

Resistance of the marine diatom *Thalassiosira* sp. to toxicity of phenolic compounds

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ABSTRACT: In many nearshore marine systems, microalgae can be chronically exposed to anthropogenic and biogenic phenolic and halophenolic compounds that accumulate in surficial sediments. Although biodegradation of some phenolic compounds has been demonstrated in freshwater algae, this capability has not been tested in marine species. We examined a ubiquitous marine diatom, *Thalassiosira* sp. HP9101, for its capacity to tolerate, and/or utilize phenol and benzoic acid. We also examined the aromatic ring cleavage reactions of this diatom and its capacity to dechlorinate chlorophenolic compounds. Axenic *Thalassiosira* sp. cultures were grown at $60 \mu\text{E m}^{-2} \text{s}^{-1}$ (12:12 h light:dark cycle) in the presence or absence of phenol, benzoate, catechol or protocatechuate and their growth kinetics and physiological responses determined. *Thalassiosira* sp. was inhibited by catechol and protocatechuate. Growth in the presence of 1 mM phenol was observed only after an extended lag and was quite slow. Growth in the presence of 0.25 mM phenol occurred after a much shorter lag and phenol was taken up by these cultures at an estimated rate of $0.08 \text{ fmol phenol cell}^{-1} \text{ h}^{-1}$. No inhibition of *Thalassiosira* sp. by 1 mM benzoate was observed. Growth stimulation by added phenolic substrates was not demonstrated, although phenol-supplemented *Thalassiosira* sp. produced substantial levels of protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase, the *ortho*- and *meta*-pathway aromatic ring cleavage enzymes, respectively. Only protocatechuate 4,5-dioxygenase activity was detected in *Thalassiosira* sp. grown with 1 mM benzoate. Phenol supplemented *Thalassiosira* sp. was also capable of dechlorinating monochlorophenols, 3,5-dichlorophenol, and 2,4,6-trichlorophenol. These reactions were NADH-dependent and were not observed in control cultures grown without phenol.

KEY WORDS: Benthic microalgae · Marine diatom · Phenol · Aromatic ring cleavage · Haloaromatics · Dehalogenation

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INTRODUCTION

Phenolic compounds are important products of the petrochemical and coal-processing industries (Reisch 1994), and are common pollutants in many nearshore marine systems. These compounds are also produced

naturally through breakdown of lignin (Wilson et al. 1986, Goni & Thomas 2000, Motamed & Texier 2000), which is often abundant in estuarine sediments. Other types of phenolic compounds that can be found in nearshore marine systems include a variety of halophenols. Chlorinated phenolic compounds, such as chlorophenols, chloroguaiacols, and chlorocatechols, have numerous anthropogenic sources, including pulp mill effluent (Kringstad & Linström 1984, Xie et al. 1986, Häggblom & Salkinoja-Salonen 1991), agricul-

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tural and residential runoff (Ahlborg & Thunberg 1980), and sewage and wastewater discharges (Chapman et al. 1996). In addition, bromophenolic compounds are natural products of a variety of marine organisms including many species of marine macroalgae (reviews by Butler & Walker 1993, Gribble 1999) and infaunal polychaetes and hemichordates (Woodin et al. 1987, Chen et al. 1991, Woodin 1991, Steward et al. 1992, 1995, Fielman et al. 1999, Gribble 1999). These anthropogenic and biogenic phenolic and halophenolic compounds can accumulate to significant levels in surficial sediments (Karickhoff et al. 1979, Schellenberg et al. 1984, King 1986, Xie et al. 1986, Lincoln et al. 2002), and at least some of these compounds, at concentrations found in sediments, are demonstrably toxic to some of the sediment biota (Woodin 1991, Woodin et al. 1993, Fielman et al. unpubl. data).

Steward et al. (1992) demonstrated that elevated bromophenol concentrations produced by the infaunal marine polychaete *Notomastus lobatus* were not inhibitory to benthic microalgal populations associated with exposed sediments in the North Inlet estuary, a salt-marsh estuary near Georgetown, South Carolina, USA. The authors concluded that the microalgae were capable of resistance to halophenol toxicity. Information on the physiological mechanisms underlying microalgal resistance to these and other phenolic compounds may have relevance to ecosystem function, because acute or chronic exposure to such compounds may be common to not only benthic microalgae but to planktonic types that, for example, are intimately associated with resuspended sediments. A pressing question is whether metabolic degradation may play a role in resistance of microalgae to toxicity of phenolic and halophenolic compounds.

Recent studies have shown significant biodegradation of phenol and its methylated homologues by the freshwater mixotrophic chrysophyte *Ochromonas danica* (Semple & Cain 1996, 1997, Semple et al. 1999). In addition, an unidentified freshwater microalga has been found to tolerate high levels of pentachlorophenol and to produce CO₂ from this compound (Tikoo et al. 1997). We have examined a marine diatom, *Thalassiosira* sp. HP9101, for its capacity to tolerate and to degrade phenolic compounds. *Thalassiosira* spp. are ubiquitous and often numerically abundant in marine systems, with over 100 species described from diverse marine environments (Round et al. 1990, Gedde 1999). Also, *Thalassiosira* spp. were a relatively abundant component of the nanophytoplankton in North Inlet (Lewitus et al. 1998), a shallow well-mixed estuary marked by high tidal scouring. We therefore consider *Thalassiosira* sp. HP9101 an appropriate model species representative of microalgae that can be intimately associated with resuspended sediments.

As a first step towards testing the potential for marine microalgae to degrade phenolic compounds, we examined the physiological and metabolic responses of *Thalassiosira* sp. to phenol, catechol, protocatechuate, and benzoate additions. We also examined this organism for metabolic activities needed for degradation of chlorophenols. We employed chlorophenols in this study due to their widespread occurrence in nearshore marine sediments and their recalcitrance relative to their brominated homologues.

MATERIALS AND METHODS

Culture source and growth conditions. *Thalassiosira* sp. HP9101 (Bacillariophyceae) was isolated in 1991 by A.J.L. from the Choptank River, a subestuary (salinity 7 to 12 ppt) of Chesapeake Bay. The culture was made axenic by aseptically picking a single cell using a capillary pipette, followed by cultivation. Batch cultures were maintained in a temperature-controlled environmental chamber (26°C) in f/2-enriched filtered seawater as in Guillard 1975, but without Si addition), adjusted to 17 ppt by dilution with ultrapure deionized water. Cultures were acclimated to growth at 60 µE m⁻² s⁻¹ (12:12 h light:dark cycle) by repeated transfers from mid-exponential growth phase, following Lewitus & Kana (1994). After acclimation to the growth irradiance, aliquots were transferred into new medium (n = 2 for Expt A, n = 3 for Expt B), with or without phenolic compound addition, at an initial population density of 5 × 10³ to 5 × 10⁴ cells ml⁻¹, depending on the experiment. Culture purity was confirmed in all experiments by periodic testing (including at the end of the experiment) using a DAPI-staining technique (Porter & Feig 1980).

Experimental protocols. The purpose of the first experiment (Expt A) was to determine if ring cleavage dioxygenase or reductive dechlorinase activity could be measured in *Thalassiosira* sp. HP9101, and whether these enzyme activities required the presence of phenolic compounds in the culture medium (i.e. were potentially inducible by phenolic substrates). Chlorophenols were not used as supplements due to their toxicity, volatility, and low solubility in seawater. Aliquots of *Thalassiosira* sp. culture were transferred into replicate 1 l polycarbonate bottles containing medium with or without 1 mM phenol, 1 mM catechol, or 1 mM protocatechuate, at initial population densities of 5 × 10³ cells ml⁻¹. After a 5 d period of exposure (that allowed an initial period of acclimation), aliquots from these cultures were again transferred to new medium to re-establish initial population densities of 5 × 10³ cells ml⁻¹. Cultures in experimental treatments, but not controls, experienced lags in growth. All cultures exhibit-

ing active population growth were harvested in exponential growth phase (verified by continued time-course cell counts of the remaining culture) at cell abundances ranging from 5×10^5 to 1.5×10^6 cells ml^{-1} . Cell abundance was monitored using a hemocytometer, following Lewitus et al. (1998).

Based on the relatively high dioxygenase and dechlorinase activity measurements from phenol-grown *Thalassiosira* sp. cultures in the first experiment, a follow-up experiment (Expt B) was designed to further examine the effects of phenol (concentration range from 0.01 to 1 mM) on the growth rate, chlorophyll *a* cell⁻¹, biovolume, and dechlorinase activity of the diatom. We also tested 1 mM benzoate as another potential phenolic substrate. Chlorophyll *a* was measured by fluorometric analysis, following extraction in 90% acetone using a freeze-thaw method (Glover & Morris 1979). Biovolume was calculated by capturing images with an image analyzer, and calculating cell volume as $\pi r^2 \times \text{length}$. Biovolume estimates were based on sample sizes of 12 to 13 cells at 0 h, or from 21 to 78 cells at other sampling times. Phenol or benzoate concentrations in the culture medium were followed by HPLC analysis of culture supernatants, taken at the same times as cell abundance was measured (1 to 3 d intervals). Culture samples (1 ml) were mixed with an equal volume of 100% acetonitrile. After centrifugation to remove precipitated proteins and polysaccharides, the samples were assayed for phenol or benzoate using a Hewlett Packard 1100 series HPLC system with a 15 cm HP C18 reversed-phase column and using a methanol:H₂O:acetic acid (50:49:1) mobile phase at a flow rate of 1.5 ml min^{-1} . Detection was by absorbance at 280 nm. The retention times of phenol and benzoate from the cultures were identical to those of the authentic compounds. Compound quantification was based on absorbances of known quantities of phenol and benzoate.

Ring cleavage dioxygenase and dehalogenase activity assays. *Thalassiosira* sp. cells grown with phenol or benzoate were homogenized by sonication. The homogenate was centrifuged at $5000 \times g$ for 5 min and the supernatant designated as crude extract. Catechol 1,2-dioxygenase activity (Nakazawa & Nakazawa 1970), catechol 2,3-dioxygenase (Nozaki 1970), protocatechuate 3,4-dioxygenase (Fujisawa 1970), and protocatechuate 4,5-dioxygenase (Ono et al. 1970) were assayed using established methods. Dechlorination of various chlorophenols by *Thalassiosira* sp. crude extracts was measured using a reaction system consisting of 10 μM NADH, 50 $\mu\text{g ml}^{-1}$ 2,4,6-trichlorophenol, 3,5-dichlorophenol, or 2-, 3-, or 4-chlorophenol, and 50 $\mu\text{g ml}^{-1}$ crude extract protein in 100 mM KH_2PO_4 , pH 4.6. Assay mixtures were incubated at 28°C for 15 min and the reactions stopped by addition of an

equal volume of acetonitrile. After centrifugation, the samples were assayed for chlorophenols using the HPLC system described above and following the methods of Watson et al. (2000). Retention times of dehalogenation products were compared to those of authentic standard compounds and compound quantification was based on absorbance of known quantities of authentic compounds. One unit of enzyme activity is defined as 1 μmol of substrate consumed per minute. Protein was assayed by the method of Lowry et al. (1951).

RESULTS

Effects of phenolic compounds on growth, biovolume, and chlorophyll *a*

In Expt A, growth was inhibited in 2 treatments (1 mM catechol or 1 mM protocatechuate, data not shown) and never recovered throughout the experiment (112 d). In the 1 mM phenol treatment, a pronounced lag in growth occurred (82 d), followed by slow growth ($\mu = 0.07 \text{ d}^{-1}$). Because phenol concentrations were not followed in Expt A cultures, it is unknown whether the end of the growth lag was due to the development of increased tolerance (e.g. acclimation) to 1 mM phenol or to a decrease in phenol concentration (e.g. through chemical conversion or uptake by *Thalassiosira* sp.).

Expt B tested the physiological response of *Thalassiosira* sp. to phenol or benzoate additions in shorter-term incubations (maximum 14 d) than Expt A. The highest phenol concentration tested (1 mM) inhibited growth throughout the course of the experiment (Fig. 1A,B), consistent with the 82 d growth lag observed in Expt A. The mean biovolume of the phenol-enriched cultures was higher than that of the controls, but not significantly so (Student's *t*-test, $p > 0.05$) due to high variability of cell size in this treatment (Fig. 1C). The reason for the relatively high cell size variability in 1 mM phenol-treated cells was that a portion of the population was elongated (Fig. 2) and appeared as if cell division, but not biosynthesis, was repressed. In contrast to the response to 1 mM phenol, cultures grown with 1 mM benzoate in the medium were not inhibited and, in fact, cell abundance (i.e. growth rate), chlorophyll *a*, or biovolume in the benzoate-enriched cultures did not differ significantly (*t*-test, $p > 0.05$) from the controls.

The inhibitory effect of phenol on *Thalassiosira* sp. growth rate increased with increasing phenol concentration, with a threshold for significant inhibition at about 0.3 mM phenol (Fig. 3). Although population density decreased during incubation after 1.0 or

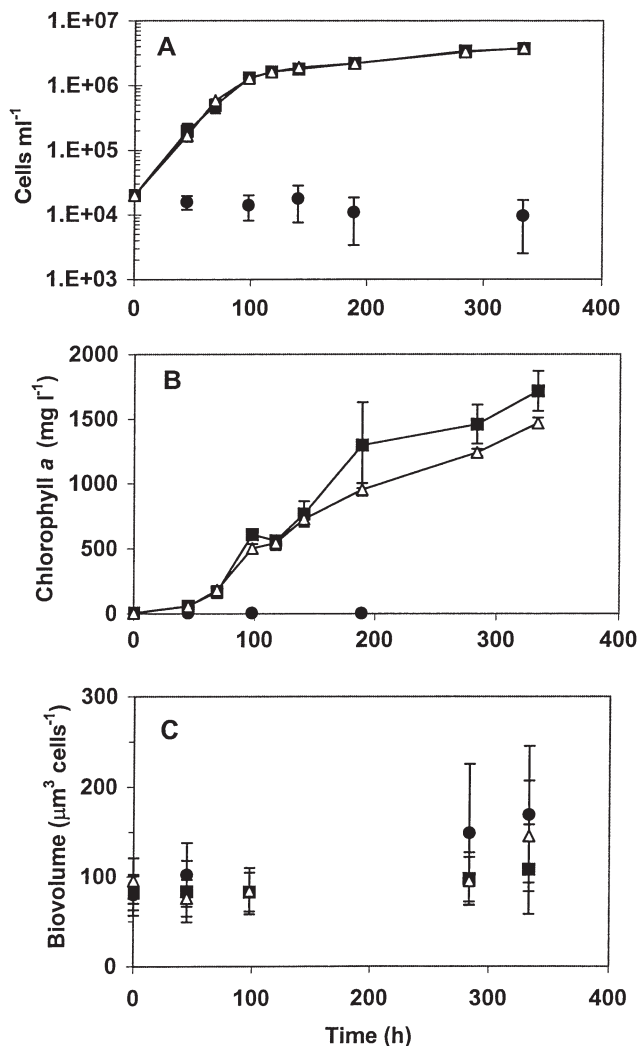


Fig. 1. *Thalassiosira* sp. Time-course changes in (A) cell abundance, (B) chlorophyll *a*, and (C) biovolume of control (■), 1 mM phenol (●), or 1 mM benzoate (△)

1.1 mM phenol addition (mean specific growth rate of -0.065 d^{-1}), the mean specific growth rate of cultures grown with 0.3 to 0.4 mM phenol was 0.66 d^{-1} , only 40% less than that of control cultures. The growth rates of cultures enriched with phenol at concentrations less than 0.3 mM did not differ significantly (t -test, $p > 0.05$) from the rates of control cultures.

In cultures grown with 0.25 mM phenol, a short lag in population doubling consistently occurred, followed by growth at rates similar to control cultures (e.g. Fig. 4A). Chlorophyll *a* cell⁻¹, however, varied significantly (t -test, $p < 0.05$) with this treatment (Fig. 4B). Initially, cellular chlorophyll content was higher in the phenol-enriched cultures, corresponding to the lag phase. Afterwards, chlorophyll *a* cell⁻¹ decreased in the phenol treatment, and remained at a significantly

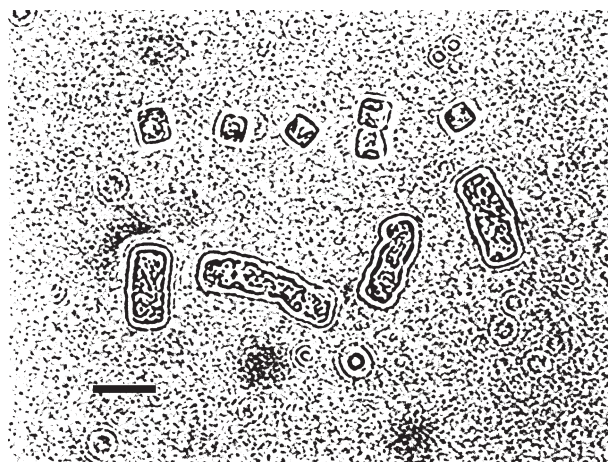


Fig. 2. *Thalassiosira* sp. Superimposed light micrographs of cells from 2 cultures. The 6 smaller cells at the top were taken from a control culture (no phenolic compound addition), and the 4 larger cells from a culture grown with 1 mM phenol. Both samples were taken at 336 h of the experimental incubation. Scale bar = 5 μm

lower level (t -test, $p < 0.05$) than in control cultures throughout the experiment. Mean biovolume was not significantly different (t -test, $p > 0.05$) between treatments (data not shown).

In cultures grown with 0.1 mM phenol, a time-course decrease in ambient phenol concentration was measured during the period of increased cell abundance (Fig. 5). Using a weighted average for population density (Frost 1972), the phenol uptake rate during the period of decreasing phenol concentration was estimated as $0.08 \text{ fmol phenol cell}^{-1} \text{ h}^{-1}$.

Production of ring cleavage dioxygenases

The capacity of the diatom to degrade phenol and other phenolic compounds was assessed by assay of enzymes involved in cleavage of the aromatic ring. *Thalassiosira* sp. grown in the presence of 1 mM

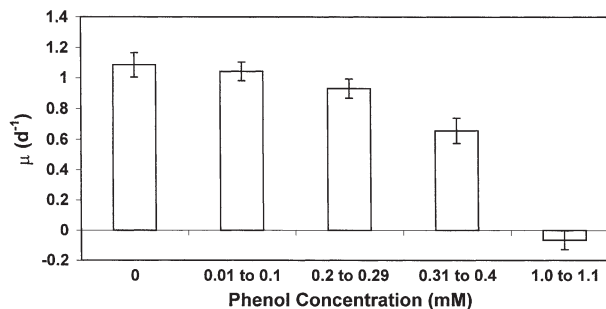


Fig. 3. *Thalassiosira* sp. Specific growth rate (μ) of replicate cultures grown without phenol (0 mM) or with phenol added at different concentration ranges

Table 1. *Thalassiosira* sp. Ring cleavage dioxygenase specific activities in cell-free extracts from cultures grown with different phenolic supplemental carbon sources. Cat 1,2-O₂ase: catechol 1,2-dioxygenase; Cat 2,3-O₂ase: catechol 2,3-dioxygenase; Proto 3,4-O₂ase: protocatechuate 3,4-dioxygenase; Proto 4,5-O₂ase: protocatechuate 4,5-dioxygenase. One unit of enzyme activity is defined as 1 μmol of substrate consumed per minute

Substrate	Specific activity (U mg ⁻¹ protein)			
	Cat 1,2-O ₂ ase	Cat 2,3-O ₂ ase	Proto 3,4-O ₂ ase	Proto 4,5-O ₂ ase
Expt A				
None	0	0	0	0
1 mM phenol	0	0	0.40	0.16
1 mM catechol	Growth inhibited, therefore not measured			
1 mM protocatechuate	Growth inhibited, therefore not measured			
Expt B				
None	0	0	0	0
1 mM phenol	Growth inhibited, therefore not measured			
1 mM benzoate	0	0	0	0.11

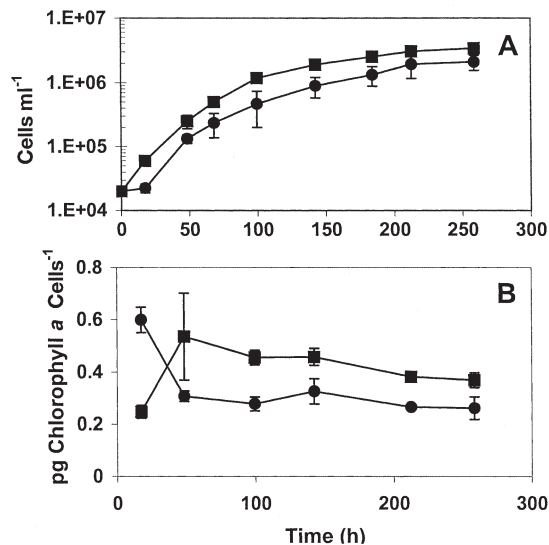


Fig. 4. *Thalassiosira* sp. Time-course changes in (A) cell abundance and (B) chlorophyll a cell⁻¹ of cultures grown without phenol addition (control, ■) or with 0.25 mM phenol (●)

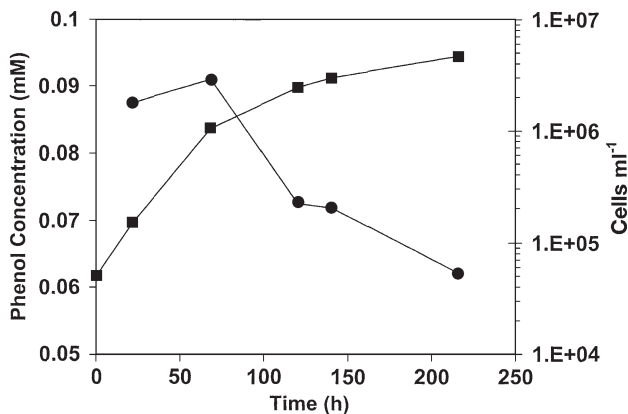


Fig. 5. *Thalassiosira* sp. Time-course changes in (■) cell abundance and (●) culture medium phenol concentration in culture grown with 0.1 mM phenol initial concentration

phenol produced substantial levels of protocatechuate 3,4-dioxygenase and protocatechuate 4,5-dioxygenase, the *ortho*- and *meta*-pathway aromatic ring cleavage enzymes, respectively (Table 1). Only protocatechuate 4,5-dioxygenase activity was detected in *Thalassiosira* sp. grown in the presence of 1 mM benzoate. No ring cleavage dioxygenase enzyme activities were detectable in control cells grown without supplemental phenolic substrates, and no catechol cleavage dioxygenases were detected.

Production of dechlorinase activity

Phenol-supplemented *Thalassiosira* sp. was also capable of dechlorinating monochlorophenols, 3,5-dichlorophenol, and 2,4,6-trichlorophenol (Table 2). These reactions were NADH-dependent and were not observed in control cultures grown without phenol. In addition, no volatile halogenated aromatic compounds were detected in control cells during HPLC assays.

Table 2. *Thalassiosira* sp. Reductive dechlorinase specific activities in cell-free extracts from cultures grown with phenol as supplemental carbon source. 2CP: 2-chlorophenol; 3CP: 3-chlorophenol; 4CP: 4-chlorophenol; 3,5-DCP: 3,5-dichlorophenol; TCP: 2,4,6-trichlorophenol; nd: not determined. One unit of enzyme activity is defined as 1 μmol of substrate consumed per minute

Phenol conc.	Specific activity (U mg ⁻¹ protein)				
	2CP	3CP	4CP	3,5DCP	TCP
Expt A					
0	0	0	0	0	0
1 mM	2.2	0.3	3.8	0.4	3.5
Expt B					
0	0	0	0	0	0
0.25 mM	nd	nd	nd	nd	5.0

DISCUSSION

This study is the first to demonstrate phenol degradation and reductive dechlorination in a marine diatom. *Thalassiosira* sp. is a ubiquitous diatom genus in marine planktonic and benthic environments, and thus understanding its role in breakdown or biotransformation of phenolic compounds may have widespread relevance in estuaries and other coastal regions influenced by anthropogenic and biogenic sources of phenolic compounds. Although the physiological response to phenol additions and catabolic enzyme activities indicate phenol degradation by *Thalassiosira* sp., it is unclear whether substrate breakdown is strictly a function of detoxification or if the diatom also uses phenol as a carbon source for growth. Although phenol enrichment did not enhance growth in any of our experiments, it is possible that mixotrophic (i.e. inorganic and phenol C use) growth rates did not exceed photoautotrophic rates at the moderate growth irradiance used. Indeed, 0.25 mM phenol addition decreased cellular chlorophyll *a* content but not growth rate. Use of phenol as a supplemental carbon source could explain the maintenance of growth despite the reduction in photosynthetic potential.

Similar to previous work with the freshwater chryso-phyte *Ochromonas danica* (Semple & Cain 1996, 1997), phenol catabolism appeared to be inducible in *Thalassiosira* sp. (i.e. ring cleavage dioxygenase or dehalogenase activity was only detected in diatom cultures incubated with phenol or benzoate), and a lag occurred in population growth which varied positively with phenol concentration. Levels of ring cleavage dioxygenases produced by phenol- and benzoate-grown *Thalassiosira* sp. were comparable to those observed in 4-hydroxybenzoate- and protocatechuate-grown *Rhizobium leguminosarum* (Chen et al. 1984, Chen & Lovell 1990, 1994) and in catechol-grown *O. danica* (Semple & Cain 1996). The specific activities of these enzymes in phenol- and benzoate-grown *Thalassiosira* sp. are certainly consistent with the capacity to use aromatic compounds as supplemental carbon sources, and in fact exceed those found in some bacteria that can utilize various aromatic compounds as their sole carbon source for growth (Chen et al. 1993).

Thalassiosira sp. produced protocatechuate 4,5-dioxygenase, the *meta*-cleavage enzyme for protocatechuate. Phenol-grown cultures also produced protocatechuate 3,4-dioxygenase, the *ortho*-cleavage enzyme. The *meta*-cleavage reactions are often considered rare in eucaryotes (Cain 1980, Jones et al. 1993, Middelhoven 1993), but have been identified in both microalgae (Semple & Cain 1996) and higher plants (Kickuth 1970, Gramss & Rudeschko 1998). The report by Semple & Cain (1996) of catechol 2,3-dioxygenase

activity in *Ochromonas danica*, was the first of a *meta*-cleavage activity in an alga. However, *Thalassiosira* sp. produced no catechol dioxygenase activities, regardless of the growth substrate. The protocatechuate 4,5-dioxygenase activity assayed in *Thalassiosira* sp. represents the first observation of this enzyme in an alga. This activity has been found previously in tissues of several vascular plants including *Mendicago sativa* (alfalfa), *Sinapis alba* (white mustard), *Lepidium sativum* (cress), and *Scripus lacustris* (rush) (Kickuth 1970, Gramss & Rudeschko 1998). Occurrence of both *ortho*- and *meta*-cleavage activities in *Thalassiosira* sp. is not unique to this organism. Several bacterial species, including *Azomonas agilis*, *Azospirillum brasilense*, *Azospirillum lipoferum*, *Azotobacter vinelandii*, and *Rhizobium leguminosarum* can also produce both enzymes (Chen & Lovell 1990, Chen et al. 1993, and references therein). The advantage gained by *Thalassiosira* sp. or other organisms from simultaneous production of the ring cleavage dioxygenases required for 2 parallel aromatic degradation pathways is not known.

Crude extracts prepared from phenol-grown *Thalassiosira* sp. also catalyzed an NADH-dependent dechlorination of various chlorophenols. The products of this reaction were consistent with a reductive dehalogenation mechanism: *ortho* and *para* carbon-chloride bonds were highly susceptible to reduction, but little activity against *meta* substituents was observed. Various bacterial dehalogenation reactions have been shown to attack different substituent positions with very different affinities (Häggbloom 1990, Mohn & Tiedje 1992, Steward et al. 1995, Watson et al. 2000). In many cases, only carbon-halide bonds at a single aromatic ring position are susceptible to catalytic cleavage.

Ochromonas danica extracts did not oxidize 4-chlorophenol or 2,4-dichlorophenol (Semple & Cain 1996), while *Thalassiosira* sp. extracts did so. An explanation for this dichotomy can be found in the different enzyme activities expressed by these organisms. As a general rule, degradation of halogenated aromatic compounds does not proceed through the *meta*-cleavage pathway (Knackmuss 1981, Rojo et al. 1987, Pettigrew et al. 1991). The observation of suicide inhibition of catechol 2,3-dioxygenase by production of an acylchloride from 3-chlorocatechol (Bartels et al. 1984) has been used as an explanation for the failure of *meta*-pathway oxidation of these compounds. This inhibition process could occur in *O. danica* and prevent oxidation of haloaromatics by this organism. It would be possible for the reductive dechlorination reaction catalyzed by *Thalassiosira* sp. to produce phenol from chlorophenols prior to catabolism of the chlorophenol aromatic ring. This sequence of reactions would avoid the issue of suicide inhibition altogether. It is also possible that the protocatechuate 4,5-dioxygenase activity

of *Thalassiosira* sp. is not as susceptible to suicide inhibition as some, but not all (Mars et al. 1997), catechol 2,3-dioxygenases. Determining the resistance of protocatechuate 4,5-dioxygenase to possible suicide inhibition will require purification of the enzyme, which was beyond the scope of the present study.

Due to the volatility, toxicity, and low solubility of these compounds, we did not attempt to grow *Thalassiosira* sp. on chlorophenols or to demonstrate complete oxidation of chlorophenols by *Thalassiosira* sp. cultures. However, our finding of both a potent reductive dechlorinase activity and aromatic ring cleavage enzymes in the same crude extracts certainly indicates that such a catabolic route is possible. If the dechlorinase and ring cleavage activities we have demonstrated for *Thalassiosira* sp. are broadly distributed among diatom and other microalgal species, they may contribute to the resistance of some natural benthic marine algal assemblages to halophenols (Steward et al. 1992).

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