cases, this approach does not incorporate the biological environment of an organism as a factor that might influence its survival. On the other hand, this factor is incorporated in studies at the community level. However, the latter, not examining the mechanisms that drive the interactions in the community, do not allow for prediction of the outcome after the addition of a stress inducer, such as trace metals. The purpose of this study was to examine the effect of the interaction of two species of marine diatoms (Skeletonema costatum (Cleve) Greville and Nitzschia thermalis (Ehrenberg) Auerswald) on their individual copper tolerances.

The two species were grown in unialgal cultures in order to determine their copper tolerance. *S. costatum* did not exhibit growth above $5 \times 10^{-7} \text{M} \ (pCu = 8.46)$ and *N. thermalis* above $6 \times 10^{-7} \text{M} \text{ added total copper} \ (pCu = 8.36)$. *Skeletonema* exhibited increased growth rate and lag phase with increasing copper concentration (and decreasing pCu). On the other hand, *Nitzschia* demonstrated decreased growth rate. No effect on lag phase was observed for this species.

No difference between the tolerances of two strains of *S. costatum* was observed. Both strains demonstrated unaffected growth at $1 \times 10^{-7} \text{M} \ (pCu = 9.16)$ and no growth at $1 \times 10^{-5} \text{M} \text{ added total copper} \ (pCu = 7.16)$.

Subsequently, *Skeletonema costatum* and *Nitzschia thermalis* were grown together at three copper concentrations ($1 \times 10^{-9} \text{M}, 4 \times 10^{-7} \text{M} \text{ and } 5 \times 10^{-7} \text{M} \text{ added total copper}$). In the unialgal cultures that were used as controls, the two species grew as predicted from their tolerance tests. However, in the mixed cultures, *Nitzschia* was the only species that exhibited growth, regardless of the copper concentration in the medium.
This inhibition of *Skeletonema* in the presence of the second diatom, was attributed to a toxic *Nitzschia* exudate. The effect of the exudate appeared to be temporary, as demonstrated by the extended lag phase and subsequent satisfactory exponential growth rate of *Skeletonema*. It is suggested that exponential growth rate was resumed because the exudate degrades within a period of five days (= lag phase).

It is shown that the interaction between the two diatom species is more important in determining the survival of *S. costatum* than its individual copper tolerance. This is not the case for *N. thermalis*. Such interactions would be unaccounted for in single-species toxicity tests. On the other hand, if they are known, prediction of how a community that includes these two species would respond to copper additions becomes possible.
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GENERAL INTRODUCTION

Copper is a trace metal that has been extensively studied in natural waters. In the marine environment, studies of this metal have been pursued in two directions. In the first, the purpose was to understand the distribution of this metal in natural seawater and in the sediments (e.g. Klinkhammer 1980, Boyle et al. 1981, Graybeal and Heath 1984, Pedersen et al. 1986) as well as its geochemical cycle (e.g. Boyle et al. 1977, Sholkovitz 1978, Windom et al. 1983, Balls 1988). In addition, its chemistry in both environments, with a special emphasis on the interaction of copper with organic compounds, has been examined (e.g. Meyers and Quinn 1974, Davis 1984, Förstner 1984, Laxen 1984/1985 and 1985, Coale and Bruland 1988, Soli and Byrne 1989). These studies have contributed to the considerable breadth of knowledge that has now been achieved regarding the behaviour of copper in the world’s oceans.

The second direction in which studies have been pursued involves the effect of copper on the biota. Studies of the biological effects of copper have centered mainly around single-species toxicity tests. The biological species that have been examined represent many trophic levels. Studies have been carried out with bacteria (e.g. Sunda and Gillespie 1979, Sunda and Ferguson 1983), phytoplankton (e.g. Anderson and Morel 1978, Morel et al. 1978, Stauber and Florence 1985a and 1985b, Pekkala and Koopman 1987), macroalgae (e.g. Forsberg et al. 1988, Ho 1988, Söderlund et al. 1988), zooplankton (e.g. Lewis et al. 1971, Gerasimov 1987, Sunda et al. 1987, Blust et al. 1988), benthic copepods and decapods (e.g. Depledge 1987, O’Brien et al. 1988, Verriopoulos and Dimas 1988), gastropods (e.g. Amiard-Triquet et al. 1987, Kitching et al. 1987, Minniti 1987), bivalves (e.g. Wright and Zamuda 1987, Harrison et al. 1988, Watkins and Simkiss 1988) and fish (e.g. Krezoski et al. 1988, Reid and McDonald 1988).
Marine phytoplankton have been studied in great detail. These studies have clearly indicated the large variability in responses to elevated copper concentrations that exists among species that belong to the same trophic level. In order to understand this variability, studies have addressed the possibility of taxonomically grouping individual species tolerances. Brand et al. (1986) examined the growth rate of 38 species of phytoplankton and discovered that three species, Skeletonema costatum, Thalassiosira pseudonana and T. oceanica, were more tolerant to copper than the other species tested. When species were grouped taxonomically, coccolithophores were found to exhibit sensitivity to a wider range of copper concentrations than diatoms. One species of dinoflagellate and a number of cyanobacterial species were the most sensitive organisms. Mandelli (1969) classified the dinoflagellates as more copper sensitive organisms than diatoms. Riley and Roth (1971) observed that, overall, diatoms accumulated more copper than chlorophytes, however, there was large variability between species and within classes. They concluded that the copper content of algae was not related to any taxonomic classification.

A different approach has been taken to group phytoplankton (and therefore generalize type-responses) with respect to copper tolerance, based on the oceanic region of origin of each species. Gavis et al. (1981) concluded that, generally, species isolated from neritic environments were more tolerant than oceanic species. Wood (1983) reached the same conclusion in an examination of phytoplankton assemblages (similar conclusions have been reached for differential tolerance to PCBs and other exotic compounds, e.g. Fisher et al. 1973, Fisher 1977). Although no clear conclusion was reached, Murphy et al. (1984) showed differences in response to metal limitation by Thalassiosira pseudonana (a neritic species) and T. oceanica (an oceanic member of the genus). On the other hand, Murphy and Belastock (1980) did not succeed in demonstrating a clear distinction in tolerance between species from the two environments. Strains from unpolluted and polluted neritic and oceanic regions were
examined for their relative tolerance to a complex industrial waste product. Although a pattern of increasing sensitivity with distance from a polluted origin was observed, the ranges of tolerance of the different groups were very broad. Therefore, solid conclusions could not be drawn due to the large overlap between groups. Although not directly relevant to copper tolerance, this study exemplifies the confusing patterns that emerge during attempts to generalize species' responses to inhibitory substances.

It therefore becomes apparent that, at least within the phytoplankton, it is very difficult to assign specific relative tolerance with respect to either taxonomic groups or environmental origin without some reservations. A single-species toxicity test may, therefore, be valid for a given phytoplankton strain of specific origin, but may not necessarily hold for that of the same or similar species from a different habitat. The differential response has been attributed, in most cases, to adaptations of the organism to chemical characteristics of the habitat (e.g. organic loading and/or high background trace metal concentration, e.g. Jensen et al. 1976, Fisher and Frood 1980). A factor that has been ignored is the adaptation of the organism to the biological component of its habitat (i.e. other species in the community and their interactions).

Different species in a community interact with each other in a particular fashion. Specific interactions determine the position of each member in the community as a whole. These interactions have resulted from a number of adaptations that allow the coexistence of different species or control the possibility of extinction of others. In the presence of a stress inducer such as copper, different species may show different types of response (ranging from no response to sublethal to lethal effects). It may be expected that the interaction of any pair of species will be affected, solely as a result of their differential tolerance. If, for example, one species of a pair of competitors is more severely affected, then the success of the other might increase. On the other hand, the type of interaction between any pair of species might determine the apparent tolerance of these organisms to the stress agent. For example, although a predator may not exhibit
signs of stress due to a physiological response to an inhibitor, its prey might. In this case, an indirect effect of reduction of food availability may result in reduced success of the predator. It becomes obvious, therefore, that a single-species tolerance test simply cannot describe the true ecological tolerance of individual species.

A number of studies has been carried out in an attempt to determine the effect of copper additions on communities. These studies have examined either the whole community (e.g. a phytoplankton assemblage) or its components (e.g. species composition). Goering et al. (1977) observed an increase in silica uptake of a phytoplankton assemblage at high copper concentrations and Harrison et al. (1977) recorded an initial reduction in nitrate assimilation rate of a phytoplankton assemblage that was, however, alleviated within a few days. Crossey and LaPoint (1988) observed an increase in chlorophyll $a$ and respiration associated with a decrease in primary production: chlorophyll $a$ and of production: respiration ratios in a polluted creek. These studies are quite helpful in assessing the overall impact on the community and the impact of the community response on their habitat. Unfortunately, they are not very explicit as to the specific changes expected within the community due to differential response of individual members.

Changes in species composition, dominance and succession have been examined for some communities. The most commonly encountered effects of increased copper levels involve a shift in species dominance and a change in the species diversity. Effler et al. (1980) observed an initial increase in cyanobacteria immediately after low copper treatment of a lake. This was accompanied by a sharp decrease in bacterial populations which, however, recovered very quickly. Daphnia also demonstrated an initial decrease followed by large oscillations. In a similar study, McKnight (1981) observed the disappearance of the dominant dinoflagellate Ceratium followed by an increase in cyanobacteria. Daphnia was replaced by a cyclopoid copepod. Yasuno and Fukushima
(1987), in an examination of polluted rivers, showed that the diatom *Acanthess minutissima* dominated all polluted areas.

Similar studies have been carried out in the marine environment. Thomas *et al.* (1980b) recorded the disappearance of *Nitzschia delicatissima* and *Chaetoceros* spp. from a phytoplankton assemblage exposed to a high metal mix concentration and a shift in dominance to *Skeletonema costatum* and *Thalassiosira* spp. Hollibaugh *et al.* (1980) recorded *S. costatum* as the dominant species in all metal concentrations of a number of different metals. The presence and density of other species depended upon the concentration of the specific metal. Sanders *et al.* (1981a) observed a shift in dominance from a *Skeletonema* and *Chaetoceros* spp. dominated assemblage to an *Amphiprora paludosa* var. *hyalina* dominated assemblage upon the addition of copper. Sanders *et al.* (1981b) examined the diatom succession in elevated copper. In the undisturbed community, a bloom of *Skeletonema* was followed by a bloom of *Chaetoceros*. Under copper additions, *Skeletonema* remained unaffected and the *Chaetoceros* bloom was replaced by a *Navicula* or *Amphiprora* bloom. Sanders and Cibik (1988) also examined in the laboratory, a shift in dominance of a phytoplankton community isolated from Chesapeake Bay. *Thalassiosira pseuodonana* increased at high arsenic concentration, compared to the control, and eventually flagellates dominated. A bloom of *Cerataulina pelagica* present in the controls never occurred at high arsenic levels. In high silver levels, *S. costatum* was more abundant and blooms were more prolonged than in the control. On the other hand, the concentration of *Rhizosolenia fragilissima* was not affected at all.

As a rule, community diversity tends to decrease in elevated metal concentrations. This has been demonstrated in a number of studies. LaPoint *et al.* (1984) and Roline (1988) have demonstrated this for aquatic macroinvertebrates in polluted streams. Yasuno and Fukushima (1987) and Crossey and LaPoint (1988) reached similar conclusions for attached algae communities from polluted streams. Finally, Stokes *et al.*
(1973) demonstrated increased microalgal biomass and diversity with increasing distance from a smelter outflow in a lake near Sudbury, Ontario.

Harrass and Taub (1985) attempted to examine the impact of copper on communities in the laboratory and compare these to community responses in the field. They used freshwater microcosms (Standard Aquatic Microcosm) that included ten algal and five invertebrate species. At the low copper concentration (0.8 x 10⁻⁵ M added copper), *Daphnia* was severely affected. This resulted in algal biomass accumulation and a rotifer increase. At 3.2 x 10⁻⁵ M Cu, all trophic levels were completely inhibited for extended periods of time. The authors concluded that these results were quite similar to community responses observed in the field. Shannon *et al.* (1985) compared the Standard Aquatic Microcosm (SAM) with the Mixed Flask Culture (MFC). The latter also includes a standard community. The difference is that the MFC examines the overall community responses, such as production and respiration, without considering the species-specific responses because it assumes that "the system level variables are species-independent" (Shannon *et al.* 1985). The authors concluded that overall MFCs demonstrated a higher sensitivity in the community response to metal additions, but lower reproducibility compared to SAM.

Studies that have been performed at the community level are certainly more realistic than single-species toxicity tests in that they take into consideration the biological complexity of a species' habitat. They do demonstrate that the entire status of the community changes with the addition of an inhibitor such as copper at high concentrations. From these studies, it is clear that it is not always the same species that dominates the phytoplankton assemblage. For example, in the studies mentioned above, *S. costatum* does dominate in some instances but is inhibited in others. The response of species that do not have high abundances is even more unpredictable. Partly, the source of this variability can be attributed to the different interactions of the species in the different habitats. The studies at the community level are unfortunately very descriptive.
Copper is added and the response of the community is recorded. In the end, community interactions appear to control the response of the organisms. For example, in the study by Harrass and Taub (1985), when *Daphnia* decreased, phytoplankton abundance increased.

The limitations of both approaches (i.e. single species and community bioassays), therefore, become obvious. A single-species toxicity test, by definition, isolates the organism from its interspecific biological environment. A study at the community level (as performed to date) describes quite precisely the effects on the overall community structure but fails to examine the mechanisms of change. In addition, no association can be made between individual species tolerances and their response at the community level. Interactions between species may play a significant role in controlling species abundance, regardless of their specific copper stress status. This effect will be particularly pronounced in the manifestation of sublethal rather than lethal effects.

Few laboratory studies have actually addressed the importance of species interactions on the metal tolerance of particular species. Rice *et al.* (1981) concluded that, for some metals (e.g. Cd, Zn, Ni), an increase in detrital food quantity resulted in a decrease in metal content in the tissue of the polychaete *Capitella capitata*. They concluded that the increase in population density (which resulted from the higher food quantity) increased competition for available metals which, in turn, decreased the average metal content per individual. The opposite was the case for Cu. No effect was observed with Fe or Mn. LeBlanc (1985) used the cladocerans *Daphnia pulex* and *D. magna*. In the absence of copper, *D. pulex* overtook the cultures. In single species cultures it was shown that with increased exposure time, *D. magna* developed copper tolerance whereas *D. pulex* did not. When cultured together in elevated copper concentrations, with no prior period of acclimatization, an initial bloom of *D. magna* was eventually replaced by *D. pulex* domination. The period before the shift in the species that controlled the cultures was extended, if the organisms were first introduced to a lower initial copper
concentration (which will then inhibit *D. pulex* and acclimatize *D. magna*). An examination of this study reveals that although *D. magna* was the most tolerant species, this tolerance was overridden by the fact that *D. pulex* was the superior competitor. Sanders (1986) observed a shift in phytoplankton dominance towards a bloom of *T. pseudonana* in increased arsenic levels. He also discovered, however, that this particular species of phytoplankton was not preferentially grazed by zooplankton. He concluded that although the zooplankton might not be directly affected at a particular metal concentration, an indirect effect would lead to a shortage of the preferred food item. The importance of food concentration to copper tolerance of *D. magna* was demonstrated by Gerasimov (1987). For all copper concentrations (below a certain level), increased food density enhanced survival and increased growth of this species. Thomas and Robinson (1987) ascertained that the presence of bacteria or the filtrate from a non-axenic culture of *Amphora coffeaeformis* enhanced metal tolerance of the diatom. They concluded that a soluble compound (< 0.22 μm) was produced by the interaction of the diatom and bacteria that alleviated copper toxicity.

The importance of the presence of other organisms for the individual tolerances of species becomes very apparent. Population responses to a toxicant may depend on both the origin of the species involved as well as the organisms with which the habitat is shared. To date, most trace metal studies have ignored this complicating factor and have concentrated on the effect on organisms in single-species cultures. The purpose of this study was to examine the effect of a simple two-species interaction on individual species copper tolerances.

The group of organisms that I chose to study were marine diatoms (Class Bacillariophyceae). They are primary producers, their response to copper has been extensively studied, they are relatively easy to culture and they have short generation times.
The two species that I used were *Skeletonema costatum* (Greville) Cleve and *Nitzschia thermalis* (Ehrenberg) Auerswald. First, I examined their individual copper tolerances using a number of bioassays. Two growth curve parameters were examined, exponential growth rate and length of lag phase (Chapter 1). Subsequently, I assessed the effect of their interaction on their predetermined tolerance. The two species were grown at a control copper concentration (1 x 10^{-9} M Cu- as suggested by Morel *et al.* (1979) for the preparation of an artificial seawater medium). In addition, they were grown at partially stressful copper concentrations. I then compared their performance when grown alone to when grown in the presence of the second species (Chapter 2A). Since the interaction between the two species overrode their copper tolerance, I examined the possible factors that could be driving the interaction and, therefore, controlling the species survival irrespective of copper concentration (Chapter 2B).

Both phytoplankton culturing and trace metal methodology involve a number of traditional, "acceptable" protocols which are not necessarily compatible. These include the use of containers made of different materials and the culturing medium preparation. Since in this study both protocols had to be incorporated, I examined the effect of the two procedures on phytoplankton growth (Appendix 3).
CHAPTER 1: Copper Tolerance of Two Species of Marine Diatoms

INTRODUCTION

The biological effects of copper in both the marine and freshwater environments have been studied extensively. These studies involve both the physiological responses of microalgae to elevated (or reduced) copper concentrations as well as the importance of chemical speciation to copper toxicity.

A copper requirement has been demonstrated for some species, *Chlorella vulgaris, Oocystis marssonii* (Manahan and Smith 1973) and *Gonyaulax tamarensis* (Schenck 1984). One of the first studies to demonstrate the detrimental effect of copper to phytoplankton was by Steeman Nielsen and Wium-Andersen (1970). These authors examined the copper tolerance of *Chlorella pyrenoidosa* and *Nitzschia palea*. Since then, a large number of studies have examined the physiological responses to increased copper concentrations. Growth rate reduction with increasing copper levels is one of the most prominent observations (e.g. Erickson 1972, Jensen et al. 1976, Morel et al. 1978, Fisher et al. 1981, Nakamura et al. 1986). For some species of algae, there have been reports of an increase in the length of lag phase in response to copper elevations (e.g. Steeman Nielsen and Wium-Andersen 1970, Bartlett et al. 1974, Bentley-Mowat and Reid 1977, Morel et al. 1978). There have also been reports of a reduction in photosynthetic rate (e.g. Steeman Nielsen et al. 1969, Steeman Nielsen and Wium Andersen 1970, Erickson 1972, Overnell 1976, Rao and Sivasubramanian 1985) and of an increase in the rate of respiration (Rao and Sivasubramanian 1985) as symptoms of copper toxicity. The latter authors also described a decrease in maximum photosynthetic rate ($P_{\text{max}}$) and a difference in the initial slopes of Photosynthesis vs. Irradiance curves.

A number of studies have examined other physiological responses of phytoplankton cells to copper toxicity. Alterations in cell morphology (e.g. Erickson
1972, Sunda and Lewis 1978, Thomas et al. 1980a, Fisher et al. 1981) and reduction in cell motility (Anderson and Morel 1978) are two examples. Pekkala and Koopman (1987) observed an increase in sinking rate with increasing copper concentration after 30 minutes of contact time. Fisher et al. (1981) examined a number of physiological effects due to elevated copper. They reported an increase in the levels of C · cell⁻¹, N · cell⁻¹, chl a · cell⁻¹ and DNA · cell⁻¹ as signs of copper elevation. Kanazawa and Kanazawa (1969) and Rice et al. (1973) suggested that copper interferes with transmembrane ion transport.

Perhaps one of the most important effects at the cellular level, especially for diatoms, is the interference of copper with silicic acid uptake. Valente et al. (1987) demonstrated an inhibition of spore formation of Chaetoceros protuberans in the presence of elevated copper, which was reversed in the presence of high silicic acid concentrations. Rueter (1983) showed that silicic acid uptake is reduced in increasing copper levels. Under Si-starvation, both the uptake rate and the content of the Si-pools in the cells were higher in lower copper concentrations. Although this was the case with Thalassiosira weisflogii, a different response was observed in T. pseudonana. The results of Rueter et al. (1981) suggested for this species a possible increase in uptake (manifested by an increase in Si-cell quota), as well as an increase in the silicon-uptake threshold, with increasing copper concentration. In addition, copper sensitivity was reduced in the presence of higher Si(OH)₄ in the medium. The authors concluded that there is competitive inhibition between the two elements and proposed a model whereby Cu and Si use the same transport site to enter the cell. On the other hand, Thomas et al. (1980a) concluded that for Skeletonema costatum, copper did not act as a silicon competitive inhibitor.

Sunda and Guillard (1976) demonstrated that the copper species that is most toxic to phytoplankton is the cupric ion (Cu²⁺). Canterford (1979) and Canterford and Canterford (1980) showed that an increase in the concentration of the chelating agent
EDTA (which complexes ionic copper) in the phytoplankton medium, enhanced the copper tolerance of *Ditylum brightwellii*. Nakamura *et al.* (1986) concluded that growth sensitivity of *Chattonella antiqua* to copper was dependent upon the EDTA concentration and not the total copper concentration. Sunda and Lewis (1978) suggested that growth rate in *Monochrysis lutheri* was negatively associated with ionic copper concentration. Gavis *et al.* (1981) examined the effect of cupric ion concentration on the growth of phytoplankton quite extensively. They determined three types of responses of growth to increase in pCu (the negative logarithm of free cupric ion activity). The "one-step" response (which has a single threshold of growth inhibition), "one-step incipient" (which is a no-response curve), and a "two-step" response (which includes a lower and a higher threshold of partial and complete inhibition respectively). However, Florence and Stauber (1986) demonstrated that some ligands (such as phenanthroline) form complexes with copper that are very toxic as well. This type of complex catalyzes the production of excited oxygen species (oxygen free radicals) from molecular oxygen (such as H$_2$O$_2$) inside the cell. These free radicals then react with most biological substances (such as amines, enzymes etc.) and may, therefore, inhibit a number of processes in the cell (e.g. lipid oxidation). Florence *et al.* (1984) suggested that the lipid solubility of the copper complexes is what is of primary importance in copper toxicity and not simply whether the ionic copper is complexed.

The cell membrane is a site of copper binding before the metal enters the cell. Gavis *et al.* (1981) suggested that negative charges on the membrane bind the positively charged cupric ion. They proposed that the cupric ions bind to carboxyl and amino groups that are present on the cell surface. Fisher and Jones (1981) concluded that the EC$_{50}$ (the concentration of metal at which growth rate is reduced to 50% of the control) of *Asterionella japonica* for several metals, including copper, was correlated with the solubility of their sulphides. A negative correlation was found with the stability constants of the metals for cysteine and methionine (which are sulfhydryl-containing
amino acids). They concluded that sulfhydryl binding of metals is the manner in which inhibition occurs.

It has been demonstrated that cells remove copper from the medium in proportion to the levels present (e.g. Mandelli 1969, Bentley-Mowat and Reid 1977). In natural seawater, from an area of upwelling, phytoplankton copper uptake was not large enough to account for any changes in copper concentration in the water (Knauer and Martin 1973). In the laboratory, an initial rapid uptake followed by copper release has been documented for some species (e.g. Mandelli 1969, Bentley-Mowat and Reid 1977, Anderson and Morel 1978). Mandelli (1969) also showed an increase in copper uptake with increasing temperature and salinity. Jones et al. (1987) demonstrated reduction (Cu(II) → Cu(I)) on the cell wall. They suggested a non-renewable cell wall component as being responsible for the reduction. They also suggested that the reduction rate of Cu(II) at the cell surface is not related to the cupric ion activity but rather to the total copper concentration and the Cu-organic complexes. The authors concluded that a trans-plasmalemma NAD(P)H-cytochrome c reductase-type enzyme was responsible for the reduction.

A few studies have addressed the mechanisms of microalgae reactions that prevent copper toxicity. Foster (1977) suggested copper exclusion. A less tolerant strain of Chlorella vulgaris showed higher copper uptake than a more tolerant one. In Amphora spp., copper was found bound on spherical, polyphosphate bodies or on electron dense, irregular-shaped bodies (Daniel and Chamberlain 1981). The latter had a high copper:sulfur ratio and were considered bodies used for the deactivation and removal of the metal from the cytoplasm. The polyphosphate bodies contained copper inconsistently and were suggested to provide indiscriminate binding sites. Cloutier-Mantha and Brown (1980) proposed that in Skeletonema, amino acids are used for detoxifying metals.
*Skeletonema costatum* is a phytoplankton species that has been used extensively for copper toxicity tests. Mandelli (1969) determined the copper level of growth inhibition in a 16:8 h L:D cycle as $8 \times 10^{-7}$ M Cu and in continuous light as 2.6 to 4 x $10^{-6}$ M. Jensen *et al.* (1976) obtained an 80% reduction in growth with the addition of $1.6 \times 10^{-7}$ M Cu to natural seawater of unknown copper background level. According to Overnell (1976), this species suffered a 50% reduction in photosynthetic rate in the presence of $5 \times 10^{-5}$ M Cu. Berland *et al.* (1977) successfully grew this species in concentrations up to $1.6 \times 10^{-6}$ M Cu added to natural seawater in the absence of chelators. In the same study, a decrease in growth (no inhibition was ever obtained) at $3.2 \times 10^{-6}$ M was observed. Fisher and Frood (1980) observed a halving in growth rate with the addition of $8 \times 10^{-8}$ M Cu to natural seawater. From the results of Khan and Saifullah (1986), *S. costatum* showed progressively decreasing growth rates with increasing copper levels with a complete inhibition around $3.2 - 4.8 \times 10^{-7}$ M (no added EDTA). Finally, perhaps the most detailed study of the copper tolerance of this species is by Morel *et al.* (1978). No apparent effect on growth was observed in copper concentrations up to $6 \times 10^{-6}$ M Cu. This study was performed in Aquil (Morel *et al.* 1979) in the presence of $5 \times 10^{-6}$ M EDTA (pCu = 8.8). The copper inhibition level lies somewhere around $1 \times 10^{-6}$ M (Fitzgerald and Faust (1963) suggested that one part EDTA immobilizes one part copper; as Motekaitis and Martell (1987) cautioned, however, this will depend on the EDTA concentration; these authors showed that at $1 \times 10^{-9}$ M EDTA less than 1% of the copper is bound whereas in $1 \times 10^{-7}$ almost all of the metal is bound). Gavis *et al.* (1981) concluded that the growth rate of *S. costatum* demonstrates a two-step response to the negative log of cupric ion activity.

Increased copper concentrations result in a number of other physiological effects for *S. costatum*. Morel *et al.* (1978) found an increase in lag phase with increasing copper concentration, however, this effect was reversed in the presence of high silicic acid. Thomas *et al.* (1980a) observed elongated *S. costatum* cells in the presence of high
copper levels but no cell aberrations were apparent. No effect on cell shape was observed by Fisher and Frood (1980). The same authors found a decrease in growth rate with increasing cellular copper load.

Beside centric diatoms, pennates are also commonly used bioassay microalgae. Mandelli (1969) demonstrated growth inhibition in the pennate diatom *Nitzschia closterium* similar to that observed for *S. costatum*. Steeman Nielsen and Wium-Andersen (1970) observed growth inhibition of *Nitzschia palea* in $2 \times 10^{-7}$ M Cu (in the absence of EDTA, iron added as a fresh precipitate) at an initial cell concentration of 10,000 cells $\cdot$ mL$^{-1}$ and in $8 \times 10^{-8}$ M Cu at an inoculum size of 100 cells $\cdot$ mL$^{-1}$. Fisher and Frood (1980) examined the effect of copper on the growth of *Nitzschia closterium* and *Asterionella japonica*. The growth of these two species was reduced to a third of the control for the former and a half for the latter in the presence of $8 \times 10^{-8}$ M Cu added to natural seawater (which averaged $\sim 1 \times 10^{-9}$ M background copper concentration). Fisher *et al.* (1981) showed a decrease in growth rate in the presence of $4 \times 10^{-7}$ M Cu in f/2 enriched natural seawater. Lumdsen and Florence (1983) demonstrated a decoupling of photosynthesis from cell division for *N. closterium*. Although their growth rate was reduced in medium containing $-4.8 \times 10^{-7}$ M Cu, their photosynthetic rate was not affected. No such effect was observed for *Asterionella glacialis*. Florence and Stauber (1986) and Stauber and Florence (1987) suggested a mechanism for such decoupling in *N. closterium*. It involved the depression of the reduced glutathione:oxidized glutathione ratio and the subsequent suppression of mitotic division (for which a high ratio is required). The oxidation of the reduced glutathione is catalyzed by cupric ions. This reaction is cytosolic. In addition, ionic copper inhibits glutathione reductase, the enzyme that could reverse the reaction (Florence and Stauber 1986).

In terms of other physiological responses, Thomas *et al.* (1980a) observed very few cell aberrations in pennate diatoms growing in elevated copper. *Nitzschia delicatissima* was sometimes present in the twinned state. *N. closterium* was sometimes
discovered with abnormally swollen cells. *N. pungens* never showed any morphological alterations. Fisher *et al.* (1981) observed an increase in cell volume for *A. japonica* in the presence of elevated copper. The same result was obtained by Fisher and Frood (1980), although no effect on cell morphology was observed for *N. closterium*. Stauber and Florence (1987) observed no copper effect on the ultrastructure of the chloroplasts, nucleus, mitochondria or the cell membrane of the same species.

The similarity in metal tolerance of different strains of the same species has been the topic of only few studies. Most of them involved two strains of *S. costatum* (Skel-0 and Skel-5) which were isolated from two different fjords in Norway. Jensen *et al.* (1976) obtained very similar growth responses to copper additions for these two strains. Both were reduced to 20% growth of the control in 1.6 x 10^-7 M added Cu (to filtered seawater with unknown copper content). For zinc, different types of growth responses were obtained for the two strains (Jensen *et al.* 1974). Skel-0 demonstrated reduced but uninterrupted growth in elevated zinc concentrations. Skel-5, on the other hand, at the same zinc levels, showed normal growth for three days followed by a rapid decline in cell numbers. Both strains demonstrated equal tolerance to zinc in terms of photosynthetic rate (Overnell 1976). Braek *et al.* (1976) examined the combined effect of copper and zinc on the same two strains of *S. costatum*. Synergism between the two metals was observed for both strains. In this study however, Skel-5 was proven slightly more sensitive to copper addition (in the absence of zinc) than Skel-0. The combined effect of cadmium and zinc was not quite so similar for the two strains (Braek *et al.* 1980). Skel-0 was more sensitive to cadmium whereas Skel-5 was more sensitive to zinc. In combination, the two metals acted synergistically in Skel-5, whereas increased zinc concentration alleviated cadmium toxicity in Skel-0. Although both strains accumulated the same amount of zinc in the course of the experiments, Skel-0 accumulated more cadmium than Skel-5 (Braek *et al.* 1980).
This chapter examines the individual copper tolerances of Skeletonema costatum and the pennate Nitzschia thermalis. The parameters of growth that I chose to observe were exponential growth rate and length of lag phase. I shall discuss the change in these growth parameters with increasing copper concentration. In addition, I will examine the similarity in copper tolerance between two strains of S. costatum.

**SELECTION OF ORGANISMS**

The diatom species that I chose to examine are species that are present in high abundances in tidepools. Tidepools can be considered, to a certain extent, small, partially enclosed ecosystems during at least the period that they remain isolated from the ocean (during low tide). During the period of complete isolation (which could vary from hours to days), the interaction of diatoms that are present there will be maintained rather than become diluted by water movement (the latter might be the case in a large, dynamic body of water). Diatom species that are found together in tidepools might interact with each other in a particular manner. Any effect that such an interaction might have on their copper tolerances will play a role in determining the structure of the community under copper stress.

Five tidepools as well as the sea-surface were sampled on five occasions during May-June, 1988, at Point Atkinson, West Vancouver, British Columbia. The sampling strategy, the enumeration procedure and a summary of the results are presented in Appendix 1. A number of patterns regarding species’ abundance emerged from this study:

1. *Skeletonema costatum* was mostly abundant at the sea-surface and in lower tidepools.
2. Pennates, as a group, were not very abundant at the sea-surface, but were the group with the highest abundance in all of the tidepools on all five occasions.
3. *Melosira* demonstrated low densities at the sea-surface. This genus reached large concentrations in most tidepools.
Genera of benthic diatoms such as *Odontella*, *Surirella* and *Gyrosigma* were almost non-detectable at the sea-surface, yet consistently appeared in the tidepools in moderate abundance.

Of the described groups, I chose *S. costatum* and the pennates to use in this study. This was due to the high abundances that both these taxa attained in most tidepools. The actual species that were used in this thesis were not isolated from tidepool samples. They were both obtained from the North East Pacific Culture Collection, University of British Columbia, Vancouver. They were isolated from local waters, *S. costatum* from the Strait of Georgia and *Nitzschia thermalis* from Jericho Beach, Vancouver. Unfortunately, I am not able to determine whether the latter was included in the group of pennates that were present in the tidepool samples. However, this species has been observed in several different areas: in the marine environment it has been found in the Bay of Fundy, New Brunswick (Linkletter *et al.* 1977) and in a marine mudflat of the Eems-Dollard estuary, in the Netherlands (Admiraal and Peletier 1979). In addition, it has been classified as a marine littoral diatom (Lewin 1963), although the location from which it was isolated is not mentioned in that study. In freshwater, it has been collected from lakes in the Swiss Alps (Brun 1880). It has also been found in high abundances on the walls of water reservoirs, and in swamps and sloughs in South Africa (Cholnoky 1968). In east Africa, it was found as a member of the lake periphyton and in rivers and ponds, with the highest relative abundance reached in a small "salted" pond (Gasse 1986). Foged (1978), in a compilation of data on the diatoms of eastern Australia, classified this species as cosmopolitan. Unfortunately, this *Nitzschia* species has been extensively misidentified (e.g. see VanLandingham 1978) and no consistent habitat pattern exists. Since this species has been found in temperate estuaries as well as in freshwater, its presence was quite likely in the brackish tidepools of Point Atkinson (with respect to its tolerance to physical factors such as salinity and temperature). I therefore felt that it was a reasonable representative of the pennate group that was present in the tidepool samples.
METHODS

Stock cultures of two strains of *Skeletonema costatum* (NEPCC 18c and NEPCC 676) and one strain of *Nitzschia thermalis* (NEPCC 608) were obtained from the North East Pacific Culture Collection, Department of Oceanography, University of British Columbia, Vancouver. Once obtained, the cultures were maintained in polycarbonate 250 mL Erlenmeyer flasks, using artificial seawater (Aquil) prepared as in Morel *et al.* (1979) except for two modifications. I discovered that under the laboratory conditions used, trace metal contamination was introduced into the Aquil when I bubbled CO$_2$ to drop the pH before autoclaving. For this reason, I only raised the pH of both the seawater and the Chelex-100 resin to 7.5 instead of 8.0 (which is recommended in Morel *et al.* 1979). In this manner, after autoclaving and allowing the medium to stand for at least 48 hrs., the pH was raised only up to ~8.4 and no noticeable precipitate was observed. The second alteration in the procedure was in the preparation of the trace metal mix. Iron chloride was added as fresh Fe-precipitate (iron was allowed to precipitate in distilled deionized water -Nanopure- for 24 hrs.) rather than a Fe-EDTA mix. The reason for this modification was the very high affinity that EDTA has for copper (99.5%, Jackson and Morgan 1978), which might influence the copper toxicity results. The metal stock solution was prepared by the addition of the remaining trace metals into the Fe-precipitate stock, allowing at least 2-3 hrs. for equilibration of the solution. Before use, the flasks were washed with Sparkleen Detergent, soaked in 1N HCL for at least 24 hrs., rinsed three times with Nanopure water, and autoclaved in a Castle standard laboratory autoclave, at 21 psi for 20 minutes. The microalgal cultures were kept in a culture chamber at ~16° C, under a 16:8 h L:D cycle, at an irradiance of ~90 μE · m$^{-2}$ · s$^{-1}$. All cultures were unialgal but not axenic (although sterile technique was used throughout).

Generally, the same procedure was followed with the replicate diatom cultures during the experiments. Both the flasks and the medium were processed in a similar
manner as for the stocks. After the medium was autoclaved and allowed to stand for at least 24 hrs., 200 mL were added to the washed flasks. Subsequently, the fresh metal and vitamin stocks were added to the appropriate concentrations. Finally, the freshly prepared copper stocks (prepared by the dissolution of CuSO₄ in DDW) were included in volumes necessary to attain the desired concentrations. The flasks were allowed to equilibrate with the medium for 24 hrs. after which they were inoculated with aliquots from exponentially growing diatom stock cultures. All transfers were performed in a class 100 laminar flow hood with all possible metal parts replaced by polypropylene.

Every 24 hrs., 10 mL aliquots were removed from the experimental flasks after gentle shaking to ensure homogeneous diatom distribution, and used for cell counts and fluorescence measurements. In vivo fluorescence was measured using a Turner Designs Model 10 fluorometer. Cell counts were performed using a Palmer Maloney counting slide under a Zeiss Standard 14 compound microscope. The lowest cell density that I could detect using the cell counts was 200 cells \( \cdot \) mL\(^{-1} \). For each replicate, three subsamples were counted using five fields per subsample. The fields represented a random cell distribution as recommended by Lund et al. (1958).

Three sets of toxicity tests were run for both \( S. \) costatum (strain NEPCC 18c) and \( N. \) thermalis. Each set was designed to examine a narrower range of copper concentrations than the preceding set. In the first set, the concentrations of added total copper were \( 1 \times 10^{-5} \text{ M}, 1 \times 10^{-7} \text{ M}, 1 \times 10^{-11} \text{ M} \) (treatments) and \( 1 \times 10^{-9} \text{ M} \). For each of the subsequent sets, the concentration that was used as the "control" was the highest from the preceding set at which no significantly different growth from the next lower concentration was observed (as demonstrated by a Student’s t- or a Wilcoxon test). The final set of toxicity tests was performed at the \( 1 \times 10^{-7} \text{ M} \) level (0.1 - 1 \( \mu \text{M} \) added Cu) for both species.

In order to compare copper tolerance between strains of the same species, I attempted the same procedure for the second stock of \( S. \) costatum (NEPCC 676). This
strain was proven very sensitive and therefore I only managed to carry out tests using only the widest range of copper concentrations (first set). As a result the comparison was accomplished at a very general level.

Three replicates were used for each copper concentration, for all tests that were carried out with all three strains. Replicates were arranged in a systematic design on shelves, ensuring equal light intensity in the interior of all the containers.

For each culture, two parameters of growth were examined, exponential growth rate and length of lag phase. Exponential growth rate was determined using $K_e$ and $k$ as in Guillard (1973) (where $K_e = [\ln (N_t/N_0)] / [t_1 - t_0]$ and $k$ (div • day$^{-1}$) = $K_e / 0.6931$). The exponential portion of the growth curve was determined as the part with the steepest overall slope. $N_0$ and $N_1$ for each replicate were chosen as the points beneath or beyond which there was an apparent deviation from this slope. The length of the lag phase was determined as the time between inoculation and the time at which $N_0$ was estimated.

The effect of both added total copper concentration and initial $pCu$ on exponential growth rate and lag phase was explored using linear regressions. Initial $pCu$ in the medium at pH 8.4 was calculated assuming only inorganic complexation of copper, primarily by carbonate ions (as suggested by Byrne and Miller (1985)). It was calculated from the following equations:

$$p[Cu^{2+}] = -\log [\text{total copper}] + \log \alpha$$

$$pCu = p[Cu^{2+}] + 0.68$$

where $p[Cu^{2+}]$ is the negative log of the free cupric ion concentration, [total copper] is the added total copper concentration, $\alpha$ is the ratio of total copper concentration to free cupric ion concentration at the pH of the medium, and 0.68 is the negative log of the activity coefficient (0.21) in seawater. The value for the activity coefficient was estimated by Byrne and Miller (1985). Byrne et al. (1988) estimated $\log \alpha$ for copper in seawater at 25°C and pH 7.6 and 8.2 (both these factors affect the ionic concentration of copper in seawater). From extrapolation and due to the linearity of the relationship
between log α and pH within this narrow range of pH values, log α at pH 8.4 was estimated to equal 1.48. By using the two equations, I was able to convert the total copper concentration to pCu.

For the regressions of growth rate against copper concentration and pCu, I used all the values that I obtained during any set of toxicity tests that included the specific concentration. For the regressions of lag phase, only the values from the last set of toxicity tests were included. The regressions were estimated using the MGLH module of SYSTAT (Wilkinson 1985) on a Commodore PC-10 II. A significance level of 0.05 was used throughout.
RESULTS

The final sets of toxicity tests for *S. costatum* (18c) and for *N. thermalis* are presented in Figs. 1.1-1.5. The results for the preliminary sets are included in Appendix 2. The copper concentrations that I used for *S. costatum* in the final set were 0.08 μM and 0.1 to 0.5 μM Cu in 0.1 μM intervals (Figs. 1.1,1.2). For *N. thermalis*, the final copper concentrations were 0.1, 0.3, 0.5 and 0.6 to 1.0 μM Cu in 0.1 μM intervals (Figs. 1.3,1.4). In addition, I ran an extra experiment at 0.3 and 0.4 μM Cu (Fig. 1.5). One of the reasons for this was to determine the reaction of this organism at a copper concentration closer to the lethal one.

*S. costatum* grew very well in concentrations of 0.08-0.4 μM Cu and did not grow at all in 0.5 μM added Cu (Figs. 1.1 and 1.2). One of the replicates of the 0.5 μM Cu treatment did, however, demonstrate signs of potential increase. Since no growth was ever observed in all the other replicates that I ever ran at this concentration, I did not use that replicate in my growth rate estimations. In addition to the inhibition of growth at 0.5 μM added total Cu, there was an increase in the length of lag phase as the latter concentration was approached. Fig. 1.6 demonstrates the change in growth rate with increasing copper concentration for both types of biomass measurements and Fig. 1.7 the change in growth with pCu. Linear regressions demonstrated a significant increase in growth rate with total copper concentration up to but excluding the lethal level of 0.5 μM (reg. coeff. = 2.13; 1.56, $r^2 = 0.681$; 0.333, $N = 24$, $p < 0.001$; <0.01 for the *in vivo* fluorescence and the cell counts estimations, respectively). An inverse relationship was observed between growth rate and pCu (reg. coeff. = -0.842; -0.582, $r^2 = 0.666$; 0.291, $N = 24$, $p < 0.001$; <0.01 for *in vivo* fluorescence and the cell counts estimations, respectively). Lag phase demonstrated a similar increase with added total copper (reg. coeff. = 10.10; 10.47, $r^2 = 0.451$; 0.422, $N = 24$, $p < 0.01$ for fluorescence and cell count measurements, respectively) and a decrease with pCu (reg coeff. = -4.27; -4.53, $r^2 =$
0.409; 0.403, N = 24, p < 0.05 for fluorescence and cell counts, respectively) (Figs. 1.10A and 1.11A).

*N. thermalis* was slightly more tolerant than *S. costatum*. This species did grow in copper concentrations of up to 0.5 μM inclusive (Figs. 1.3-1.5). In these results, I have included the final set of toxicity tests as well as a last experiment that I conducted in order to add the results concerning growth at a concentration of 0.4 μM added total Cu (Fig. 1.5). Unlike *Skeletonema*, this species showed a different type of growth response to increasing added total copper concentrations and pCu (Fig. 1.8). For the fluorescence measurements, a significant decrease was observed between 0.1 and 0.5 μM added total Cu (reg. coeff. = -0.710, \( r^2 = 0.211 \), N = 23, p < 0.05). The relationship was even stronger when the growth rates were examined between 0.3 and 0.5 μM (reg. coeff. = -2.25, \( r^2 = 0.598 \), N = 14, p = 0.001). The relationship of growth rate with pCu was only present between 8.66 and 8.16 which corresponds to 0.3 and 0.5 μM total copper (reg. coeff. = 2.25, \( r^2 = 0.598 \), N = 14, p = 0.001) (Fig. 1.9). No significant pattern was observed for the cell counts, probably because of the larger error associated with the growth rate at 0.5 μM added total Cu and pCu = 8.56. The length of the lag phase was not affected by increasing total copper concentration or decreasing pCu (Figs. 1.9B, 1.10B).

As mentioned in the methods section, the second strain of *S. costatum* (NEPCC 676) did not grow after the first set of tolerance tests (1 x 10^-5, 1 x 10^-7, 1 x 10^-9 and 1 x 10^-11 M added total copper). As a result, I only present the results from that set (Figs. 1.12-1.13). This strain (NEPCC 676) grew adequately up to 1 x 10^-7 M Cu and did not grow at all at 1 x 10^-5 M Cu. This is in agreement with the results for the first strain (18c), as demonstrated in Figs. 1.14-1.15. Therefore, in general, the two strains showed similar copper tolerance, at least within the same two orders of magnitude. They both grew at copper concentrations up to 1 x 10^-7 M and neither grew at 1 x 10^-5 M added total copper.
From the fluorescence measurements, the second strain (676) demonstrated a much lower growth rate (Figs. 1.14A, 1.15A). This was suspected to be primarily due to a poor physiological state of this strain during the experiments. In addition, a significant increase in growth rate with both total copper concentration and pCu was observed in the cell count estimates (Figs. 1.14B, 1.15B) (reg. coeff. = 0.223; -0.223, $r^2 = 0.630$; 0.630, N = 8, p < 0.05 for total copper and pCu respectively).
FIGURE 1.1: Growth curves of *Skeletonema costatum* (NEPCC 18c) at $5 \times 10^{-7}$, $4 \times 10^{-7}$ and $3 \times 10^{-7}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 1.2: Growth curves of *Skeletonema costatum* (NEPCC 18c) at $2 \times 10^{-7}$, $1 \times 10^{-7}$ and $8 \times 10^{-8}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 1.3: Growth curves of *Nitzschia thermalis* (NEPCC 608) at $1 \times 10^{-6}$, $9 \times 10^{-7}$, $8 \times 10^{-7}$, $7 \times 10^{-7}$ and $6 \times 10^{-7}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. $N_0$ and $N_1$ were not estimated because no apparent exponential growth occurred.
FIGURE 1.4: Growth curves of *Nitzschia thermalis* (NEPCC 608) at $5 \times 10^{-7}$, $3 \times 10^{-7}$ and $1 \times 10^{-7}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 1.5: Growth curves of *Nitzschia thermalis* (NEPCC 608) at $4 \times 10^{-7}$ and $3 \times 10^{-7}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 1.6: Effect of added total copper concentration on the growth rate of *Skeletonema costatum* (NEPCC 18c). (A) Fluor = *in vivo* fluorescence; (B) Cell no. = cell counts. Error bars represent ± one standard deviation (N=3 except at 0.1 μM, N=9 and at 0.5 μM, N=6).
FIGURE 1.7: Effect of free cupric ion activity on the growth rate of *Skeletonema costatum* (NEPCC 18c). (A) Fluor = *in vivo* fluorescence; (B) Cell no. = cell counts. Error bars represent ± one standard deviation (N=3 except at pCu=8.56, N=5 and at pCu=9.16, N=9).
FIGURE 1.8: Effect of added total copper concentration on the growth rate of *Nitzschia thermalis* (NEPCC 608). (A) Fluor = *in vivo* fluorescence; (B) Cell no. = cell counts. Error bars represent ± one standard deviation (N=3 except at 0.1 μM, N=9, at 0.3 μM, N=6 and at 0.5 μM, N=5).
FIGURE 1.9: Effect of free cupric ion activity on the growth rate of *Nitzschia thermalis* (NEPCC 608). (A) Fluor = *in vivo* fluorescence; (B) Cell no. = cell counts. Error bars represent ± one standard deviation (N=3 except at pCu=8.46, N=5, at pCu=8.66, N=6 and at pCu=9.16, N=9).
FIGURE 1.10: Effect of added total copper concentration on the length of lag phase of (A) *Skeletonema costatum* (NEPCC 18c) and (B) *Nitzschia thermalis* (NEPCC 608). Error bars represent ± one standard deviation (N=3 except in (B) at 0.5 μM, N=2).
FIGURE 1.11: Effect of free cupric ion activity on the length of lag phase of (A) *Skeletonema costatum* (NEPCC 18c) and (B) *Nitzschia thermalis* (NEPCC 608). Error bars represent ± one standard deviation (N=3 except in (B) at pCu=8.46, N=2).
FIGURE 1.12: Growth curves of *Skeletonema costatum* (NEPCC 676) at $1 \times 10^{-5}$ and $1 \times 10^{-7}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 1.13: Growth curves of *Skeletonema costatum* (NEPCC 676) at $1 \times 10^{-9}$ and $1 \times 10^{-11}$ M Cu. (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 1.14: Effect of added total copper concentration on the growth rate of *Skeletonema costatum* clones NEPCC 18c (= S. costatum) and NEPCC 676 (= S. costatum*). (A) *In vivo* fluorescence; (B) Cell counts. Error bars represent ± one standard deviation (N=3).
FIGURE 1.15: Effect of free cupric ion activity on the growth rate of *Skeletonema costatum* clones NEPCC 18c (= *S. costatum*) and NEPCC 676 (= *S. costatum*). (A) *In vivo* fluorescence; (B) Cell counts. Error bars represent ± one standard deviation (N=3).
DISCUSSION

The results that I obtained generally agree with those of other researchers. The tolerance of both species to added total copper is within the 0.1-1.0 μM range. Most of the studies that involved either *S. costatum* or pennate diatoms obtained reduction in growth within the same range of copper concentrations.

For *Skeletonema*, the copper level of growth inhibition that I obtained (5 x 10^{-7} M Cu) is quite similar to the one obtained by Mandelli (1969) and very similar to that of Khan and Saifullah (1986). The similarity with the latter study is of great importance because the authors did not include EDTA in the medium preparation. The results of Jensen *et al.* (1976) demonstrate a lower copper tolerance level for this species (1.6 x 10^{-7} M). These authors however, used copper additions to natural seawater while the background concentration remained unknown. Since neither total copper nor cupric ion activity is included in their study, I cannot compare their results to mine. On the other hand, Berland *et al.* (1977) obtained higher copper tolerance for the same species (inhibition at 1.6 x 10^{-6} M). Again these authors added copper to natural seawater. The background concentration is not known and neither is the degree of complexation. Once again, no conclusion can be made by comparison of the two studies. Morel *et al.* (1978) concluded that the level of inhibition for *Skeletonema* was 6 x 10^{-6} M Cu in the presence of 5 x 10^{-6} M EDTA. This corresponded to pCu = 8.8 which is slightly higher than 8.46, the value of inhibition in my study.

In this study, *Nitzschia thermalis* demonstrated slightly higher toxicity tolerance (inhibition at 6 x 10^{-7} M) than *Skeletonema costatum*. Once again, the copper levels that inhibited *N. thermalis* in this study are similar to those that have been shown to inhibit other species of pennates (e.g. Steeman Nielsen and Wium-Andersen 1970, Fisher *et al.* 1981, Lumdsen and Florence 1983).
There are problems associated with determining the copper concentration at which inhibition of growth occurs for a particular species. One of the major difficulties relates to the composition of the medium used. High organic complexation decreases copper bioavailability (e.g. Anderson and Morel 1978, Jackson and Morgan 1978, Sunda and Lewis 1978). If the medium that is used for culturing microalgae is natural seawater (unfiltered or not UV-treated to remove the organics) then the amount of copper that has to be added to the medium in order to make inhibition apparent ought to be quite large. On the other hand, if natural seawater is used, with undetermined copper concentration, one will only obtain relative values of copper that might induce toxicity. Finally, the presence of an artificial chelator will complicate measurement of the amount of copper present in a form that will affect the organism. As mentioned earlier, EDTA (as well as NTA) has a very high affinity for copper and will complex it in the medium. A number of studies (e.g. Steeman Nielsen and Wium-Andersen 1970, Khan and Saifullah 1986, Kazumi et al. 1987), including this one, avoid the addition of EDTA and include the iron as a fresh precipitate. Although this procedure avoids the very strong binding of copper to EDTA, it introduces a new site for copper adsorption which also binds copper quite strongly (suggested by Jackson and Morgan 1978, Laxen 1984/1985, Stauber and Florence 1985a). In addition, as an experiment progresses, organics that are released from the organisms (e.g. organic exudates by algae and lysis of dead cells) will complex copper and thus render it unavailable to the organisms (e.g. Steeman Nielsen and Wium-Andersen 1971, McKnight and Morel 1979, Fisher and Fabris 1982). So, over the course of a one-week algal bioassay, the chemical speciation in the medium will change in such a way that it makes it impossible to determine precisely how much of the copper is still present in a form that is available to the organism.

A number of methods have been developed to establish either the degree of copper complexation or the ionic copper concentration in the medium. These involve modelling of the chemical species present in seawater (e.g. Zirino and Yamamoto 1972,
Westall et al. 1976, Borgmann 1981, Sunda and Huntsman 1983, Zuehlke and Kester 1983, Brand et al. 1986), equilibration with MnO₂ (Van den Berg 1982), various types of ion-exchange resins (e.g. Mills et al. 1982, Mackey 1983, Zorkin et al. 1986, Sunda and Hanson 1987), determination of the labile fraction by Anodic Stripping Voltammetry (e.g. Florence et al. 1982), bacterial bioassays (e.g. Gillespie and Vaccaro 1978, Sunda and Ferguson 1983, Sunda et al. 1984, Morel et al. 1988), copper ion-specific electrodes (Davey et al. 1973) or a combination of the above (e.g. Batley and Florence 1976, Figura and McDuffie 1980, Buckley and Van den Berg 1986, Hering et al. 1987). All of these methods have their limitations. Some are based on assumptions that are too simplistic, others do not have the sensitivity required to determine the species of copper that are present in very low concentrations. With others, the species that are measured are not exactly known.

For all these reasons, comparisons between studies for specific levels of copper that cause growth inhibition are very difficult. The fact that in most cases the results of different studies, using different media and generally slightly different culturing conditions, are within the μMolar level of each other is very encouraging.

The increase in lag phase of S. costatum that was observed during this study with increase in copper concentration agrees with the results obtained by Morel et al. (1978). These authors also concluded that there was an increase in length of lag phase at copper concentrations within the same order of magnitude of the level that caused complete growth inhibition (at the 1 μM level for their study, compared to the 0.1 μM for my study or at pCu values between 9.5 and 8.5 for both studies). Morel et al. (1978) suggested "conditioning" of the medium and/or adaptation of the cells to the high copper concentrations as possible mechanisms for the effect on lag phase. A number of studies have demonstrated the excretion of organic exudates by microalgae (e.g. McKnight and Morel 1979, Fisher and Fabris 1982). Although these substances may not be excreted specifically in response to metals they nevertheless have an affinity for some metals,
including copper. The organics will alter the copper speciation in the medium by binding
the metal and may render the metal less bioavailable. One can, therefore, suggest that the
medium becomes "conditioned" by the cells themselves and that the conditioning occurs
during the extended lag phase. Another possibility is that the surviving cells may
become "adapted" to the high copper concentration during the extended lag phase. This
adaptation may implicate an intracellular physiological response, for example the
activation of detoxification processes.

One of the most interesting results in this study is the increase of growth rate of
Skeletonema with increasing copper concentration, in a manner similar to the lag phase.
Although I cannot suggest a probable mechanism for this, it is possible that during the
extended lag phase the physiology of the organism changes in a way that enhances its
growth rate. It is also possible that a selection mechanism for more tolerant cells takes
place. As the inhibitory copper level is approached, it may be expected that a larger
portion of the population will be removed due to copper stress. If this is the case, the
population that does remain is going to be smaller but able to withstand higher copper
concentrations. It is also possible that the more tolerant cells also demonstrate higher
growth rates. If this is the case and as the copper tolerance threshold is being
approached, the proportion of more "fit" cells (with higher copper tolerance and division
rates) will increase in relation to the cells that are "unfit" and so the overall growth rate of
the population will increase.

*N. thermalis* did not demonstrate an increase in lag phase. There are no known
studies that suggest an increase in lag phase for any pennate diatoms. On the other hand,
this species showed a decrease in growth rate with increasing copper concentration. This
also is consistent with most toxicity studies.

From these results, it is suggested that copper toxicity is invoked in different
manners in the two species. Lumdsen and Florence (1983) demonstrated a decoupling of
photosynthesis from cell division under copper stress for *N. closterium*. During such
decoupling, photosynthetic rate is not affected whereas cell division is inhibited. No such effect has been demonstrated for *S. costatum*. The proposed mechanism that caused toxicity to *Nitzschia* was attributed to cytosolic reactions. Although such a mechanism has never been suggested for *Skeletonema*, it has not been tested either. It is known, however, that no decoupling occurs (e.g. Overnell 1976). In this study, no difference was observed between the growth measurements carried out using *in vivo* fluorescence or cell counts. Although not tested directly, no decoupling of the two processes is suggested for either of the species used. The mechanism of copper toxicity for *S. costatum* has been explained as competitive inhibition of silicate uptake (Morel et al. 1978). Fisher et al. (1981) demonstrated copper interference with silicic acid uptake in the pennate diatom *Asterionella japonica*. Also, a number of studies have demonstrated interactions between copper and other metals. Manganese has been shown to reduce copper toxicity; increased copper concentrations have been shown to result in lower cellular manganese content (e.g. Sunda et al. 1981, Sunda and Huntsman 1983, Stauber and Florence 1985b, Kazumi et al. 1987). Rueter and Morel (1981) concluded that increased zinc concentration in the medium decreased copper inhibition of silicic acid uptake. These studies have been carried out with both centric and pennate diatoms and there is no reason to believe that any distinction should be made based on groups of diatoms. However, it is possible that in one of the two species used in this study, the amount of a certain metal required to outcompete copper successfully for the binding sites was higher than in the other, therefore causing a different physiological response. Murphy et al. (1984) demonstrated that varied iron, manganese and copper concentrations invoked different responses in *Thalassiosira pseudonana* and *T. oceanica*. Each of these species demonstrated manganese limitation in different copper concentrations. The former required manganese in low copper and high iron levels, while the latter did not. On the other hand, *T. oceanica* required higher manganese concentrations in elevated copper than *T. pseudonana*. 
Any one or a combination of these toxicity mechanisms may be operating in each of the two species that I examined in this chapter. Since I did not evaluate the means by which growth inhibition was invoked I cannot determine the operating factors. The only conclusion that I can reach is that the results strongly suggest that these are different for the two species.

The results of the comparison between the copper tolerances of the two strains of *S. costatum* are very general. The second strain (NEPCC 676) appeared to be in very poor physiological state, and did not survive for a long enough period. As a result I was not able to carry out very detailed bioassays and I can only conclude that the tolerance level of this strain is between 1 x 10^{-7} M Cu and 1 x 10^{-5} M Cu. This is the same range that I obtained from the first set of toxicity tests for strain NEPCC 18c (Appendix 2). The similarity in copper tolerance between strains is in agreement with the results of Jensen *et al.* (1976). The two strains used in my study were isolated from adjacent bodies of water, Burrard Inlet and the Strait of Georgia. It is quite likely that these two strains belong to the same population (i.e. *S. costatum* in the Strait of Georgia) and, therefore, there is no reason to believe that they should have different physiological responses to copper stress. The only difference perhaps arises from the fact that one of the strains (676) was isolated from more coastal waters which might demonstrate elevated copper levels compared to the more open waters of the Strait of Georgia (such as found for the North Pacific Ocean - Bruland 1980, by other authors for the East Coast of N. America- Boyle *et al.* 1981, and for the Northwestern Atlantic - Huizenga and Kester 1983). Murphy and Belastock (1980), in their examination of tolerance to chemical stress of 11 strains of *T. pseudonana* and 6 of *S. costatum*, concluded that strains isolated from coastal polluted waters were more tolerant than strains isolated from open waters. On the other hand, it has been demonstrated that strains of species (such as *Chaetoceros compressum*, *S. costatum*, *N. closterium* and *A. japonica*) isolated from open ocean water were more tolerant to metal stress than strains from coastal waters with
elevated metal levels (Fisher and Frood 1980). This was attributed to the adaptation of the open ocean species to the lower complexation capacity of the water (and therefore to higher metal bioavailability).

It can be concluded from this chapter that although the tolerances of the two species, *S. costatum* and *N. thermalis*, are within 0.1 μM Cu, *Nitzschia* is slightly more tolerant than *Skeletonema*. In addition, it was observed that the two species demonstrated different responses in growth rate and lag phase to elevated copper concentrations. It is, therefore, suggested that the mechanism of toxicity and/or the detoxification process is different in the two species. I suggest that the copper concentrations that would be of primary interest in examining the tolerance of these two species when grown together are 0.4 μM and 0.5 μM Cu. At the first copper level, *Skeletonema* has demonstrated increased growth rate and lag phase (both of which I consider as signs of copper stress) and at the second level, *Skeletonema* demonstrates no growth and *Nitzschia* shows signs of copper stress by a reduction in growth rate. In the next chapter, I shall examine the effect of the interaction with a second species that demonstrates different stress responses, on the individual tolerance of each species at these "sensitive" copper concentrations.
CHAPTER 2: Effect of the interaction of two species of marine diatoms on their individual species copper tolerance

INTRODUCTION

Most of the laboratory studies that have been carried out to assess the physiological and ecological effects of toxicity on microalgae have used unialgal cultures. The few studies that have involved more than one species did not in all cases demonstrate similar responses for a species growing alone and in the presence of a second one.

A number of general physiological studies have examined the interaction of two species that were co-cultivated in the same cultures. One of the first was by Rice (1954) who examined the interaction between *Chlorella vulgaris* and *Nitzschia frustulum*. A similar study was performed by Pratt (1966) on the interaction of *Skeletonema costatum* and *Olisthodiscus luteus*. Other studies involve the interaction of *Chlamydomonas globosa* and *Chlorococcum ellipsoideum* (Kroes 1971), of *S. costatum* and *Thalassionema nitzschioides* (Fedorov and Kustenko 1972), and of *Thalassiosira pseudonana* and *Dunaliella tertiolecta* (Mosser et al. 1972). Lange (1974) examined the interaction of three species of cyanobacteria, *Microcystis aeruginosa*, *Nostoc muscorum* and *Phormidium foveolarum*, Sze and Kingsbury (1974) that of *Chlamydomonas* spp. and *Staurastrum paradoxum* and Elbrächter (1977) that among *Biddulphia* (= *Odontella*) *regia*, *Coscinodiscus concinnus*, *Ceratium horridum* and *Prorocentrum micans*. Additional studies include the interactions of *P. micans*, *S. costatum* and *Chaetoceros didymus* (Uchida 1977), of *Thalassiosira pseudonana* and *Pheaodactylum tricornutum* (Sharp et al. 1979) and the competitive interaction among *Scripsiella faeroense*, *Prorocentrum micans* and *Gymnodinium splendens* (Kayser 1979). In addition, De Jong and Admiraal (1984) examined the interactive growth of *Navicula salinarum*, *Nitzschia*
closterium and Amphipora cf. paludosa and Rijstenbil (1989) that of S. costatum and Ditylum brightwellii.

In most of the studies mentioned, the outcome of the interaction is inhibitory for at least one of the species involved. Nitzschia inhibits the growth of Chlorella whereas the opposite does not occur (Rice 1954). Olisthodicus overtook Skeletonema in most cases (Pratt 1966) and so did Ditylum (Rijstenbil 1989). Finally, Prorocentrum inhibited both Skeletonema and Chaetoceros (Uchida 1977).

A number of observers have concluded that the outcome of the interaction depends upon the initial cell number ratio of the species involved. In all species pairs examined by Kayser (1979) in batch cultures, the species with the highest initial cell density was the one that dominated the culture at the end of the experimental period. This was also the case in studies between A. cf. paludosa and N. closterium (De Jong and Admiraal 1984). The species with the higher inoculum size was the one that overtook the cultures. Fedorov and Kustenko (1972) discovered that Thalassionema overtook the bialgal cultures, regardless of nutrient concentration, when its inoculum size was larger than Skeletonema. The reverse was not achieved. When the phosphate : nitrate ratio was too low for Skeletonema, this species did not overtake the cultures regardless of the respective inocula. In the study by Rice (1954), the initial cell concentration was important as well. However, a much larger concentration of Chlorella was necessary to inhibit Nitzschia growth than of Nitzschia to inhibit Chlorella. In the studies by Lange (1974) and Sze and Kingsbury (1974), the outcome of species interactions was not altered with different inoculum sizes.

The conclusion of a number of the studies that examined the interaction of species in culture is one of "allelopathy" (as in Sharp et al. 1979). This involves the negative effect of compounds excreted into the medium by one microalgal species on another species present. The effect is usually determined by observing the growth of an organism in culture medium that contains filtrate (to remove the cells) from a culture of the
inhibitory species. Rice (1954) observed a decrease in both growth rate and maximal yield for both species when grown in each other’s filtrates. Although some inhibition did occur by their own filtrate, this was not as pronounced as the effect by the other species. Olisthodiscus demonstrated satisfactory growth in Skeletonema filtrate whereas the opposite was not achieved (Pratt 1966). The growth of Skeletonema was least inhibited in the highest dilution of the O. luteus filtrate. Chlamydomonas was inhibited by the addition of Chlorococcum filtrate into its own medium (Kroes 1971). On the other hand, Chlamydomonas has been shown to induce similar inhibition to Staurastrum (Sze and Kingsbury 1974). Both S. costatum and C. didymus were inhibited in P. micans filtrate (Uchida 1977) and T. pseudonana demonstrated increased lag phase and reduced maximal yield in the presence of Pheodactylum filtrate (Sharp et al. 1979). Similar conclusions were reached by Kayser (1979) for the filtrates of P. micans and G. splendens which increased the length of lag phase and by Rijstenbil (1989) for both species used in his study.

In an attempt to characterize the inhibitory phytoplankton exudates, Rice (1954) and Sharp et al. (1979) concluded that the detrimental effect is removed by autoclaving the filtrate. In addition, Uchida (1977) suggested that the size of the exudates of the species that he used must have been quite large because their effect was removed by filtering the medium through a cellulose filter. Pratt (1966) indicated that the effects are maximal at the beginning of a filtrate experiment because of subsequent degradation of the exudate.

In some instances, the effect of exudates appears to depend upon the phase of growth during which it was excreted. Sharp et al. (1979) found that the inhibitory substance was excreted during the stationary phase of P. tricornutum. Similar results were obtained by Kayser (1979). Fedorov and Kustenko (1972) found an inhibitory effect of the senescence exudates of Thalassionema on Skeletonema. However, for Thalassionema, the inhibitory effect was pronounced for Skeletonema exudates of
exponentially growing cells. No difference between exudates of these two phases was recorded for *Ditylum* on *Skeletonema* (Rijstenbil 1989).

In some of the aforementioned studies, other factors that might influence the outcome of bialgal culture growth were examined. Nutrient exhaustion by one of the species in a multialgal culture was suggested as an alternate explanation. For example, Rice (1954) added nutrients to the cultures to reverse the outcome but was unsuccessful. This was also the case in the studies by Lange (1974) and by Sze and Kingsbury (1974). Uchida (1977) added nutrients to the filtrates but the inhibitory effect persisted. As mentioned earlier, in the study by Fedorov and Kustenko (1972), nutrient levels controlled the outcome in the cases of *S. costatum* dominance but not with *T. nitzschioide*es. These two species demonstrated growth enhancement in the presence of different nutrients (nitrate for *Skeletonema* and phosphate for *Thalassionema*). In the presence of a low nitrate : phosphate ratio in the medium, *Skeletonema* could not overtake the cultures (Fedorov and Kustenko 1972). In all other cases, it did. Elbrächter (1977) concluded that nutrient limitation was responsible for all the species inhibitions that he observed (*Biddulphia* by *Coscinodiscus*, *Ceratium* by *Biddulphia* and *Prorocentrum* by *Biddulphia* and *Coscinodiscus*). Kayser (1979), using marine dinoflagellates, observed very little inhibition in filtrate cultures and no effect at all in continuous cultures and concluded that the effect that was observed in batch cultures was due to nutrient exhaustion.

It has thus been demonstrated that although species exhibit particular growth characteristics when grown alone, they may be severely affected (to the point of complete growth inhibition) in the presence of a second species. It may, therefore, be suggested that growth under metal stress may be influenced by the presence of a second species.

One important common aspect between the results obtained from studies of species interactions in multialgal cultures and individual species’ metal tolerances is the importance of organic exudates. So far, I have discussed their importance in species
interactions. However, their importance in phytoplankton metal tolerance requires a little more attention. Stolzberg and Rosin (1977) concluded that extracellular, metal-binding, organic matter was produced by Skeletonema costatum. These exudates had a complexing capacity of 3 to 4 x 10^-7 M. Swallow et al. (1978) examined the effect of extracellular organic exudates of nine species of phytoplankton (known to produce organic exudates) on copper complexation in the medium. Of these, only the freshwater species Gloeocystis gigas was proven to produce organic compounds that do actually complex copper. The authors suggested that the sensitivity of the ligand measurement technique was not low enough to detect potential ligands in the other species. McKnight and Morel (1979) examined the production of copper complexing organic ligands in a number of freshwater phytoplankton. The results were quite variable. Diatoms did not produce any ligands, five chlorophytes and two chrysophytes produced measureable concentrations (a weak organic acid-type ligand) and four cyanophytes produced very strong ligands (probably more than one ligand). McKnight and Morel (1980) subsequently determined copper complexation by hydroxymate siderophores produced by certain species of cyanobacteria. In cultures of Anabaena cylindrica, increased cupric ion activity was observed in cultures of high iron content. The authors concluded that when iron is in low concentration in the medium, copper binds onto the siderophore. When the iron concentration increases, this metal displaces copper on the compound. They detected two types of ligands, weak ones in lower iron concentrations and stronger ones in higher iron levels. Cultures of Synechococcus leopoliensis, on the other hand, did not demonstrate increased cupric ion concentration in elevated iron. Fisher and Fabris (1982) examined the copper complexing ability of exudates produced by Skeletonema costatum, Asterionella japonica and Nitzschia closterium. Copper was the metal that was preferably bound by the exudates produced by all of these species during exponential growth, and zinc during senescence. They did not find any differences in complexing capacity between the different diatom species exudates and suggested that these ligands
are stronger than refractory dissolved organic carbon found in natural seawater (e.g. humic acids). *S. costatum* was found to produce two ligands that bind zinc, a weaker and a stronger one (Imber *et al.* 1985). The conditional stability constants of the ligands excreted by the cyanobacterium, *Microcystis aeruginosa* differed, depending on the period of growth during which they were produced. The ligand concentration (complexation capacity) and the stability constants increased with aging of the cultures (Ogiwara and Kodaira 1989).

Although studies have been performed on the complexing ability of organic exudates for copper, it still remains unclear as to whether their production is stimulated by increased copper levels in the surrounding medium. Steeman Nielsen and Wium-Andersen (1971) and Florence *et al.* (1982) have suggested stimulation by ambient concentrations for *Nitzschia palea* and *S. costatum*, and *Nitzschia closterium* respectively.

Lange (1974) attributed the inhibition by *Phormidium foveolarum* of *Nostoc muscorum* and *Microcystis aeruginosa*, to exudates that remove metals from the medium. Murphy *et al.* (1976) demonstrated that a hydroxamate chelator produced by the cyanobacterium *Anabaena flos-aquae* (or its associated bacteria) removed iron from the medium during a specific period in nitrogen fixation. This in turn inhibited *Scenedesmus* due to iron limitation. Van Den Berg *et al.* (1979) determined that *Anabaena cylindrica*, *Navicula pelliculosa* and *Scenedesmus quadricauda* produce copper ligands of different strengths and concentrations. However, the final ionic copper concentration in the medium did not differ between species. They subsequently discovered that in the absence of copper, *Chlorella vulgaris* grew better in the *Anabaena* filtrate and worse in the *Scenedesmus* filtrate. In the presence of elevated total copper, the filtrates with the stronger ligands were more beneficial. Brown *et al.* (1988) demonstrated that the copper tolerance of *Thalassiosira profunda* was slightly enhanced in the presence of *Amphora coffeaeformis* exudate. The authors suggested that copper is bound onto the mucilage
that is excreted by the pennate. These are the few studies that attempted to link the excretion of an exudate by a phytoplankter and the different manners in which it may affect the survival of a second species in different copper concentrations.

A second outcome of multialgal cultures that may affect the apparent tolerance of an organism to a metal is nutrient limitation. In the interactions that are examined in the laboratory this factor will come into play only in batch cultures. As shown in a number of studies, nutrient exhaustion by one species was the factor that limited the growth of other species. Metals have been shown to increase the length of lag phase in some species (see Chapter 1). If one of the species demonstrates an increase in lag phase then, by the time it starts growing, the medium might be nutrient-deplete. In this case, although exponential growth will be observed when the organism is grown alone, no such growth will be observed in the bialgal cultures. This type of outcome will depend upon the individual nutrient requirements and nutrient uptake rates of all species involved.

It becomes apparent, therefore, that organisms may modify the medium as well as alter its chemical speciation. This in turn will affect their metal tolerance directly. It has also been demonstrated that the complexing capacity of excreted organics varies between species. Some species produce very strong ligands, some produce weak ones, some do not produce any at all. In addition, it has been shown that the interaction between species that are cultured together affects their individual growth. In a number of studies, the inhibitory effect was attributed to exudates of the species that dominated. In others, it has been attributed to nutrient depletion which will become more likely in the case of extended lag phase of a species upon copper addition. It can, therefore, be expected that the apparent metal tolerance of a species may be altered in the presence of a second species. This may be achieved either by inhibition, as demonstrated by the bialgal experiments, or by a medium modification, as demonstrated by the different complexing capabilities of organic exudates of different species and by nutrient depletion.
Very few studies of this type have been carried out. Mosser et al. (1972) examined the interaction between *Thalassiosira pseudonana* (a sensitive species) and *Dunaliella tertiolecta* (a tolerant species) in the presence of PCBs and DDT. *Thalassiosira* was the dominant species in the control mixed cultures, demonstrating very high growth rates compared to *Dunaliella*. In high toxicant levels, this species was more inhibited in the presence of *Dunaliella* than in unialgal cultures. The result was attributed to competition for nutrients. Goldman and Stanley (1974) examined the relative growth of *Phaeodactylum tricornutum*, *Thalassiosira pseudonana* and *Dunaliella tertiolecta* in wastewater-seawater mixtures. In this study, the authors demonstrated that the single species bioassays (which determined *Phaeodactylum* as the dominant species) did predict the outcome of the mixed cultures (in which *Phaeodactylum* dominated within a few days). A study carried out by Dashora and Gupta (1978) suggested an effect of chlorine and copper on species combinations (of *Scenedesmus obliquous*, *Selenastrum minutum* and *Phormidium luridum*). However, it is difficult to assess the value of this study to my research, since no examination of the individual species tolerances was included.

In the previous chapter of this thesis, I discussed the specific copper tolerances of *Skeletonema costatum* and *Nitzschia thermalis*. It was demonstrated that *Nitzschia* is slightly more tolerant than *Skeletonema* and the copper effect ("stress") was manifested differently in the two species. For this part of the study, the two species were cultured together in order to examine the effect of the presence of a second species (that might be responding differently to copper stress, as well as modifying the medium) on their apparent individual copper tolerances. The chapter is composed of two parts. In the first part, I discuss the effect of the second species on the expected growth and survival at three copper concentrations for each of the two organisms. In the second section, I discuss possible factors that may play a role in the interaction of the two species and may, therefore, determine the apparent copper tolerance of individual species.
PART A: Interaction of Skeletonema costatum with Nitzschia thermalis in three copper concentrations.

METHODS

The same general experimental procedure was followed during these experiments as in Chapter 1. The only difference with the experiments described in this section is that the stock cultures from which the inocula were removed were in late exponential phase to the beginning of senescence. The large number of replicates used during the experiments required a large number of cells for inoculation that could only be obtained from dense cultures. Enough cells were available by inoculating from stocks that were late in the exponential phase or early senescence.

Aliquots were removed when the stock cultures reached an appropriate concentration. I subsequently determined the number of cells $\cdot$ mL$^{-1}$ in the stock cultures using a Palmer Maloney counting slide under a Zeiss Standard 14 compound microscope. Then, the volume of the inoculum necessary to obtain a density of $\sim$500 cells $\cdot$ mL$^{-1}$ in the first experiment and $\sim$1,000 cells $\cdot$ mL$^{-1}$ in the second one was calculated. The actual population sizes that I did obtain using this method were 400 $\pm$ 60 and 800 $\pm$ 80 cells $\cdot$ mL$^{-1}$ for the first and second experiments respectively (mean $\pm$ S.E., N = 45).

Three treatments were used with respect to the biological species. One unialgal treatment for each of the two species was included. These served as controls for the growth of Skeletonema and Nitzschia in the absence of a second species. In addition, a third treatment was included in which S. costatum and N. thermalis were inoculated simultaneously. This treatment was used to determine the effect that the second species had on the growth and survival of the other species. The inoculum size was the same for both species and all treatments. As a result, the mixed cultures received twice the number of cells compared to the unialgal flasks.
Each of these treatments was examined at three copper concentrations. The first, $1 \times 10^{-9}$ M Cu, served as a control. It has been demonstrated (Chapter 1) that, in this concentration, both species show satisfactory growth. Therefore, no effect on their growth should be observed as a result of copper. The second copper concentration that was used was $4 \times 10^{-7}$ M (= 0.4 $\mu$M) Cu. From the previous chapter, it was expected that *Nitzschia* should grow equally well with the control treatment at this level. On the other hand, *Skeletonema* should demonstrate a prolonged lag phase and an increase in growth rate due to the copper stress. The last copper level that was used was $5 \times 10^{-7}$ M (= 0.5 $\mu$M). In this concentration, *Skeletonema* should not grow at all. *Nitzschia* should either grow equally well with the control ($1 \times 10^{-9}$ M) or show a slight decrease in growth rate compared to the control. Any deviations from these expectations should be attributed to the interaction with the second species present in the same batch culture.

Five replicates were used for each of the above nine treatments and all treatments were run simultaneously. A combination of random and systematic designs was used in order to arrange the positions of the flasks on the culture chamber shelves. Three shelves with a total of four light sources were available for use. I assigned a replicate per light source for each of the nine treatments. I then randomly assigned the fifth replicate to one of the light sources. Once all flasks were associated with a light replicate, they were randomly positioned within each source using a random number table.

The experiment was run twice in order to verify the consistency of the results. As mentioned earlier, during the first run the inoculum provided a cell concentration of $\sim$500 cells $\cdot$ mL$^{-1}$ for each species. In the course of the experiment, I observed slightly extended lag phases which I attributed to the unusually low inoculum size. During inoculation, a number of cells might die due to the mechanical process of transferring them, as well as due to the change in medium composition. If the inoculum size is not large enough, the number of cells that do survive might be too small to reflect an increase in population size (given the accuracy of my counting procedure which cannot detect
densities below 200 cells · mL⁻¹). If the population increase is not detectable, it will be perceived as an extended lag phase. Therefore, to overcome such problems in the second experiment, the inoculum was increased to provide an initial concentration of ~1,000 cells · mL⁻¹.

Within each copper concentration, the growth rate and lag phase were compared between two treatments (species growing alone and in the presence of the second species). All comparisons were performed using a Student’s t-test. In cases where no growth was obtained in one of the treatments, a Wilcoxon rank sum test was used instead. In cases where no growth was observed in either of the two treatments, no statistical comparison was carried out. The Student’s t- and the Wilcoxon rank sum tests were carried out using EPISTAT (Gustafson 1984) on a Commodore PC-10 II. A significance level of 0.05 was used throughout.
RESULTS

The results are shown in Figs. 2.1-2.3 for experiment 1 and Figs. 2.4-2.6 for experiment 2. Skeletonema did grow satisfactorily in 1 x 10^{-9} M Cu, exhibited longer lag phase and good growth rate in 4 x 10^{-7} M and did not grow at all in 5 x 10^{-7} M Cu. On the other hand, this species did not show any growth in the presence of Nitzschia (with the exception of one replicate in 1 x 10^{-9} M Cu in the first run of the experiment). Nitzschia grew in all copper concentrations whether in uni- or bialgal experimental flasks.

The statistical comparisons between the two treatments (single species versus double species additions), for each copper concentration and both experiments, are shown in Tables 2.1-2.8. S. costatum demonstrated significantly higher growth in all copper concentrations when grown alone than when grown in the presence of Nitzschia. The only exception was in 4 x 10^{-7} M Cu during the second run of the experiment (Table 2.5). In this case, there was no significant difference between the two treatments. No statistical comparisons to determine differences in lag phase were possible since no growth at all was observed in the treatments that included Nitzschia.

For N. thermalis, there was no significant difference in growth rate between the two treatments in any copper concentration, in both runs of the experiment (Tables 2.3 and 2.7). The same was true for the lag phase (Table 2.4 and 2.8), with one exception. The treatment that included Skeletonema in 1 x 10^{-9} M added Cu demonstrated an extended lag phase compared to the single species addition (Table 2.8).

As may be noted from the tables, a number of replicates were not incorporated in the growth rate and lag phase estimations on a number of occasions. In experiment 1, at the 4 x 10^{-7} M Cu level, replicate 4 of Skeletonema growing alone did not come out of lag phase before the termination of the experiment (Table 2.2). In the same experiment, in 1 x 10^{-9} M Cu, replicate 3 of N. thermalis did not demonstrate a pronounced
exponential growth rate pattern during any stage of the experimental period (Tables 2.3 and 2.4). During experiment 2, one of the replicates of *S. costatum*, growing alone in $4 \times 10^{-7}$ M Cu, became contaminated with *Nitzschia* on day 12 and was completely excluded from the calculations (Tables 2.5 and 2.7). In the same treatment, a second replicate did not show exponential growth (although it demonstrated signs of increase) before the end of the experiment.
FIGURE 2.1: Growth curves of *Skeletonema costatum* (A, B) and *Nitzschia thermalis* (C, D) grown in unialgal (A, C) and bialgal (B, D) cultures, in $5 \times 10^{-7}$ M Cu (Experiment 1). Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2.2: Growth curves of *Skeletonema costatum* (A, B) and *Nitzschia thermalis* (C, D) grown in unialgal (A, C) and bialgal (B, D) cultures, in $4 \times 10^{-7}$ M Cu (Experiment 1). Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2.3: Growth curves of *Skeletonema costatum* (A, B) and *Nitzschia thermalis* (C, D) grown in unialgal (A, C) and bialgal (B, D) cultures, in $1 \times 10^{-9}$ M Cu (Experiment 1). Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2.4: Growth curves of *Skeletonema costatum* (A, B) and *Nitzschia thermalis* (C, D) grown in unialgal (A, C) and bialgal (B, D) cultures, in 5 x 10^{-7} M Cu (Experiment 2). Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2.5: Growth curves of *Skeletonema costatum* (A, B) and *Nitzschia thermalis* (C, D) grown in unialgal (A, C) and bialgal (B, D) cultures, in 4 x 10^-7 M Cu (Experiment 2). Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2.6: Growth curves of *Skeletonema costatum* (A, B) and *Nitzschia thermalis* (C, D) grown in unialgal (A, C) and bialgal (B, D) cultures, in $1 \times 10^{-9}$ M Cu (Experiment 2). Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
TABLE 2.1: Statistical comparisons between growth rates of *Skeletonema costatum* growing in unialgal cultures and in the presence of *Nitzschia thermalis*, in three different copper concentrations (Experiment 1). – = species grown alone; + = species grown in the presence of *N. thermalis*; N = sample size; S.D. = standard deviation; N.D. = not determined; * = Wilcoxon test used instead.

<table>
<thead>
<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Growth Rate (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^{-7}</td>
<td>–</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>N.D.</td>
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<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td>N.D.</td>
</tr>
<tr>
<td>4 x 10^{-7}</td>
<td>–</td>
<td>0.97</td>
<td>0.18</td>
<td>* &lt;0.050</td>
<td>&lt;0.005</td>
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<td></td>
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<td>(4)</td>
<td></td>
<td></td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>1 x 10^{-9}</td>
<td>–</td>
<td>1.37</td>
<td>0.34</td>
<td>4.31</td>
<td>&lt;0.005</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td>&lt;0.050</td>
</tr>
<tr>
<td>1 x 10^{-9}</td>
<td>+</td>
<td>0.22</td>
<td>0.49</td>
<td></td>
<td>&lt;0.050</td>
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<td></td>
<td></td>
<td>(5)</td>
<td></td>
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<td>&lt;0.050</td>
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TABLE 2.2: Statistical comparisons between lengths of lag phase for *Skeletonema costatum* growing in unialgal cultures and in the presence of *Nitzschia thermalis*, in three different copper concentrations (Experiment 1). – = species grown alone; + = species grown in the presence of *N. thermalis*; N = sample size; S.D. = standard deviation; N.D. = not determined.

<table>
<thead>
<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Lag Phase (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^{-7}</td>
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<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<td></td>
<td></td>
<td>(5)</td>
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<tr>
<td>+</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<td></td>
<td></td>
<td>(5)</td>
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<td></td>
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<tr>
<td>4 x 10^{-7}</td>
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<td>14.4</td>
<td>3.9</td>
<td>N.D.</td>
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<td></td>
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<td>(4)</td>
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<tr>
<td>+</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
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<td>(5)</td>
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<tr>
<td>1 x 10^{-9}</td>
<td>–</td>
<td>8.6</td>
<td>4.7</td>
<td>N.D.</td>
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<td>(5)</td>
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<td>+</td>
<td></td>
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<td>N.D.</td>
<td>N.D.</td>
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<td>(5)</td>
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TABLE 2.3: Statistical comparisons between growth rates for *Nitzschia thermalis* growing in unialgal cultures and in the presence of *Skeletonema costatum*, in three different copper concentrations (Experiment 1). – = species grown alone; + = species grown in the presence of *S. costatum*; N = sample size; S.D. = standard deviation.

<table>
<thead>
<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Growth Rate</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
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<td>+</td>
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<tr>
<td>4 x 10^{-7}</td>
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<td>0.42</td>
<td>0.050</td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>1.21</td>
<td>0.31</td>
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<tr>
<td>1 x 10^{-9}</td>
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<tr>
<td></td>
<td>+</td>
<td>1.13</td>
<td>0.68</td>
<td>0.155</td>
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TABLE 2.4: Statistical comparisons between lengths of lag phase for *Nitzschia thermalis* in unialgal cultures and in the presence of *Skeletonema costatum*, in three different copper concentrations (Experiment 1). – = species grown alone; + = species grown in the presence of *S. costatum*; N = sample size; S.D. = standard deviation.

<table>
<thead>
<tr>
<th>Copper Treatment Mean Lag Phase</th>
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<th>P</th>
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<td>Copper Concentration (M added Cu)</td>
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<tr>
<td>5 x 10^{-7}</td>
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<tr>
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<tr>
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TABLE 2.5: Statistical comparisons between growth rates for *Skeletonema costatum* growing in unialgal cultures and in the presence of *Nitzschia thermalis*, in three different copper concentrations (Experiment 2). – = species grown alone; + = species grown in the presence of *N. thermalis*; N = sample size; S.D. = standard deviation; N.D. = not determined; * = Wilcoxon test used instead.

<table>
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<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Growth Rate (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
</tr>
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<tbody>
<tr>
<td>5 x 10^-7</td>
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</tr>
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<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
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<tr>
<td>4 x 10^-7</td>
<td></td>
<td>1.34</td>
<td>0.77</td>
<td></td>
<td>*</td>
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<tr>
<td></td>
<td></td>
<td>(3)</td>
<td></td>
<td>=0.100</td>
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</tr>
<tr>
<td></td>
<td>+</td>
<td>0</td>
<td>0</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>(5)</td>
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<tr>
<td>1 x 10^-9</td>
<td></td>
<td>1.26</td>
<td>0.13</td>
<td></td>
<td>*</td>
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<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td>&lt;0.010</td>
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<td>+</td>
<td>0</td>
<td>0</td>
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<tr>
<td></td>
<td></td>
<td>(5)</td>
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</tbody>
</table>
TABLE 2.6: Statistical comparisons between lengths of lag phase for *Skeletonema costatum* growing in unialgal cultures and in the presence of *Nitzschia thermalis*, in three different copper concentrations (Experiment 2). = species grown alone; + = species grown in the presence of *N. thermalis*; N = sample size; S.D. = standard deviation; N.D. = not determined.

<table>
<thead>
<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Lag Phase (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 x 10^{-7}</td>
<td>-</td>
<td>N.D.</td>
<td>N.D</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x 10^{-7}</td>
<td>-</td>
<td>7.1</td>
<td>0.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(3)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 x 10^{-9}</td>
<td>-</td>
<td>4.1</td>
<td>1.6</td>
<td></td>
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</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>N.D.</td>
<td>N.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>(5)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 2.7: Statistical comparisons between growth rates for *Nitzschia thermalis* in unialgal cultures and in the presence of *Skeletonema costatum*, in three different copper concentrations (Experiment 2).  - = species grown alone;  + = species grown in the presence of *S. costatum*;  N = sample size;  S.D. = standard deviation.

<table>
<thead>
<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Growth Rate (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10(^{-7})</td>
<td>-</td>
<td>1.18 (5)</td>
<td>0.31</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.72 (5)</td>
<td>0.55</td>
<td>1.94</td>
<td>&gt;0.050</td>
</tr>
<tr>
<td>4 x 10(^{-7})</td>
<td>-</td>
<td>1.07 (5)</td>
<td>0.48</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.24 (4)</td>
<td>0.31</td>
<td>0.611</td>
<td>&gt;0.100</td>
</tr>
<tr>
<td>1 x 10(^{-9})</td>
<td>-</td>
<td>1.57 (5)</td>
<td>0.39</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>2.09 (5)</td>
<td>0.87</td>
<td>1.23</td>
<td>&gt;0.100</td>
</tr>
</tbody>
</table>
TABLE 2.8: Statistical comparisons between lengths of lag phase for *Nitzschia thermalis* growing in unialgal cultures and in the presence of *Skeletonema costatum*, in three different copper concentrations (Experiment 2). – = species grown alone; + = species grown in the presence of *S. costatum*; N = sample size; S.D. = standard deviation.

<table>
<thead>
<tr>
<th>Copper Concentration (M added Cu)</th>
<th>Treatment</th>
<th>Mean Lag Phase (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 x 10^-7</td>
<td>–</td>
<td>0.92 (5)</td>
<td>0.95</td>
<td>1.32</td>
<td>&gt;0.100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.5 (5)</td>
<td>0.50</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x 10^-7</td>
<td>–</td>
<td>1.5 (5)</td>
<td>0.9</td>
<td>0.050</td>
<td>&gt;0.100</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.6 (4)</td>
<td>0.6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 x 10^-9</td>
<td>–</td>
<td>0.58 (5)</td>
<td>0.53</td>
<td>2.58</td>
<td>&lt;0.050</td>
</tr>
<tr>
<td></td>
<td>+</td>
<td>1.48 (5)</td>
<td>0.57</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
DISCUSSION

One common pattern arose from both experiments. Regardless of the copper concentration in which these two species were growing, *Nitzschia thermalis* had an inhibitory effect on *Skeletonema costatum*. The opposite interaction does not appear to occur. If it did occur, the effect was not strong enough to reverse the outcome of the experiments.

The two species, when in unialgal cultures, behaved as expected. A few points on the behaviour of individual replicates, however, require clarification. As noted earlier in this chapter, extended lag phases were observed during the first run of this experiment. This was corrected by increasing the inoculum size during the second run. If the lag phases from both species are compared between experiments, a decrease in length of lag phase can be seen (Table 2.2 with 2.6 and Table 2.4 with 2.8 for *S. costatum* and *N. thermalis* respectively).

In addition, several replicates were not included in the growth calculations because of the lack of demonstration of exponential growth during the experimental period. For *Skeletonema*, there were two such occasions. Both of these were growing in $4 \times 10^{-7}$ M Cu. In this concentration, this species demonstrated extended lag phase as a sign of copper stress. Both the experiments were terminated when there were 50 mL of medium remaining in the experimental flasks (this decision was reached in order to avoid any artifacts due to the increased surface : volume ratio of the medium when such a small volume remained). As a result, in both these instances, the experiment was terminated before the flasks demonstrated exponential increase.

On one occasion, one *Nitzschia* replicate that was growing in $1 \times 10^{-9}$ M Cu did not show strong exponential increase at any point on its growth curve (Fig. 2.3B). The cell counts largely oscillated around 1,000 cells • mL$^{-1}$ and it was not possible to decide on a particular point of inflection of the growth curve.
Skeletonema did not demonstrate a significant difference in growth between treatments in one of the replicates growing in $4 \times 10^{-7}$ M added copper (Table 2.5). From the actual growth rates, however, the unialgal cultures did show growth ($1.34$ divisions $\cdot$ day$^{-1}$) whereas in the presence of Nitzschia no replicates grew at all. I believe that the lack of significance involves the large variance associated with the mean growth rate. I consider this lack of significance a statistical artifact and I conclude that in all cases Skeletonema grew better in the absence of the pennate.

N. thermalis demonstrated a peculiar pattern of growth in both uni- and bialgal cultures. This species had very short lag phases (0.58-1.48 days, Tables 2.4 and 2.8). Once out of lag phase, it grew at a very rapid rate for 1-2 days, reached a plateau of cell numbers and then maintained its population size for the remainder of the experimental period. This becomes apparent from the high growth rates that were obtained for this species during the second experiment, especially in $1 \times 10^{-9}$ M added copper (Table 2.7). Most of these values were obtained from 1-2 day exponential growth, as calculated from the growth curves.

Nitzschia thermalis overtook all the bialgal cultures. Since the control, unialgal cultures grew as expected, one might expect that Nitzschia has an advantage over Skeletonema. This could be a competitive advantage in nutrient uptake. Skeletonema, as a rule, demonstrates longer lag phase than Nitzschia. It is therefore possible that by the time the lag phase of S. costatum is over, Nitzschia, which has reached stationary phase, has consumed most of the nutrients in the medium. This will prevent Skeletonema from attaining any significant growth rate. The possibility of nutrient depletion by one of the species grown in multialgal cultures was concluded by Kayser (1979) on the interaction of marine dinoflagellates and by Elbrächter (1977) for marine diatoms.

Nitzschia may modify the medium by affecting the carbon-source for Skeletonema specifically. De Jong and Admiraal (1984) suggested that the depletion of HCO$_3^-$, which will subsequently result in high $O_2$ and high pH, may act as a growth
limiting factor in the field. It has been demonstrated in the laboratory that increased carbonate addition in the medium enhances photosynthetic rate (Admiraal et al. 1982). This, in turn, might determine the dominant species in an interaction depending upon the rate of carbon-uptake and the growth-limiting carbon concentrations for the different species involved. One may then suggest that *Nitzschia* can remove the source of carbon dioxide from the medium during its growth before the lag phase of *Skeletonema* is over. In this fashion, sufficient carbon for exponential growth may never become available to *Skeletonema*. A decrease in the carbonate concentration in the medium should be manifested by an increase in pH (as in De Jong and Admiraal 1984).

An alternative explanation for the results obtained in this series of experiments is the production of exudates by *Nitzschia* that are inhibitory to *Skeletonema*. As described earlier, such a type of interaction has been commonly found among species of microalgae (e.g. Rice 1954, Pratt 1966, Fedorov and Kustenko 1972, Sharp *et al.* 1979, Rijstenbil 1989).

A third explanation involves the production of an organic exudate by *Nitzschia* that complexes metals in the medium. This substance might be a siderophore-type, high molecular weight (M.W. ~ 1,000), organic compound, secreted to complex metals in the medium. The existence of such compounds is not known for marine diatoms. If it does exist, it might be secreted in order to remove iron from the culture medium. However, organic compounds of lower molecular weight have also been demonstrated to complex metals. For example, Fisher and Fabris (1982) have documented the excretion of such compounds by *Skeletonema*, *Asterionella* and *Nitzschia*. As a result, the presence of such *Nitzschia* exudates in the culture medium may lead to a specific metal limitation in *Skeletonema* which will result in growth inhibition.

From this experiment, *Nitzschia* was the organism that grew successfully in all the bialgal cultures whereas *Skeletonema* did not. I concluded that *Nitzschia* has an inhibitory effect upon *Skeletonema*. This, however, does not preclude the reverse from
occurring. It is possible that Skeletonema may be inhibiting Nitzschia in a similar fashion, but that the effect is not strong enough to override its own inhibition. This may be observed in two instances. The first was during experiment 1, in replicate 1 of the cultures growing in $1 \times 10^{-9}$ M Cu. This Skeletonema replicate did grow at a satisfactory growth rate (1.10 div \cdot day$^{-1}$). Nitzschia grew in the same flasks as well, although at a relatively low growth rate (0.51 div \cdot day$^{-1}$). This was the only occasion during which Skeletonema grew at all in the mixed cultures. Its lag phase in that replicate is much shorter than the mean lag phase demonstrated by the unialgal cultures in the same copper concentration (1.2 versus 8.6 days respectively). Therefore, it appears that the inhibitory effect might depend on the species that exits lag phase early enough that, given its growth rate, it can dominate the culture. Rice (1954) discovered a similar situation between Chlorella and Nitzschia frustulum. In this situation, Chlorella was never observed to dominate a mixed culture. However, when this species was allowed to condition the medium before Nitzschia was added to inhibit it, Chlorella was able to cause a reduction in the Nitzschia growth. In addition, its own growth was not reduced at all, even after the addition of the second species.

The second occasion during which the negative effect of Skeletonema became apparent was during the second experiment. Although there was no sign of increase for Skeletonema, Nitzschia demonstrated increased lag phase in the presence of the second species compared to when grown alone in $1 \times 10^{-9}$ M added Cu. Rijstenbil (1989) observed a negative effect of both Ditylum and Skeletonema exudates on each other. However, the inhibition on Ditylum by exponential exudates of Skeletonema was stronger than the inhibition of Skeletonema by any type of Ditylum exudates. It is, therefore, quite possible that both species affect each other but that, overall, Nitzschia is the least sensitive one.

In the next section of this chapter, I shall attempt to determine whether any of the above factors explain the inhibitory effect that Nitzschia imposes upon Skeletonema. I
shall examine the possibility of alterations in the pH of the cultures due to carbonate depletion, the possibility of nutrient (other than carbon) depletion, inhibitory exudates in Nitzschia cultures, and finally the possibility of organic exudates acting as compounds that complex and remove metals from the medium, leading to trace metal limitation.
PART B: Possible factors driving the interaction between *Skeletonema costatum* and *Nitzschia thermalis*

METHODS

The first factor examined was a differential change in pH, measured at the end of growth, between the unialgal and the mixed cultures within each copper concentration. A differential increase in pH was used as an indicator of differential carbonate depletion by the end of the experimental period.

At the end of experiment 2, (see Part A) I determined the pH in all the experimental flasks using a Fisher Accumet® pH Model 140 meter. Three treatments were used, *S. costatum* cultures, *N. thermalis* cultures and mixed cultures within each of the copper concentrations. Subsequently, I compared the pH values between the three treatments using a one-way analysis of variance. In cases where a significant difference was obtained, a Tukey "honestly significant difference" test was used to determine which pairs of means were different.

Other factors that were examined were the possible excretion of an inhibitory compound by *Nitzschia*, nutrient depletion of the medium by *Nitzschia*, and finally trace metal limitation induced through metal binding by a *Nitzschia* exudate.

Five treatments were used in this experiment, all of which were run at 1 x 10⁻⁹ M Cu medium: (a) *Skeletonema* grown in filtrate from *Nitzschia* cultures; (b) *Skeletonema* grown in *Nitzschia* filtrate, enriched with all nutrient stocks (nitrate, phosphate, silicate, trace metals and vitamins); (c) *Skeletonema* in filtrate enriched with trace metal stock only; (d) *S. costatum* in fresh medium, serving as a control.

All experimental procedures in this section were similar to those described in Part A. A stock culture of *N. thermalis* that was in late exponential to early senescent phase was used to inoculate 15 experimental flasks at an initial concentration of ~1000 cells · mL⁻¹. The inoculated flasks, as well as five flasks containing Aquil that had not been
inoculated, were then incubated as in Part A. Two light sources were used for this experiment. Two replicates of each treatment were assigned to each light source. The fifth replicate of all treatments was assigned to lights randomly. When all cultures were associated with a light source, their arrangement within sources was randomized. When *Nitzschia* reached stationary phase, I gently filtered the cells out of each flask. For filtering, I used 0.45 μm Millipore type HA filters, mounted on 25 mm Swinnex disc filter holders. Aliquots of the culture medium were drawn into a 60 mL polypropylene sterile syringe and gently pushed through the filters into clean flasks. The filters, filter holders and syringes were acid-washed prior to use. The rubber plunger-tips of the syringes were wrapped in teflon tape, in order to avoid introduction of toxic substances that might leach from the rubber. The filtering was performed under class 100 air conditions.

Each of the treatments (a) to (c) were then randomly assigned to five of the fifteen flasks that contained the *Nitzschia* filtrates. For treatment (a), the filtrate was not modified. For treatment (b), all the Aquil nutrients were added to the filtrate. Finally, for treatment (c), only the metal stock was added to the filtrate. The five replicates that just contained Aquil medium underwent the same filtration procedure and no subsequent additions.

All the flasks were allowed to equilibrate for 24 hrs. after the appropriate additions were completed. Subsequently, they were all inoculated from an exponentially growing *Skeletonema* stock culture to an initial concentration of ~1000 cells · mL⁻¹. The experimental cultures were incubated under the same conditions as in Part A. They were arranged in a fashion similar to the *Nitzschia* cultures mentioned above.

Only daily *in vivo* fluorescence measurements were used to monitor growth. These were carried out using a Turner Model 111 fluorometer.
I used Mann-Whitney U-tests to compare growth rates and length of lag phase between treatments because I could not alleviate heteroscedacity by transformation. A significance level of 0.05 was used throughout.

The one-way analyses of variance and the Mann-Whitney U- and Tukey tests were carried out using the NPAR and STATS modules of SYSTAT (Wilkinson 1985) on a Commodore PC-10 II.
RESULTS

The pH was not significantly different between the two unialgal and the mixed cultures at $4 \times 10^{-7}$ and $1 \times 10^{-9}$ M Cu (Table 2.9). At $5 \times 10^{-7}$ M Cu, the unialgal *Skeletonema costatum* cultures exhibited a lower final pH value than the mixed ones. No other differences were detected.

The results from the experiment that examined the effect of medium modification by *Nitzschia* on *Skeletonema* growth are presented in Fig. 2.7. The latter species demonstrated satisfactory growth in the control medium (Fig. 2.7A). Growth rate was $1.28 \pm 0.05$ div · day$^{-1}$ and a lag phase of $1 \pm 0$ day (mean ± standard deviation, $N = 5$) was observed. *S. costatum* also exhibited growth in the *Nitzschia* filtrate that was enriched with nutrients (Fig. 2.7C). The growth rate in this treatment was $1.03 \pm 0.19$ div · day$^{-1}$ and the length of lag phase was $5.4 \pm 0.89$ days (mean ± standard deviation, $N = 5$). The cultures that were growing in nutrient-enriched filtrate exhibited significantly lower growth rate and longer lag phase than the control (Mann-Whitney U-test, $p < 0.05$ and $<0.01$ respectively). No growth was observed in the *Skeletonema* cultures that were growing either in unenriched *Nitzschia* filtrate or in filtrate that was enriched with the metal stocks only (Figs. 2.7B and 2.7D). It should be noted that replicate 5 of the unenriched *Nitzschia* filtrate was contaminated with *Nitzschia* cells. This can be observed in the abnormally high (compared to the rest of the replicates) fluorescence throughout the experimental period (Fig. 2.7B).
TABLE 2.9: Statistical comparisons for differences in pH between *Skeletonema costatum* (=Skel), *Nitzschia thermalis* (=Nitz) and mixed cultures at the end of Experiment 2. m = mean (N = 5); S.D. = standard deviation; Tukey = critical range for pairs of means (in pH units).

<table>
<thead>
<tr>
<th>Copper Concentration (in Molar Cu)</th>
<th>Treatment</th>
<th>pH</th>
<th>S.D.</th>
<th>ANOVA</th>
<th>Tukey</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>m</td>
<td>S.D.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5 x 10^{-7}</td>
<td>Skel</td>
<td>7.88</td>
<td>0.10</td>
<td>P&lt;0.05</td>
<td>0.50</td>
</tr>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitz</td>
<td>8.31</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>8.53</td>
<td>0.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4 x 10^{-7}</td>
<td>Skel</td>
<td>8.02</td>
<td>0.12</td>
<td>P&gt;0.05</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Nitz</td>
<td>8.13</td>
<td>0.15</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>8.36</td>
<td>0.34</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 x 10^{-9}</td>
<td>Skel</td>
<td>8.32</td>
<td>0.45</td>
<td>P&gt;0.10</td>
<td></td>
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<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Nitz</td>
<td>8.08</td>
<td>0.18</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>Mixed</td>
<td>8.22</td>
<td>0.37</td>
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</table>
FIGURE 2.7: Growth curves of *Skeletonema costatum* grown in filtrate from cultures of *Nitzschia thermalis*. (A) Control; (B) Filtrate; (C) Filtrate enriched with macro- and micronutrients; (D) Filtrate enriched with metal stocks. Circles, triangles, squares, inverted triangles and diamonds represent replicates 1-5 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2.7 (continued)
DISCUSSION

From the first part of this study, one may conclude that there is no difference between the three treatments in final pH. The only difference observed was between the *S. costatum* unialgal and the mixed cultures grown at 5 x 10^-7 M Cu. The lower pH in the *Skeletonema* cultures can be explained easily. The starting pH of Aquil before inoculation was 8.4. In addition, although axenic technique was used throughout this study, the cultures were not bacteria-free. As a result, and since *Skeletonema* did not grow at 5 x 10^-7 M Cu, one may expect an actual drop in pH in these cultures. This may occur since the phytoplankton is not removing carbonate by photosynthesis and bacterial respiration is adding CO₂ to the medium.

At the remaining two copper concentrations, there was no difference in pH between any of the cultures. This indicates that the degree of carbonate depletion was similar in the three treatments. This result does not necessarily exclude the potential of carbonate limitation of *Skeletonema* by *Nitzschia* uptake in the mixed cultures. Since the pH measurements were made at the end of the experimental period for all cultures, it is possible that carbonate limitation occurred in all treatments. However, I do not believe that this is the case, for two reasons. First, pH did not increase during the experiment. In most cases, the mean final pH was lower than, or similar to, the initial value. This suggests that the carbonate level did not change during the experimental period. Even if it did, but the pH meter was not accurate enough to reflect the increase or other factors such as metal speciation were also acting and masking the increase, the changes certainly do not appear to be large enough to inhibit growth. Most of the pH values were ~8.2 which is very close to the level recommended by Morel *et al.* (1979) for Aquil. The second factor that suggests the absence of carbonate limitation is that the culture vessels were not sealed. The caps were screwed on the flasks very loosely in order to allow for gas exchange in the cultures during the experiments. In addition, the flasks were shaken
and opened daily which would allow for a rapid diffusion of CO₂ into the culture medium.

The type of inhibition that Nitzschia induces upon Skeletonema becomes clearer when one considers the filtrate experiments. From the four treatments that I used, it becomes apparent that Nitzschia modified the culture medium in a manner inhibitory to Skeletonema. The medium was filtered when the Nitzschia cultures were reaching senescence. Therefore the concentrations of one or more nutrients were close to depletion in the initial medium. In this case, if all the nutrients were added back to the culture medium, and assuming no other modification had occurred, Skeletonema should have grown in this treatment.

As expected, no growth was observed in the unenriched filtrate. This treatment was stripped of nutrients by Nitzschia and was not, therefore, expected to support growth, regardless of other modifications by the pennate. In the filtrate with all the macro- and micronutrients added, growth was indeed observed but was negatively affected. The extended lag phase and the reduced growth rate, compared to the control, suggest the possibility that some factor (other than nutrient limitation) was retarding growth. This factor was mostly active in the first five days of the experiment during the extended lag phase of Skeletonema. The subsequent exponential growth that S. costatum exhibited suggests that this factor did not persist for longer than the period of lag phase. I suggest that the observed inhibition can be attributed to a compound that is excreted by Nitzschia during some stage of growth and is toxic to Skeletonema. Such inhibitory compounds have been reported by a number of authors (e.g. Rice 1954, Pratt 1966, Fedorov and Kustenko 1972, Uchida 1977, Sharp et al. 1979, Rijstenbil 1989). In all these studies, inhibition of one species by another was attributed to a toxic exudate.

A point of interest is the extended lag phase that Skeletonema exhibited in this treatment. This type of response has also been recorded in other studies (e.g. Kayser 1979, Sharp et al. 1979, Rijstenbil 1989). It has been suggested by Pratt (1966) that an
inhibitory effect should be more prominent at the beginning of a "filtrate" experiment. The same author suggested that the magnitude of the inhibition should decrease with time as a result of degradation of the exudate due to oxidation, polymerization or bacterial decomposition. In my experiments, it is possible that after ~5 days (Skeletonema lag phase) most of the exudate was degraded thus allowing Skeletonema to attain exponential growth.

Exponential growth rate was significantly lower in the nutrient-enriched filtrate medium compared to the control. One possible explanation is that the compound was not completely degraded by the end of the five day period, thus affecting the growth rate. Alternatively, the role of bacterial abundance in the two treatments must be considered. One may assume that, at the time of inoculation, the density of bacteria was higher in the filtrate of an algal culture that had reached senescence compared to autoclaved medium (control). The medium in which Nitzschia had been growing was passed through a 0.45 μm filter before it was inoculated with Skeletonema. This procedure probably removed a portion of the bacteria but probably allowed a large number of them to pass through the filter. On the other hand, in the control, which was probably not completely sterile, the bacterial numbers were expected to have been lower. It is, therefore, possible that the larger bacterial numbers were somehow responsible for the reduction of the growth rate of S. costatum. This could have been achieved in one of two ways. It is possible that the bacteria themselves are excreting a substance that is toxic to Skeletonema. Since in Part A of this chapter, no such inhibition was observed in unialgal Skeletonema cultures, one must assume that such species of bacteria have a specific association with Nitzschia. A second possibility is competition between bacteria and the diatom for a nutrient. The higher bacterial abundance in the nutrient-enriched filtrate medium may account for a more rapid removal of a nutrient which in turn may result in growth rate reduction for Skeletonema.
All studies that have examined the effect of algal exudates have not characterized the compounds except at a very superficial level (i.e. they are removable by autoclaving or they are large because they do not pass through a cellulose filter). Pratt (1966) suggested that the substance that *Olisthodiscus* excreted which inhibited *Skeletonema* was a tannin-like compound. However, no evidence for this was provided. Thus, it becomes very difficult to assess what component of the organic molecule causes the inhibition or even whether all microalgae excrete similar substances.

From this study, I cannot conclude whether the compound that is excreted by *Nitzschia* acts as a metal-complexing agent. Growth was not observed in the filtrate that was enriched only with the metal stock. However, this suggests that the medium was either stripped of nutrients other than metals and/or included a toxic substance. The lack of growth in this treatment indicates that regardless of whether or not the excreted compound bound metals, this is not what limited the growth of *Skeletonema*.

*N. thermalis* inhibits the growth of *S. costatum*, at least temporarily. It was demonstrated that such an inhibition is probably the result of an exudate which is toxic to a certain extent to *Skeletonema*. The toxicity appears to be partially alleviated in the absence of the organism that produces it after a number of days, presumably by the degradation of the compound. However, in the presence of *Nitzschia* this compound will be continuously produced, thus inhibiting *Skeletonema*, unless it becomes diluted (for example, in a chemostat). Dilution of the filtrate of cultures of algae that excrete inhibitory compounds has been shown to enhance growth (Pratt 1966, Sharp *et al.* 1979). On the other hand, if this compound is produced during a specific stage of *Nitzschia* growth, *Skeletonema* may enter exponential growth before *Nitzschia* produces the exudate. However, it will still become inhibited as soon as the concentration of the substance increases. If, however, *Skeletonema* does not enter exponential growth in time, it will be inhibited while *Nitzschia* utilizes the nutrients in the culture. By the time that *Nitzschia* ceases producing it and/or the toxic substance degrades, the medium will be
depleted of nutrients and *Skeletonema* will not be able to exhibit exponential growth at all.

It, therefore, becomes clear that the interaction between these two species is quite complex. It has been demonstrated that such an interaction will override the specific copper tolerance of *Skeletonema*. In this study, I demonstrated that regardless of the copper concentration at which *Skeletonema* exhibited satisfactory growth, this species was inhibited simply by the presence of *Nitzschia*. On the other hand, the single-species tolerance tests for *Nitzschia* quite adequately predicted the tolerance of this species in the presence of *Skeletonema* (except for a slight inhibition at 1 x 10^{-9} M Cu). Although not examined in detail, a slight retardation of growth of *Nitzschia* is suggested by the longer lag phase exhibited at that copper level. However, in all cases *Nitzschia* did overtake the batch cultures regardless of the copper concentration in the medium and of the copper tolerance of the individual species involved.
GENERAL DISCUSSION

In this study, the copper tolerances of *Skeletonema costatum* and *Nitzschia thermalis* were examined. *S. costatum* was inhibited at $5 \times 10^{-7}$ M and *N. thermalis* at $6 \times 10^{-7}$ M added total copper. *Skeletonema* exhibited an increase in both growth rate and lag phase with increasing copper concentration. On the other hand, *Nitzschia* exhibited a decrease in growth rate. No effect on the lag phase of this species was observed. When these two species were grown together, their growth response (as predicted given the copper concentration in the medium) was altered. For *Nitzschia*, the alteration was not very pronounced. This species showed an increase in length of lag phase at $1 \times 10^{-9}$ M Cu. The growth response of *Skeletonema*, on the other hand, was severely altered. This species did not show any growth in the presence of *Nitzschia*. In an attempt to explain this inhibition, it was concluded that it could be attributed to a compound excreted by *Nitzschia* that is toxic to *Skeletonema*. It was also suggested that the effect of such a compound was temporary, as indicated by extended lag phase followed by exponential growth rate of *Skeletonema* in nutrient-enriched filtrate from senescent *Nitzschia* cultures. It was, therefore, demonstrated that the interaction between these two species was too strong to allow for the copper tolerance of *S. costatum* to dictate its survival in mixed cultures, regardless of the copper concentration in the medium.

Interactions such as these are expected to occur in the natural environment as well. *S. costatum* is a diatom that is commonly collected in the plankton of many different bodies of water (e.g. Harrison *et al.* 1983, Sakshaug and Andresen 1986, Sakshaug and Olsen 1986, Tont 1987, Mortain-Bertrand 1989). It has also been collected in the estuarine benthos (see Admiraal 1984 for review). On the other hand, *Nitzschia thermalis* (although not commonly mentioned in the literature) has been described as one of the dominant pennates in late April in the Eems-Dollard estuary, in the Netherlands (Admiraal *et al.* 1982). It has also been collected in the plankton in the Bay of Fundy,
New Brunswick (Linkletter et al. 1977) and near Vancouver, British Columbia (isolate 608 of the North East Pacific Culture Collection). It is therefore very likely that these two species share the same habitat on certain occasions. If this is true, it can be expected that interactions, such as the one observed in this study, might affect their coexistence in the field. The magnitude of the effect of the interaction will depend upon the type of habitat that these two species share. If, for instance, they share a body of water with very strong circulation patterns, one might expect that the effect on each other will be alleviated by the dilution of inhibitory compounds that they may produce. On the other hand, in areas of little water exchange such dilution will not occur and an inhibitory interaction may arise. Two examples of such areas come to mind. One may be the benthos of coastal waters. As mentioned, *N. thermalis* is one of the dominant benthic species and *S. costatum* has been found in the benthos (presumably after sinking out of the plankton). It is, therefore, quite likely that these species will share a portion of the same habitat. In addition, since the benthic pennates might form a thick mat at the bottom, *Skeletonema* cells that sink out of the plankton may encounter a high concentration of toxic exudates overlying the mat.

A second example of a habitat where such an interaction may become very important is tidepools. In this case, water exchange will depend upon the frequency of flushing of the pools. Tidepools that are higher in the intertidal will be flushed less frequently than lower-level tidepools. In such pools, any exudates that are produced will accumulate until the water is replaced by tidal flushing. In addition, *Skeletonema* will tend to sink towards the bottom of the tidepool after the nutrients close to the surface are depleted. This species may then encounter increased levels of toxic exudates. It is quite likely that *N. thermalis* is present in the tidepools that I sampled. However, since the pennate diatoms were not identified, no conclusions can be drawn regarding the possibility of these two species interacting. On the other hand, other pennate diatoms have been observed to produce filtrates that are inhibitory to other algae. For example,
Thalassionema nitzschioides inhibited *S. costatum* (Fedorov and Kustenko 1972) and *Nitzschia frustulorum* inhibited *Chlorella vulgaris* (Rice 1954). It is, therefore, quite likely that other pennates that are present in the tidepools might exude substances that inhibit *Skeletonema*.

Pennate diatoms are not the only group of microalgae that have been shown to produce inhibitory exudates. Similar inhibition can be induced by groups of microalgae such as green algae, dinoflagellates and centric diatoms (e.g. Kroes 1971, Pratt 1966, Uchida 1977). Rijstenbil (1989) demonstrated that compounds produced by *Skeletonema* were inhibitory to *Ditylum brightwellii*. It becomes quite clear that such interactions are quite common among microalgae. It has been proposed that interactions of this type might become especially important during phytoplankton blooms and may determine the dominant species (Pratt 1966, Rijstenbil 1989).

In this study, it has been demonstrated that the interaction between *S. costatum* and *N. thermalis* was the determining factor in the survival of *Skeletonema*, irrespective of the copper concentration in the medium. Although *Skeletonema* should have exhibited growth in two of the concentrations used, given its predetermined copper tolerance, it did not, in the presence of the second species. This type of study demonstrates the limitations of single-species toxicity tests, which consider the specific tolerances of an organism to an inhibitor, in most cases, however, ignore the biological interactions of the species. The chemistry of the medium and the physiological response of a particular species play a very important role in the determination of its tolerance. However, it has been demonstrated that the apparent tolerance of a species might also depend on the other organisms that share its habitat.

Studies on the impact of a stress inducer at the community level, have attempted to incorporate the biological environment in the responses of each species-member of that community. These studies, however, are descriptive and, thus, do not allow for prediction of the outcome of stress induction. Such prediction may only be possible if
the mechanisms that control community structure are known. If the mechanisms are understood, one may be able to determine the types of interactions that do occur and their potential impact on the population abundance of different species. It becomes obvious that, if one attempts to describe population responses of a particular species, an understanding of the ways in which it interacts with other species is absolutely essential. Furthermore, if one attempts to understand the population response of the same species to a stress inducer, one must first ensure an understanding of the population dynamics in the absence of the "disturbing" factor.

A single-species toxicity test will determine the response of each species to the stress-inducer. It will not, however, show whether the response will be manifested as such in the community. For example, in my study, *Skeletonema* showed increased lag phase and increased growth rate at $4 \times 10^{-7}$ M added Cu. If only this information is used, one would assume that in the natural environment at that copper concentration, *Skeletonema* would show similar responses. However, from this study, this will depend on whether *N. thermalis* is also present. According to the results of Rijstenbil (1989), the apparent response will also depend on whether *Ditylum brightwellii* is present in the same habitat. If, at any point, *Nitzschia* is present then the copper tolerance of *Skeletonema*, as demonstrated by the toxicity test, will be irrelevant. On the other hand, if the habitat is shared with *Ditylum*, *Skeletonema* will not be inhibited by the other species and its response to copper might be manifested as in the single-species test. In addition, the inhibition of *Skeletonema* by *Nitzschia* (and probably that of *Ditylum* by *Skeletonema*) will depend on the nature of the habitat (which may or may not allow for dilution of the inhibitory compound). Such predictions are only possible because of an understanding of species interactions and their mechanisms. It is in this manner that I argue that species interactions will determine the "apparent" copper tolerance of an organism.
Single-species toxicity tests can adequately describe physiological responses (e.g. nutrient uptake mechanisms, cell morphology, cell quotas). Unfortunately, they cannot be used alone to determine the importance of the physiological response to either the population response of a single species or the overall community response. The latter can be described using community studies (as carried out to date). However, community responses to a stress-inducer can only be predicted if the species interactions are understood and taken into consideration.
REFERENCES


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METHODS

The sea-surface and five tidepools were sampled at Point Atkinson (49° 20' N, 123° 15' W), Vancouver, British Columbia, Canada on five occasions. The sampling dates were May 15, June 3, June 13, June 28 and July 12, 1988.

Salinity was recorded using an Endeco Type 102 refractometer and temperature measurements were taken using a mercury thermometer, for both the sea-surface and each of the tidepools on each date. No temperature measurements were taken on 3-6-88 because the thermometer broke during transport. The depth of each tidepool was recorded and the height above sea-level was estimated by eye.

Sampling of microalgae included two water column samples from the sea-surface, as well as two water column and two bottom samples from each of the tidepools. Only one sample was taken from the sea-surface on 15-5-1988. In addition, the bottom samples of tidepool 2 for 3-6-1988 went anoxic due to inadequate preservation and are, therefore, not included. The water from tidepool 1 had completely evaporated on 12-7-1988, so no samples were collected.

Water column samples were collected using a 60 mL polypropylene syringe at mid-depth. After each 60 mL sample was placed into a bottle, the syringe was rinsed into the sample bottle using 30 mL of distilled water.

For the bottom samples, a bottomless cup was inverted and placed tightly on the floor of each tidepool. The floor was then gently scraped using a dull knife in order to remove the epibenthic microalgae. Subsequently, a 60 mL polypropylene syringe was used to remove all the resuspended algae from inside the cup. The volume of sample that was removed was determined in either of two ways. When the tidepool had a shallow portion, the cup was placed on the floor in a manner that its top was exposed. In such
cases, I removed all the water from inside the cup. When the tidepool was deep (and the cup was therefore submerged), I removed water until an obviously large dilution had occurred to the water inside the cup. Since the layer of epibenthic algae in all deep tidepools was thick, the amount of resuspended algae inside the cup was very large compared to the surrounding water. It was, therefore, very apparent when most of the sample was removed from inside the cup by the clarity of the water replacing the sample. After the transfer of the sample into the sample bottle, the syringe was thoroughly rinsed into the same bottles using distilled water.

All samples were preserved using Lugol’s solution and stored in the dark until they were counted. After intense shaking of the sample bottles (100 inversions) a subsample was removed and allowed to settle overnight in 50 mL settling chambers. The volume of the subsample that was placed in the chamber depended on the density of the sample and varied from 0.5 to 50 mL. The settling chambers were subsequently filled with distilled water to 50 mL, thus diluting the subsample. The settled subsamples were subsequently used for diatom identification and enumeration. Diatom identification was performed according to Cupp (1943).

All centric diatoms were identified to genus and the majority to species. Due to the poor resolution of the microscope that was available, however, it was very difficult to separate the different pennate genera and species which were consequently lumped into one group. Some exceptions with distinct frustule shape were identified to genus and in some occasions to species. For each subsample, I counted all the diatoms in five fields or 400 individuals. All counts were performed using a Wild M40 inverted microscope.

The diatom abundances in the tidepools were transformed in one of two ways. For the water column samples, after all the dilutions were taken into account, the abundance (in cells · cm⁻³) was divided by the depth of the tidepool to obtain number of cells per unit surface area of the water column. The cell concentration in the bottom samples was divided by the surface area covered by the bottom of the sampling cup, in order to yield
cells per unit bottom surface area. All concentrations at the sea-surface are presented as cells \cdot \text{mL}^{-1}.

The tidal height at the time of sampling was calculated as recommended in the Canadian Tide and Current Tables (1988). By adding the former to the height above sea-level, as assessed for each of the tidepools, I estimated the height above chart datum for each of the tidepools on each sampling date. The values of the five dates were then averaged and the mean height was used for the remaining calculations. I counted the number of low and the number of high tides with sufficient height to cover the individual tidepools during the period May 14-July 15th 1988. Subsequently, I expressed this as a percentage of the total number of low and high tides during the same period. In this manner, I obtained a rate of flushing for each of the pools during the two month sampling period. Lastly, I determined the length of time during which the tidepools had been isolated from the sea-surface prior to sampling. I estimated the time at which the tide had last attained the height of the tidepool before the sampling time. I then determined the elapsed time as the isolation period of the tidepools.
RESULTS

Tidal effect on the tidepools

The average height of each tidepool above chart datum is given in Table 1. Tidepools 1 and 2 are of similar height (given the accuracy of the height estimation), whereas the remaining are of very different heights. This is also apparent in the flushing frequency of the tidepools (Table 2). While tidepools 1 and 2 are always flushed at high tide, tidepool 3 is flushed 80% of the time. On the other hand, while tidepools 1 and 2 are flushed 60% of the time at low tide, tidepool 3 is never flushed. Tidepools 4 and 5 are never flushed at either low or high tide and may, therefore, be considered splashpools.

The temperature and salinity gradients up the intertidal are presented in Figure 1. Temperature did not appear to vary with the flushing rate of the tidepool. This is not surprising at the air temperatures that sampling occurred. These varied between 16° C and 27° C during the study. Given the small depth of the tidepools (4 to 34.5 cm over all sampling dates) and the isolation period before sampling (Table 3), the water temperature could have increased to reach similar levels in all tidepools simply by high heat transfer from the air. Salinity, however, appeared to always drop to very low levels for tidepool 5 (<5 %o). The only exception to this was on 28-6-1988 (Fig. 1D). This could have been the result of a recent storm during which sea-water was splashed into the tidepool. In all other cases, however, tidepool 5 appeared to receive mostly freshwater input.

Diatom distribution

The diatom distributions for all sampling dates are shown in Figs. 2-6. At the sea-surface, for the first three dates, Skeletonema costatum was the most abundant species. Pennates, Thalassiosira nordensiöldii and Chaetoceros compressus were also present in
TABLE 1: Height above chart datum of five tidepools located at Point Atkinson, Vancouver, British Columbia, Canada, sampled on five occasions in 1988 (c.d. = chart datum).

<table>
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<th>Tidepool</th>
<th>Date</th>
<th>Time</th>
<th>Tidal height above c.d. (m)</th>
<th>Tidepool height above sea-level (m)</th>
<th>Tidepool height above c.d. (m)</th>
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<td>0.1</td>
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Mean height: 6.5
TABLE 2: Frequency of flushing for the period between May 14 and July 15, 1988 for five tidepools located at Point Atkinson, Vancouver, British Columbia, Canada.

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<th>Tidepool</th>
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<th>Number of tides with height above tidepool height</th>
<th>Flushing frequency (%)</th>
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1 Flushing Frequency = \( \frac{\text{Number of tides with height above tidepool height}}{\text{total number of tides}} \times 100 \)
TABLE 3: Period of isolation from the ocean prior to sampling for five tidepools located at Point Atkinson, Vancouver, British Columbia, Canada, sampled on five occasions in 1988.

<table>
<thead>
<tr>
<th>Tidepool</th>
<th>Date</th>
<th>Time when tidepool last flushed (hr:min)</th>
<th>Sampling time (hr:min)</th>
<th>Isolation period (hr:min)</th>
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<td>09:05</td>
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high numbers. For the last two dates, there was a shift in dominance (in terms of cell numbers). *S. costatum, T. nordensiöldii* and *C. compressus* decreased in abundance. The pennates, on the other hand, were the most dominant group with *Melosira moniliformis* and *Cerataulina pelagica* (on 12-7-1988) present in high concentrations.

The pattern of dominance observed at the sea-surface was not followed tightly in the tidepools. On the three dates that it was dominant at the sea-surface, *S. costatum* was present in concentrations greater, equal or lower than the pennates in tidepools 1 and 2. In tidepool 3, its concentration was lower than the pennates and in tidepools 4 and 5, it was either lower or non-existent. On the two dates that it was not very abundant at the sea-surface, this species was present in very low concentrations in tidepools 1, 2 and 3 and absent from tidepools 4 and 5.

A similar pattern of disappearance with increasing height up the intertidal was observed with the other species that attained the highest abundance at the sea-surface, on later dates. *C. pelagica*, although in similar concentrations to the pennates at the sea-surface, had much lower concentrations in tidepools 2 and 3 and disappeared in tidepools 4 and 5 (Fig. 6). The only exception appears to be *M. moniliformis*. This species had a distribution similar to the pennates both at the sea-surface and in the tidepools, being present even in tidepool 5 on 12-7-1988.

On the other hand, the pennates presented a different distribution relative to the other groups. Regardless of their abundance at the sea-surface relative to the other diatoms, they were always one of the most or the most abundant group in the tidepools. They were always in concentrations greater than 100,000 cells · cm\(^{-2}\) both in the water-column and the bottom samples in tidepools 1-5. The concentrations were much higher in some cases, for example in the bottom samples and during the dates of their highest abundance in the sea-surface samples. In addition, they were always present in tidepool 5. In most instances, they were present in concentrations greater than 100,000 cells · cm\(^{-2}\), when the few other species present were at densities below 10,000 cells · cm\(^{-2}\).
A number of species not abundant at the sea-surface, were also present in the tidepools. Examples of these are *Odontella* (mostly *aurita*), *Climacosphenia moniligera*, *Surirella fastuosa* var. *recedens* and *Gyrosigma spencerii*. *Odontella* was found almost exclusively in the bottom samples. Even when present in the water-column, it was in much lower concentrations. It was always present in tidepool 1, its abundance increased in tidepool 2 to decrease or disappear above tidepool 3. It was almost never present in tidepool 5 (with the exception of very low abundance on 13-6-1988). *C. moniligera* and *S. fastuosa* var. *recedens* followed a similar pattern of distribution except that they demonstrated a higher upper limit. On most sampling dates, their distribution extended to tidepool 4 and, in some cases, tidepool 5 in low abundances. *Gyrosigma spencerii* was present at its highest concentrations mostly in tidepool 3 and, in some cases, in tidepools 2 and 4. It was usually abundant in the bottom samples.

On all sampling dates, tidepool 4 had a lower number of "groups" than tidepools 1, 2 and 3 and tidepool 5 had the lowest "group" diversity of all (Fig. 7) (Since not all groups of diatoms were identified to species, I have used the term "group" to signify identifiable groups. For example, both the species *Skeletonema costatum* and the group pennates were used as "groups".).
Explanation of abbreviations used in the Figures

A.lon = Acnanthes longipes
C.co = Chaetoceros compressus
Cer = Cerataulina pelagica
Cha = Chaetoceros spp.
Cli = Climacosphenia moniligera
Cos = Coscinodiscus spp.
Dit = Ditylum brightwellii
Gyr = Gyrosigma spencerii
L.bor = Lauderia annulata
Lic = Licmophora abbreviata
Mel = Melosira moniliformis and M. nummuloides
Nit = all pennates that could not be assigned easily to genera or species (mostly Naviculoideae and Nitzschioideae - see text)
Odo = Odontella (mostly aurita)
SEA = sea-surface samples
Skel = Skeletonema costatum
Sur = Surirella fastuosa var. recedens
T. gra = Thalassiosira gravida
T.nor = Thalassiosira nordenskiöldii
TP1-TP5 = tidepool 1 - tidepool 5 respectively
FIGURE 1: Temperature and salinity measurements at the ocean surface and in five tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on five occasions: (A) May 15, (B) June 6, (C) June 13, (D) June 28 and (E) July 12, 1988.
FIGURE 1 (continued)
FIGURE 2: Diatom abundance at the ocean surface waters and in five tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on May 15, 1988. (A) Ocean surface and (B)-(F) Tidepools 1-5 respectively. Errors bars represent standard error of the mean (N=2).
FIGURE 2 (continued)
FIGURE 3: Diatom abundance at the ocean surface waters and in five tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on June 3rd, 1988. (A) Ocean surface and (B)-(F) Tidepools 1-5 respectively. Error bars represent standard error of the mean (N=2).
FIGURE 3 (continued)
FIGURE 4: Diatom abundance at the ocean surface waters and in five tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on June 13, 1988. (A) Ocean surface and (B)-(F) Tidepools 1-5 respectively. Error bars represent standard error of the mean (N=2).
FIGURE 4 (continued)
FIGURE 5: Diatom abundance at the ocean surface waters and in five tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on June 28, 1988. (A) Ocean surface and (B)-(F) Tidepools 1-5 respectively. Error bars represent standard error of the mean (N=2).
FIGURE 5 (continued)
FIGURE 6: Diatom abundance at the ocean surface waters and in four tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on July 12, 1988. (A) Ocean surface and (B)-(E) Tidepools 2-5 respectively. Error bars represent standard error of the mean (N=2).
FIGURE 6 (continued)
FIGURE 7: Diatom diversity at the ocean surface and in five tidepools of increasing height in the intertidal at Point Atkinson, British Columbia on five occasions: (A) May 15, (B) June 6, (C) June 13, (D) June 28 and (E) July 12, 1988.
FIGURE 7 (continued)
REFERENCES


APPENDIX 2: Growth curves of *Skeletonema costatum* and *Nitzschia thermalis* during the preliminary sets of toxicity tests
FIGURE 1: Growth curves of *Skeletonema costatum* at $1 \times 10^{-5}$ and $1 \times 10^{-7}$ M Cu (first set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2: Growth curves of *Skeletonema costatum* at $1 \times 10^{-9}$ and $1 \times 10^{-11}$ M Cu (first set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 3: Growth curves of *Skeletonema costatum* at $5 \times 10^{-7}$ and $1 \times 10^{-7}$ M Cu (second set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 4: Growth curves of *Skeletonema costatum* at $5 \times 10^{-8}$ and $1 \times 10^{-8}$ M Cu (second set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 5: Growth curves of *Nitzschia thermalis* at $1 \times 10^{-5}$, $1 \times 10^{-7}$, $1 \times 10^{-9}$ and $1 \times 10^{-11}$ M Cu (first set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 6: Growth curves of *Nitzschia thermalis* at $1 \times 10^{-5}$, $5 \times 10^{-6}$ and $1 \times 10^{-6}$ M Cu (second set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 7: Growth curves of *Nitzschia thermalis* at $5 \times 10^{-7}$ and $1 \times 10^{-7}$ M Cu (second set of toxicity tests). (A) *In vivo* fluorescence; (B) Cell counts. Circles, triangles and squares represent replicates 1-3 respectively. Filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
APPENDIX 3: The effect of polycarbonate containers and medium chelexing on the growth of marine diatoms.

INTRODUCTION

The suitability of various materials used in the culturing of marine organisms has long been of concern (e.g. Bernhard et al. 1966, Bernhard and Zattera 1970, Justice et al. 1972, Price et al. 1986). Glass has been the traditional container for phytoplankton studies (except studies involving silicon). However, glass is not suitable for trace metal studies because of a continuous interaction of its ionic surface with cations in the medium (see Brookes 1969). This interaction disrupts the metal equilibrium of the aqueous solution. Such disruptions have been demonstrated by Robertson (1968) and Fitzwater et al. (1982). As a result, trace metal studies are now being conducted in either silanized glass (e.g. Schenck 1984) or plastic containers. Of the plastics, teflon is the most favored material; however, due to its high cost, polycarbonate is more commonly used.

Bernhard and Zattera (1970) tested the effect of polycarbonate containers after autoclaving on the growth of three species of marine phytoplankton and found no effect with the exception of the coccolith *Emiliana huxleyi* (Lohmann) Hay and Sandberg (as *Coccolithus huxleyi*). However, only exponential growth rate was examined. In addition, the level of the growth effect was presented in a qualitative manner. Fitzwater et al. (1982) and Wong et al. (1986) demonstrated increased primary productivity in polycarbonate flasks. In contrast, Marra and Heinemann (1984) found no significant difference in primary productivity between glass and polycarbonate containers when "noncontaminating" procedures were used for both treatments.

Due to the variability in sensitivity to culture materials reported for different diatoms (e.g. Price et al. 1986), the effect of glass and polycarbonate on the growth of *Nitzschia thermalis* (Ehrenberg) Auerswald, *Skeletonema costatum* (Greville) Cleve and
*Thalassiosira pseudonana* (Hustedt) Hasle and Heimdal was tested. One purpose of this study was to test the hypothesis that there is no significant difference in diatom growth between the two types of containers.

Chelating resins are used quite extensively to quantify trace metal content in seawater. They are used for one of two purposes. One purpose is to concentrate the metal and alter the matrix in which the metal is embedded. The other is to measure a specific fraction of the metal.

Atomic absorption spectrophotometry (AAS) is one of the most common techniques used for the determination of trace metals. However, its sensitivity and reproducibility may be quite variable with seawater samples. This is attributed to interference with the sodium chloride matrix. In addition, due to the low concentration of trace metals in seawater, the sensitivity of the AAS is sometimes not adequate for metal detection, even in the absence of salt-matrix problems. As a result, in a number of studies the seawater is passed through a Chelex-100 column in order to remove the salts and concentrate the trace metals (e.g. Kingston *et al.* 1978, Bruland and Franks 1979, Sturgeon *et al.* 1980).

Resin chelation has also been used to determine specific fractions of the metal in seawater. Usually, it is used in combination with other techniques in order to determine metal speciation, for example photooxidation, anodic stripping voltammetry, acid digestion, organic extraction etc. (e.g. Figura and McDuffie 1980, Mackey 1983). In addition, it has been used to measure seawater complexing ability by stripping metals released from organics after seawater treatment with ultraviolet irradiation (e.g. Batley and Florence 1976).

Because of the affinity that Chelex resin has for trace metals, it has been used for removing metal impurities from media used for marine cultures. Davey *et al.* (1970) developed the technique. They obtained excellent metal removals with the sodium form of Chelex-100 from both artificial and natural seawater; 99% of 65Zn, 115mCd, 54Mn
and $^{64}\text{Cu}$; 95% of $^{210}\text{Pb}$; 92% of $^{63}\text{Ni}$ and $^{59}\text{Fe}$; but only 32.6% of $^{110}\text{mAg}$. In addition, they concluded that no toxic components of the resin leached out during the passing through the Chelex column. They observed satisfactory phytoplankton growth in the eluted medium for a number of species. These results were verified by Florence and Batley (1976). The determined 99.5% retention of Cu, Zn, Pb and Cd by a Chelex column. They also observed that Cu-organic complexes and labile Cu and Zn are quantitatively removed by the column after 1 L of effluent natural seawater. In addition, metal impurities were completely removed by Chelex-100 from artificial seawater.

Aquil (Morel et al. 1979) is an artificial chemically defined medium that is used for phytoplankton culturing. It was developed specifically for trace metal studies due to its known composition. It is prepared in a metal-clean fashion. Part of the procedure involves the passing of the artificial seawater and its enrichment stocks through a sodium form Chelex-100 column to remove trace metal impurities. Since this medium was used during my study, I determined the impact that chelexing the medium has on the growth of $\text{Nitzschia thermalis}$ and $\text{Skeletonema costatum}$. The second purpose of this study was to test the hypothesis that there is no difference in growth these two species cultured in chelexed compared to unchelexed Aquil.
METHODS

Part 1: Effect of containers

Stock cultures of *Nitzschia thermalis* (NEPCC 608), *Skeletonema costatum* (NEPCC 18c) and *Thalassiosira pseudonana*, clone 3H (NEPCC B58) were obtained from the North East Pacific Culture Collection, Department of Oceanography, University of British Columbia, Vancouver. Glass and polycarbonate 250 mL Erlenmeyer flasks were 'aged' prior to use for this experiment. They were washed with Fisher Sparkleen detergent, rinsed three times with distilled water, soaked in 1N HCl for 48 h, rinsed three times with distilled deionized water and autoclaved in a Castle standard laboratory autoclave. The procedure was repeated five times before using the flasks for the study. In addition, this cleaning process was repeated (but only run once) between experiments. Artificial seawater (Aquil) was prepared as described in Morel et al. (1979). It was autoclaved in a polycarbonate carboy, and 200 mL aliquots of the same batch of medium were added to all flasks. The stock culture of each species was transferred to both glass and polycarbonate flasks and incubated under the experimental conditions for two weeks. Subsequently, the stocks were transferred to clean flasks of both materials containing fresh medium. When they reached exponential growth, aliquots were removed to inoculate the experimental flasks that were made of the same material. There was no significant difference in the initial inoculum size between the two treatments (Student’s t- or Wilcoxon rank sum test, P>0.05). Five replicate flasks of each treatment were used. All transfers were performed in a class 100 laminar flowhood with all metal parts replaced by polypropylene. The batch cultures were then placed in racks that ensured equidistance from the light source for all replicates of both treatments. Flasks were randomized within each set of racks to avoid any systematic pattern (Runs test for randomness, P>0.05 for all experiments). The experiment was conducted at ~ 16°C, under a 16:8h L:D cycle and an irradiance of ~ 90 μE . m⁻² . s⁻¹. Irradiance was measured inside the culture vessels with a Li-Cor Model LI-185 meter (2π collector); no
significant difference was detected between irradiances inside the two types of flask (Wilcoxon rank sum test, \( P=0.310 \)). Every 24 h, 10 mL aliquots were removed from the cultures after gentle shaking to ensure homogeneous diatom distribution, and used for cell counts and fluorescence measurements. *In vivo* fluorescence was measured using a Turner Designs Model 10 fluorometer. Cell counts were performed using a Palmer Maloney counting slide under a Zeiss Standard 14 compound microscope for *N. thermalis* and *S. costatum*, and an Olympus model BHA phase contrast microscope for *T. pseudonana*. For each replicate, three subsamples were counted using five fields per subsample. The fields represented a random cell distribution as recommended by Lund et al. (1958).

For each culture, three parameters of the growth curve were examined, exponential growth rate, length of lag phase and maximal yield. Exponential growth rate was calculated using \( K_e \) and \( k \) as described in Guillard (1973). The exponential portion of the growth curve was determined to be the part with the steepest overall slope. \( N_0 \) and \( N_1 \) for each replicate were chosen as the points beneath or beyond which there was an apparent deviation from this slope. The length of the lag phase was determined as the time between inoculation and the time at which \( N_0 \) was estimated. The maximal yield was determined as the highest value of cell density or fluorescence that each of the replicates of each species attained during the experiment.

All comparisons between treatments were done using a Student’s \( t \)-test. In cases where heteroscedasity was observed and could not be alleviated by transformation, a non-parametric Wilcoxon rank sum test was used to compare the two treatments. A significance level of 0.05 was used throughout.
Part 2: Effect of chelexing

The general experimental procedure was similar between the two experiments. For this part, only polycarbonate flasks were used for both the stock and the experimental cultures. The flasks were cleaned in the fashion described above.

Twenty litres of standard ocean water (SOW) was prepared as in Morel et al. (1979). They were divided in two equal portions. One portion was passed through a Chelex-100 column (as described in Morel et al. 1979). In addition, nitrate, phosphate and silicate stocks were also prepared and divided in two portions. One of them was also passed through the Chelex. The chelexed nutrient stocks were subsequently added to the chelexed seawater (Chelexed treatment). The unchelexed stocks were added to the unchelexed SOW (Unchelexed treatment). The two types of media were then processed identically (as described in Part 1).

The remainder of the experimental protocol was similar to that of Part 1. Only two parameters of the growth curve were examined, exponential growth rate and length of lag phase.
RESULTS AND DISCUSSION

*Nitzschia thermalis* did not demonstrate any difference in exponential growth rate between the two types of container. The lag phase of this species was significantly higher in polycarbonate according to the cell counts but not according to the fluorescence estimates (Fig. 1, Table 1). The maximal yield for this species was not incorporated in the comparisons between treatments, due to a significant difference in the initial population size. In polycarbonate, *Skeletonema costatum* exhibited a significantly lower growth rate (established using the cell counts) and a longer lag phase than in glass, as shown by both the fluorescence measurements and the cell counts (Fig. 2, Table 1). For this species, maximal yield also showed a significant decrease. *Thalassiosira pseudonana* demonstrated a reduced growth rate, as shown by fluorescence, and a decreased maximal yield, shown both by fluorescence and cell counts, when growing in polycarbonate flasks (Fig. 3, Table 1). This study was preceded by a number of preliminary experiments which demonstrated similar results. Problems encountered during these experiments and corrected for in the present study were the inoculum size (a high inoculum size subsequently resulted in a short exponential growth of two days), adequate shaking before daily removal of aliquots, and the inoculum state. For example, inocula of *S. costatum* that were in stationary phase resulted in low growth rate and extended lag phase of up to 10 days that could not necessarily be attributed to the container material. However, in those cases the effect of the container was still present, if not more pronounced. The mean growth rates were 0.93-0.95 div • day\(^{-1}\) in glass and 0-0.50 div • day\(^{-1}\) in polycarbonate (N = 5, Student’s t-test, P<0.05). In this study, I ensured that the inoculum size was appropriate, the distribution inside the flasks was homogeneous, and the inoculum was always in exponential growth. In addition, the five replicates of both treatments were processed identically throughout the experiment. I, therefore, conclude that the observed effect was the result of the container material.
Polycarbonate has been considered a plastic that has little or no effect on phytoplankton growth compared to glass (Bernhard and Zattera 1970). From my experiments, it appears to be a material that can affect growth rate, length of lag phase and maximal yield. In the present study, statistically significant (rather than qualitative) differences for each specific growth parameter were observed. Growth in polycarbonate containers appears to be associated with a larger degree of variability between replicate flasks than growth in glass (Fig. 2, Table 1). Therefore, studies with low replication of treatments may not detect a significant difference in growth.

The observed lower growth in polycarbonate containers compared to glass could be due to a number of factors. I found no difference in light intensity between the two types of flasks. However, Wong et al. (1986) observed a 15.1% reduction in photosynthetically available energy inside polycarbonate flasks. This could potentially result in differential growth. A second factor could be the plastic itself. Despite extensive soaking and rinsing of the flasks before use, some toxic agent, such as the plasticiser, may have been present in high enough concentrations to induce an inhibitory effect. Dyer and Richardson (1962) demonstrated this for type II polyvinyl chloride rods. Lastly, the observed effect may be a growth enhancement in glass containers rather than a growth inhibition in polycarbonate. The continuous interaction of the glass surface with the medium could enhance growth through leaching of a nutrient or buffering of a metal. For example, Price et al. (1987) demonstrated a selenium requirement for T. pseudonana. This nutrient is absent from Aquil and could have possibly been introduced to the medium in the glass flasks through leaching of the glass surface. However, it will remain absent from the polycarbonate containers. On the other hand, the medium was autoclaved in a standard laboratory autoclave which could provide a source of metal contamination. The effect will be reduced in the glass containers since a portion of the contaminant will be removed through adsorption on the surface walls.
The results of the effect of chelexing the medium on phytoplankton led to quite different conclusions. The growth rate of *Nitzschia thermalis* was higher in chelexed medium according to the fluorescence measurements but not according to cell counts (Fig. 4, Table 2). This species demonstrated no effect on the length of lag phase according to the cell counts. It did however show an increased lag phase in chelexed medium according to the fluorescence estimates (Fig. 4, Table 3). It can be observed in Fig. 4 that, although all five chelexed replicates exhibited high growth during the second day, there was a sudden cessation on the third day for three of the replicates. No such pattern was apparent for the unchelexed replicates.

*Skeletonema costatum* demonstrated higher growth rate in the chelexed medium according to the cell counts (but not according to fluorescence) (Fig. 5, Table 2). This species also demonstrated longer lag phase in the unchelexed medium (Fig. 5, Table 3).

Growth medium is chelexed in order to rid it of trace metal impurities and in order to prevent contamination from unknown sources (Davey *et al.* 1970, Morel *et al.* 1979). Its effect on phytoplankton growth was tested and found satisfactory (Davey *et al.* 1970). In my experiment, growth was enhanced in most cases. This can be attributed to a number of factors. It could be the result of high metal impurities in the salts, chemicals and/or distilled water used for the preparation of the medium all of which will be removed by the Chelex column. A second possible source of contamination is the glass carboy that was used for the mixing of the salts. The preparation procedure of the medium is not carried out under class 100 conditions but rather on a laboratory bench. Aerosols from the laboratory could be contributing to the metal contamination as well. In all these cases, metals will be added to the seawater and, unless they are removed by chelexing the medium, may become inhibitory.

*N. thermalis* demonstrated increased lag phase in the chelexed medium. This could be attributed to the absence of a required metal due to removal by the resin. Aquil has been tested as a growth medium for a number of species and has been considered
adequate. However, it is possible that this is the case for most species but not *Nitzschia thermalis*. Perhaps this species requires one of the metals in higher concentrations than those added to the chelexed SOW and may therefore become limited. A different possibility is that this species may have a requirement for a metal that is never added to Aquil, such as selenium (see Price *et al.* 1987).

Lower growth in polycarbonate containers or unchelexed medium was not consistently demonstrated for both biomass measurements. In some cases, it was exhibited when using cell counts as a biomass index but not when using fluorescence. This could be explained if an inhibitory agent affects some aspect of algal physiology (e.g. pigment production) but not another (e.g. cell division; as for copper in *S. costatum*, see Morel *et al.* 1978). However, the discrepancy between the two biomass indices is probably the result of different within treatment variance for each index. In most of the cases where there was a discrepancy, no treatment effect was observed for the index with the highest variability. With *Nitzschia* in the container experiment, the discrepancy arose as a result of the peculiar shapes of the growth curves. Not much growth was observed with the fluorescence measurements (Fig. 1). In glass, the fluorescence of this species increased exponentially for 1-2 days, very late in the experiment. The initial increase in cell number is not reflected by the fluorescence estimates. Due to the peculiar shape of these growth curves, I can only accept the result obtained by the increase in cell concentration.
TABLE 1. Comparison of growth parameters of species grown in glass and polycarbonate containers (m = mean (N = 5); sd = 1 standard deviation; cell # = cells . mL⁻¹ medium; fluor = fluorescence; PC = polycarbonate; * = Wilcoxon test.)

<table>
<thead>
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<th>Growth parameter (units)</th>
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<td>Glass</td>
<td>0.536</td>
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<td>PC</td>
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<td>Glass</td>
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<td>0.894</td>
<td>0.133</td>
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<td></td>
<td><em>T. pseudonana</em></td>
<td>Cell #</td>
<td>Glass</td>
<td>1.42</td>
<td>0.100</td>
<td>&gt;0.05</td>
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<td>Glass</td>
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### TABLE 1 (continued)

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<th>Fluor</th>
<th>Glass</th>
<th>PC</th>
<th>Fluor</th>
<th>Glass</th>
<th>PC</th>
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<td>Maximal yield</td>
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<td>(cells/mL)</td>
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<th>PC</th>
<th>Fluor</th>
<th>Glass</th>
<th>PC</th>
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<td>&lt;0.05</td>
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<td>&gt;0.05</td>
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<td>S. costatum</td>
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<td>23,100</td>
<td>&lt;0.05</td>
<td>&lt;0.05</td>
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<td>PC</td>
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<td>33,600</td>
<td>&gt;0.05</td>
<td>&gt;0.05</td>
<td>*</td>
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<td>Cell #</td>
<td>Glass</td>
<td>Fluor</td>
<td>PC</td>
<td>Glass</td>
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TABLE 2: Effect of chelexing artificial seawater medium on the growth rate (in div \cdot day^{-1}) of Skeletonema costatum (= Skel) and Nitzschia thermalis (= Nitz). (cell # = cells \cdot mL^{-1} medium; fluor = fluorescence; Chelex = chelexed medium; Unchel = unchelexed medium; N = sample size; S.D. = standard deviation.)

<table>
<thead>
<tr>
<th>Species</th>
<th>Biomass Index</th>
<th>Treatment</th>
<th>Mean Growth Rate (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
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<td></td>
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<tr>
<td>Nitz</td>
<td>Cell #</td>
<td>Chelex</td>
<td>1.42 (5)</td>
<td>0.43</td>
<td>1.06</td>
<td>&gt;0.100</td>
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<tr>
<td></td>
<td></td>
<td>Unchel</td>
<td>1.20 (5)</td>
<td>0.20</td>
<td></td>
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</tr>
<tr>
<td></td>
<td>Fluor</td>
<td>Chelex</td>
<td>1.35 (5)</td>
<td>0.09</td>
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<tr>
<td></td>
<td></td>
<td>Unchel</td>
<td>0.89 (5)</td>
<td>0.02</td>
<td>12.7</td>
<td>&lt;0.001</td>
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<td>Skel</td>
<td>Cell #</td>
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<td>1.71 (5)</td>
<td>0.18</td>
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<td></td>
<td></td>
<td>Unchel</td>
<td>1.05 (4)</td>
<td>0.21</td>
<td>4.98</td>
<td>&lt;0.010</td>
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<tr>
<td></td>
<td>Fluor</td>
<td>Chelex</td>
<td>0.96 (5)</td>
<td>0.10</td>
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<td></td>
<td>Unchel</td>
<td>0.86 (4)</td>
<td>0.12</td>
<td>1.48</td>
<td>&gt;0.100</td>
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</table>
TABLE 3: Effect of chelexing artificial seawater medium on the length of lag phase (in days) of *Skeletonema costatum* (= Skel) and *Nitzschia thermalis* (= Nitz). (cell # = cells · mL⁻¹ medium; fluor = fluorescence; Chelex = chelexed medium; Unchel = unchelexed medium; N = sample size; S.D. = standard deviation; * = Wilcoxon test used instead.)

<table>
<thead>
<tr>
<th>Species</th>
<th>Biomass Index</th>
<th>Treatment</th>
<th>Mean Lag Phase (N)</th>
<th>S.D.</th>
<th>t-value</th>
<th>P</th>
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<tbody>
<tr>
<td><em>Nitz</em></td>
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<td>1.2 (5)</td>
<td>1.1</td>
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<td>1.0 (5)</td>
<td>1.0</td>
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<tr>
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<td>Fluor</td>
<td>Chelex</td>
<td>3.0 (5)</td>
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<td></td>
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<td>1.0 (5)</td>
<td>0.0</td>
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</tr>
<tr>
<td><em>Skel</em></td>
<td>Cell #</td>
<td>Chelex</td>
<td>0.8 (5)</td>
<td>0.5</td>
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<tr>
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<td>Chelex</td>
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<td></td>
<td>Unchel</td>
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<td>0.86 (4)</td>
<td>0.1</td>
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</table>
FIGURE 1: Growth curves of *Nitzschia thermalis*. A = *in vivo* fluorescence; B = cell counts. Solid lines = glass containers; dashed lines = polycarbonate containers; circles, triangles, squares, inverse triangles and diamonds = replicates 1-5 respectively; filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 2: Growth curves of *Skeletonema costatum*. A = *in vivo* fluorescence; B = cell counts. Solid lines = glass containers; dashed lines = polycarbonate containers; circles, triangles, squares, inverse triangles and diamonds = replicates 1-5 respectively; filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 3: Growth curves of *Thalassiosira pseudonana*. A = *in vivo* fluorescence; B = cell counts. Solid lines = glass containers; dashed lines = polycarbonate containers; circles, triangles, squares, inverse triangles and diamonds = replicates 1-5 respectively; filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 4: Growth curves of *Nitzschia* thermalis. A = *in vivo* fluorescence; B = cell counts. Solid lines = chelexed medium; dashed lines = unchelexed medium; circles, triangles, squares, inverse triangles and diamonds = replicates 1-5 respectively; filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
FIGURE 5: Growth curves of *Skeletonema costatum*. A = *in vivo* fluorescence; B = cell counts. Solid lines = chelexed medium; dashed lines = unchelexed medium; circles, triangles, squares, inverse triangles and diamonds = replicates 1-5 respectively; filled symbols represent the points at which $N_0$ and $N_1$ were estimated.
REFERENCES


