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Evidence for the importance of catechol-type siderophores in the iron-limited growth of a cyanobacterium

Abstract—To compensate for low levels of available iron, cyanobacteria may produce siderophores to assist in the scavenging of iron from the environment. In this paper we examine the role of catechol-type siderophores produced by the halo-tolerant cyanobacterium *Synechococcus* sp. PCC 7002 in the acquisition of iron from a chelated source. To inhibit catechol-type siderophore mediated iron transport, bovine serum albumin (BSA) was added to iron-deficient and replete cultures. Batch culture growth rates and cellular photosynthetic pigments decreased markedly in iron-limited populations in the presence of BSA, with no apparent decreases in growth rate in the iron-replete cultures. These results are supported by experiments with continuous culture chemostats where the addition of BSA to steady-state cultures leads to the washout of cells from low-iron chemostats, indicating that the cellular growth rate was reduced. The addition of BSA to short-term iron assimilation experiments further demonstrates that the presence of BSA can induce uptake kinetics consistent with the activity of an “iron-shuttle,” while BSA itself has no affinity for iron. These results demonstrate that catechol-type siderophores associated with the surface of the cell play an important role as “iron custodians.” While the presence of these catechols introduces complexity in the iron-transport mechanism and decreases the maximum velocity of iron uptake during episodic pulses of iron, the presence of the catechol associated with the cell surface functions to increase the overall cellular affinity for iron in low-iron environments.

The availability of iron to aquatic primary producers has received considerable attention since the revelation that iron may regulate productivity in areas of the world's oceans where the availability of iron is low (Martin 1994; Landry et al. 1997). Primary producers in these regions often are forced to scavenge iron at concentrations as low as 10^{-11} – 10^{-10} M (Bruland et al. 1991). These regions are commonly dominated by chroococcoid cyanobacteria of the genus *Synechococcus*, indicating that these organisms must have evolved strategies for surviving at low ambient iron concentrations. Alterations in the physiology of marine cyanobacteria allow these organisms to subsist on lower levels of cellular iron (Brand 1991; Wilhelm et al. 1996), compared with eukaryotic phytoplankton. Cyanobacteria adapt to the low-iron environments through the induction of active iron-

transport systems that allow the cyanobacteria to increase their ability to assimilate extracellular iron from the low-iron medium (Wilhelm and Trick 1994, 1995).

Iron-assimilation systems in heterotrophic bacteria commonly involve the production of low-molecular-weight chelators (“siderophores”), which are regulated by and specific for Fe^{3+} in the system (Neilands 1995). These chelators function utilizing different chemical moieties to bind iron, most commonly catechol and hydroxamate groups (Matzanke 1991). Siderophores act as high-affinity iron transporters in concert with membrane-specific receptor proteins, which transport the ferrisiderophore complexes into the cell at the cost of cellular energy (Winkelmann 1991). These transport systems also appear to function in cyanobacteria but to date have been studied to a lesser extent (Trick and Wilhelm 1995; Wilhelm 1995).

Recently, several research groups have observed that dissolved oceanic iron exists primarily chelated to organic ligands (Gledhill and van den Berg 1994; Rue and Bruland 1995; van den Berg 1995). These ligands have stability constants consistent with microbially produced siderophores (Lewis et al. 1995; Rue and Bruland 1997). The existence of oceanic iron in the chelated state dramatically alters our understanding of the availability of iron for primary producers in specific oceanic waters (Rue and Bruland 1997) and opens questions as to the origin of these ligands.

In this paper we examine the role of the catechol siderophore in the acquisition of iron from a chelated iron source. The growth of *Synechococcus* sp. PCC 7002 under low-iron conditions has been well studied and has been demonstrated to include the production of four siderophores, two of which are catechol-type in nature (Armstrong and Van Baalen 1979; Wilhelm and Trick 1994). We have previously proposed that, in aquatic environments, catechol-type siderophores will remain associated with the surface of the cell, and that iron transport will be a two-step process; iron bound to extracellular hydroxamates and atypical-type siderophores will be scavenged by the catechols (owing to the higher affinity for iron in catechols), and the catechol-siderophore complex will then be transported into the cell (Wilhelm and Trick 1994).

To determine the importance of catechol-type sidero-

phores to cyanobacterial iron transport, we have utilized bovine serum albumin (BSA) to interrupt the association and dissociation of iron from the catechol-type siderophores. BSA has been demonstrated to effectively inhibit the catechol-type siderophore Enterobactin, while not affecting the activity of the hydroxamate-type siderophore Aerobactin (Konopka and Neilands 1984).

Strains and media—*Synechococcus* sp. PCC 7002 (*Agmenellum quadruplicatum* PR6) was grown and maintained axenically in 50-ml cultures of modified A⁺ medium (Wilhelm and Trick 1995) at 30°C in acid-washed, 250-ml Erlenmeyer flasks. Cultures were maintained on an orbital shaker and illuminated by cool-white fluorescent lights at 120 mmol photons m⁻² s⁻¹.

All water and nutrient stocks with the exception of iron, vitamin B₁₂, and the trace metals were first treated with Chelex-100 (Price et al. 1989) to remove residual iron. Modified A⁺ medium was prepared in stock components, sterilized, and combined as previously described (Wilhelm and Trick 1995). Iron was added from a stock solution of filter sterilized FeCl₃ (5.83 mM in 0.1 N HCl) to generate final concentrations of 4.2 × 10⁻⁵ M (iron-replete medium) and 5.1 × 10⁻⁹ M (iron-deficient medium) total iron (Wilhelm et al. 1996).

Analysis of growth rate—Cells were preconditioned for at least two periods of 72 h at the appropriate iron level prior to their subculture (5.0%) into fresh medium for growth studies. Bovine serum albumin (Sigma) was added to the medium at the appropriate concentration prior to inoculation with preconditioned cells. Cell densities were estimated spectrophotometrically every 6 h at 750 nm with an LKB Biospec II spectrophotometer and correlated with an established cell density-to-absorbance ratio to estimate cell number (Wilhelm et al. 1996). Cell growth was monitored for at least 120 h to ensure cells had reached stationary phase. Regression analysis of logarithmically transformed data was used to determine the maximum growth rate for each sample within each group of treatments.

Chemostat studies—The effect of BSA on iron-limited chemostats was determined using our previously established chemostat techniques (Wilhelm and Trick 1995). Thermostated chemostats were established at appropriate iron levels (4.2 × 10⁻⁵ or 5.1 × 10⁻⁹ M total iron) and filter-sterilized BSA added to the medium reservoir at 0.1 μg liter⁻¹ when the cultures had reached steady-state. Cells densities were strictly monitored to determine the effect of BSA on the cultures, and maximum growth rates for cell within the chemostat were calculated for chemostats where washout occurred. The reservoir was replaced with BSA-free medium when it appeared that the chemostat was almost completely void of cells, and the recovery of the culture was also monitored.

Pigment extraction—Exponentially growing cells (60 h after inoculation) were harvested by centrifugation and resuspended in 500 ml of 100% acetone. Cells were disrupted with 0.1 mm glass beads by three 30-s pulses in a Biospec

Mini-Beadbeater and stored overnight at -20°C. Prior to pigment analysis, cells were again subjected to a 30-s pulse in the Mini-Beadbeater, the glass beads and cellular debris were removed by a 5-min centrifugation, and samples were stored under nitrogen at -20°C until analysis. Pigments were separated by high-performance liquid chromatography (HPLC) with a Beckman System Gold Solvent Module 126 as previously described (Wilhelm et al. 1996). Chlorophyll *a* and β-carotene standards were obtained from Sigma. Zeaxanthin was prepared from barley and purified by thin-layer chromatography (Diaz et al. 1990).

Iron uptake rates—Exponentially growing cells were diluted 50% with fresh medium 72 h before iron uptake rates were to be determined. Filter-sterilized BSA was added at the appropriate concentration 24 h prior to uptake studies in order to ensure its complete dissolution throughout the medium. For high-iron-grown cells, cultures were harvested by centrifugation and resuspended in fresh or "spent" low-iron medium. Spent medium is defined as A⁺ medium that is rendered cell free (by centrifugation) after 72 h of cyanobacterial growth. This leaves ambient concentrations of extracellular products in the medium for the initiation of experiments. The use of spent medium thus allows for an analysis of high-iron-grown cells in the presence of extracellular siderophores.

Immediately prior to uptake, cell densities were assayed and 40 ml of culture was transferred to thermostated, water-jacketed reaction vessels (30°C). After a 1-h stabilization period in the reaction vessels, 3.47 μg of ⁵⁵FeCl₃ was added and iron uptake monitored over the next 45 min. Intracellular levels of iron were determined using the titanium(III)-citrate-EDTA method of Hudson and Morel (1989). The cells from 1 ml of culture were collected on polycarbonate filters and rinsed with 2 ml of saline solution, the titanium(III)-citrate-EDTA wash, and again with a saline wash (Wilhelm and Trick 1994). This technique removes all Fe superficially associated with the surface of the cell, and allows for a determination of iron incorporation rates. Once rinsed, filters were placed in scintillation vials and 10 ml of Ecoscint scintillation fluid (Diamed) was added. Incorporated radiolabel was monitored after 12 h with a Beckman IC 6000 LS scintillation counter, and results were converted to rates of iron incorporation.

Iron-binding capacity of BSA—To determine the iron-binding capacity of BSA, we used the chrome azural S assay (CAS) of Schwyn and Neilands (1987) and compared the results to compounds of various iron-binding capacities. Disodium EDTA, NTA, sodium citrate, and sodium acetate all have established conditional stability constants for Fe³⁺ (ranging from 10²³ for EDTA to 10⁴ for acetate) and were used as a series of comparative chelators in the CAS. Samples were prepared in concentrations of 1, 2, 5, 10, 25, and 50 mM in Chelex-100-treated water, and exposed to the CAS assay solution for 2 h. Results are reported as the ratio of the absorbances at 630 nm (A₆₃₀ control : A₆₃₀ sample). This results in a positive linear relationship between CAS activity and iron chelation, with a result of 1.0 equal to no detectable chelation.

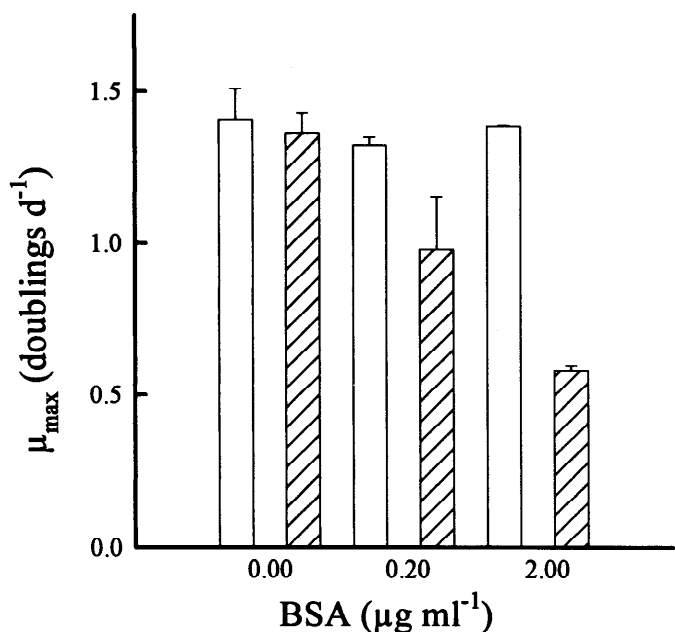


Fig. 1. Effect of BSA on the maximum growth rate of a cyanobacterium in high- (open bars) and low-iron (hatched bars) media. Growth rates (\pm SD) of *Synechococcus* sp. PCC 7002 were determined from the exponential phase of growth (over 72 h) from regression analysis of cell densities determined every 6 h.

Effect of BSA on growth rate and cell pigments—The introduction of BSA to low-iron cultures decreased maximum growth rate while having no effect on cultures under iron-replete conditions (Fig. 1). Under low-iron condition without BSA, the high-affinity iron acquisition system supplies the cyanobacteria with sufficient iron to maintain a growth rate comparable to the high-iron-grown cells. However, the addition of BSA at 2 mg ml⁻¹ decreased the growth rate of the low-iron cells by ~57% while having no effect on the iron-replete cultures. Increasing the concentration of BSA in the low-iron culture further decreased the maximal growth rate of the population.

Changes in cellular pigment levels were detected in both high- and low-iron-grown cultures when BSA was added (Fig. 2). Although greater changes were demonstrated in low-iron-grown *Synechococcus* in the presence of BSA, changes in Chl *a*, zeaxanthin, and β -carotene levels were seen at the lowest level of added BSA. Levels of pigment decreased by >50% with increased concentrations of BSA.

Effect of BSA on chemostat cultures—Continuous culture of *Synechococcus* sp. PCC 7002 under low-iron conditions demonstrated that BSA at concentrations of 0.1 mg ml⁻¹ are sufficient to reduce growth rate below 0.6 d⁻¹ (Fig. 3). At this level of BSA, growth rate can be calculated from cell loss processes to be 0.37 (\pm 0.008) d⁻¹, which ultimately results in the washout of cells from the chemostat as the cellular growth rate becomes less than the chemostat dilution rate. Chemostats with high levels of iron also showed initial decreases in cell density after the addition of BSA, but the

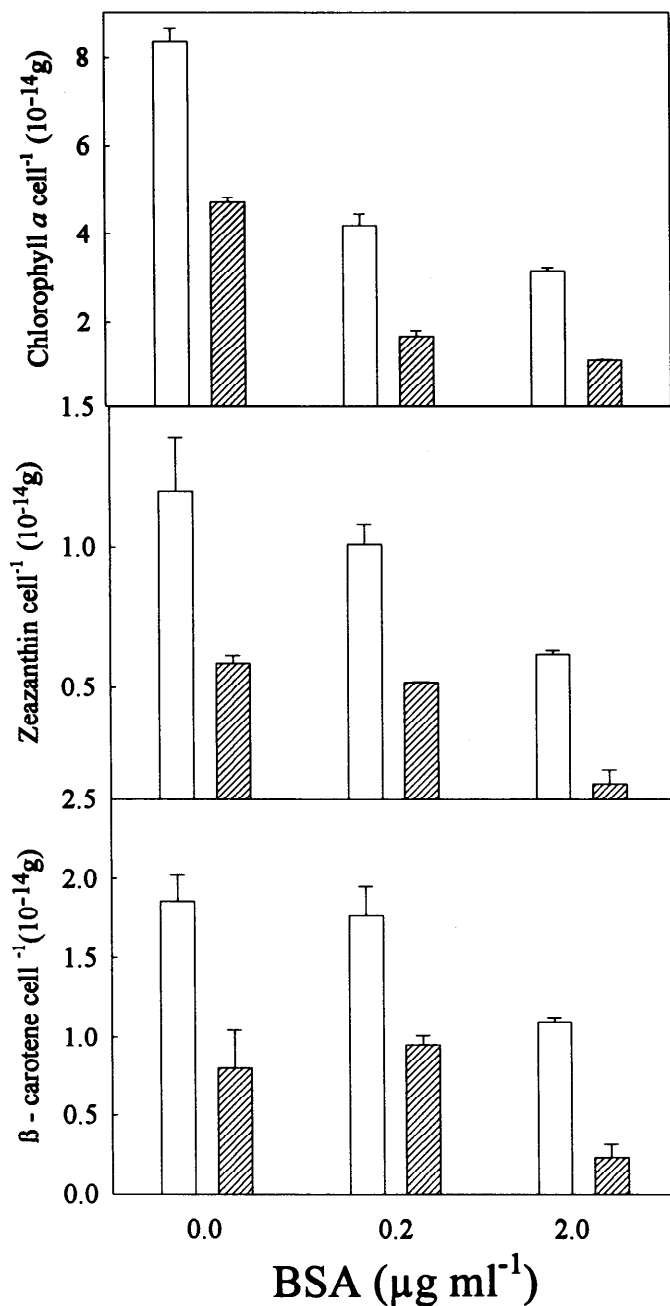


Fig. 2. Cellular pigment levels for Chl *a*, zeaxanthin, and β -carotene in *Synechococcus* sp. PCC 7002 cells grown over a range of BSA concentrations. Results are presented for iron-replete (open bars) and iron-deficient (hatched bars) cultures (\pm SD) for cellular pigment concentrations determined by HPLC analysis.

chemostats stabilized (at lower cell densities) after 2 d (data not shown).

Iron-binding capability of BSA—The CAS assay demonstrated that BSA does not bind iron substantially (Fig. 4). All other compounds demonstrated some binding of iron, and this appeared to be related to their specific binding affinity constant, with EDTA being the most effective at bind-

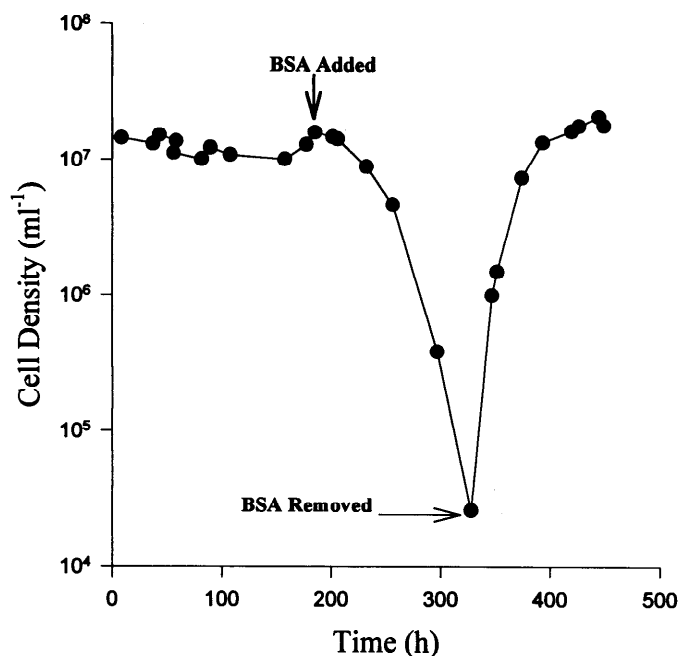


Fig. 3. Effect of BSA on *Synechococcus* sp. PCC 7002 cells in an iron-limited chemostat. BSA was added or removed from steady-state chemostats at the indicated point.

ing iron and acetate being the least effective. The results from BSA-treated samples, in fact, suggest that the BSA stabilizes the CAS iron-dye complex, as reflected in the negative relationship between BSA concentration and detected CAS activity (Fig. 4).

Effect of BSA on iron uptake rates—Iron uptake rates were monitored in low-iron-grown *Synechococcus* sp. PCC 7002 over a range of BSA concentrations (Fig. 5). Results demonstrate that as BSA concentrations were increased from 0.2 through 200 mg ml⁻¹ the rate of iron assimilation decreased. However, rates of iron uptake were significantly lower for control trials (where no BSA was added) in comparison to samples where low levels of BSA were added (3.87×10^{-19} g cell⁻¹ min⁻¹). In samples where 2.0 mg of denatured BSA ml⁻¹ was added, iron uptake rates were less than the control rates (2.5×10^{-19} g cell⁻¹ min⁻¹). The decrease of iron uptake rates for high-iron-grown cells in spent medium was less dramatic in the presence of BSA (1.9×10^{-19} g cell⁻¹ min⁻¹).

Uptake rates were also determined for high-iron-grown cells in fresh low-iron medium and spent low-iron medium. These iron-replete cells demonstrated ~5-fold higher rates of iron uptake in fresh medium (2.5×10^{-20} g cell⁻¹ min⁻¹) relative to spent medium where extracellular siderophores should be present (5.3×10^{-20} g cell⁻¹ min⁻¹).

Implications—Siderophores have been demonstrated to be crucial to the survival of many eubacteria during prolonged exposure to low levels of biologically available iron (Winkelmann 1991). The results from both batch cultures and chemostats suggest that this is also the case with *Synecho-*

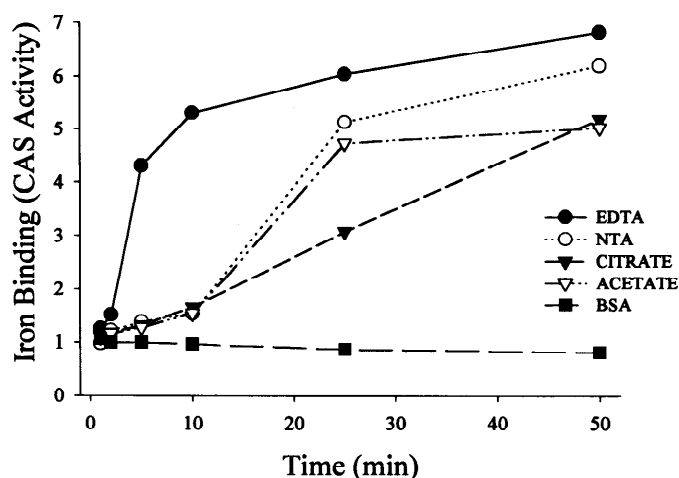


Fig. 4. Iron-binding capacity of BSA. The iron-binding capacity of BSA as determined with chrome azural S relative to chemicals of known iron-binding ability. Iron-binding capacity is demonstrated as the A_{630} ratio between the control and the sample. Na₂EDTA ($-\log K_A = 26$), NTA ($-\log K_A = 18$), sodium citrate ($-\log K_A = 13$), and sodium acetate ($-\log K_A = 4$) all demonstrate higher iron-binding capacities than did BSA based on their higher control: sample absorbance ratio.

coccus sp. PCC 7002—the inhibition of catechol-type siderophores leads to a marked decrease in growth rate. Inhibition of the system not only decreases growth, but also appears to have a significant effect on cellular pigment levels and thus by inference on photosynthesis.

How BSA inhibits siderophore activity is not yet understood, but the data from the CAS assay demonstrate that BSA is not acting by sequestering iron. The two catechol-type siderophores produced by *Synechococcus* sp. PCC 7002 have previously been demonstrated to have extremely high affinities for iron with respect to Fe³⁺ ($-\log K_A = 43$ and

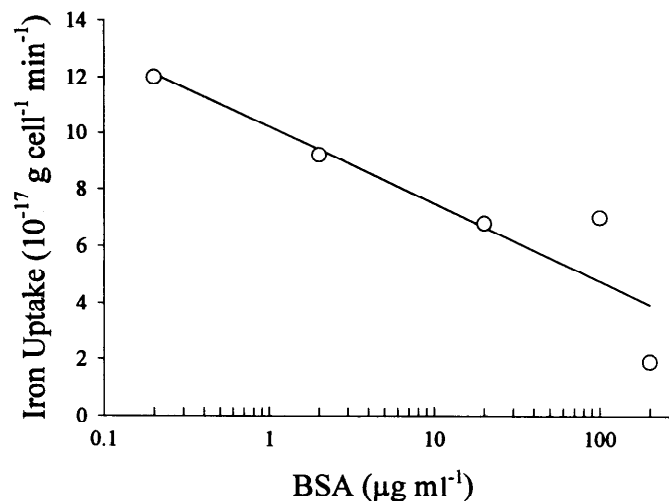


Fig. 5. Effect of BSA on iron assimilation of iron-limited *Synechococcus* sp. PCC 7002. Iron uptake rates were determined by regression analysis and are presented against BSA concentration.

38; Lewis et al. 1995). This suggests that the mechanism of BSA action is indeed inhibitory and not competitive.

The perplexing results here are found in an analysis of the iron uptake rates, where the initial addition of BSA leads to an increase in iron acquisition rates, but increases in the concentration of BSA decrease this effect. This suggests that the catechol-type siderophores may be playing a role as iron regulators or custodians during prolonged periods of low iron availability. The levels of iron added for these uptake experiments were ~2 orders of magnitude higher than those utilized to culture the cells under low-iron conditions. This "pulse" event may provide sufficient iron so that the system would be more effective in the absence of the catechols. The catechols appear to provide a greater avidity to the cell for iron, but at a cost of reduced iron-transport velocity. Thus, when BSA is present, the high-affinity, low-velocity catechol-mediated system is blocked, and the higher velocity, lower affinity hydroxamate-type siderophores regulate the influx of iron, leading to a short-term increase in iron uptake. The catechols, however, must still play a role owing to the fact that as the concentration of BSA increases, the rate of iron uptake decreases to a point below that of the control. These results also suggest that the hydroxamates in themselves can function independent of the catechol-type siderophores, an issue that has been questioned previously in examinations of the system (Wilhelm and Trick 1994).

This study also supports previous work demonstrating that the siderophores do increase the overall velocity of iron transport into the cell (Wilhelm and Trick 1994). Iron uptake by high-iron-grown cells placed in spent medium decreased by 21% due to the presence of the extracellular and cell-bound siderophores in the system. This decrease in uptake rate is substantially altered (to ~76% of the iron uptake rate of the control culture) by the addition of BSA to inhibit catechol-type siderophores. In other studies this reduction has been attributed to the absence of ferrisiderophore receptors on the surface of iron-replete cells. In the absence of these receptors, the siderophores chelate iron away from the cell and decrease iron-assimilation rates (Wilhelm and Trick 1994).

It remains to be determined if proteins other than BSA can interfere with catechol-mediated iron transport. Because much of the dissolved organic material (DOM) in aquatic systems may be proteinacious (Long and Azam 1996), albeit at concentrations in natural systems ~10–100-fold lower than those in this study, interference from other proteins may significantly reduce the efficiency of catechol-mediated iron transport. One may propose, however, that this may provide for some advantages to the cyanobacterium as increases in available iron will lead to increases in total community productivity, which will increase the amount of DOM (and thus potentially inhibitory protein) in the system. This would lead to an inhibition of the catechol-transport system when iron was the most available, and potentially lead to savings in cellular energy in the form of the ATP utilized for ferrisiderophore transport. In contrast, low levels of iron would lead to decreases in DOM and ultimately increased catechol-mediated iron transport. This mechanism is theoretical, however, and requires examination in natural systems.

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The influence of fish-exuded chemical signals on the carbon budget of *Daphnia*

Abstract—Respiration and carbon assimilation rates were measured in juvenile instars of *Daphnia magna* cultivated either in fish-treated or in control medium without fish exudates. Respiration and carbon assimilation rates increase proportionally to *Daphnia* body size. This relationship did not differ between fish-influenced and control daphnids throughout juvenile development. The results imply that observed life-history shifts in *Daphnia* exposed to fish exudates are facultative changes and not the result of changes in the carbon budget induced by other predator-induced defense mechanisms or experimental setups.

Predator-induced phenotypic plasticity is an effective way to increase fitness in a variable environment (Stearns 1989). In aquatic environments a variety of examples show that behavior, morphology, and life-history of prey organisms can be phenotypically altered in response to stimuli from predators (*see* recent reviews by Larsson and Dodson [1993] and Dodson et al. [1994]). These stimuli are often predator-borne chemicals released into the water.

Life-history changes may be facultative processes that prey organisms use to lower the risk of predation. A second possibility is that the costs of other predator-induced effects create differences in important life-history traits such as growth and fecundity (Ball and Baker 1996). Such factors can be differences in behavior, feeding rates, or stress effects due to exposure to predator stimuli in unnatural experimental environments.

A common problem appears in experiments using kairomones from aquatic predators; that is, the chemical structures of the substances are unknown and it is not possible to chemically detect, purify, or synthesize them. This problem restricts fieldwork, so most of the work is done in lab-

oratory experiments. Hence, a mixture of unknown substances is added to experimental treatments with chemical signals from predators. It is therefore not possible to predict any effect of these signals on the physiology of the prey organisms, whether those substances are toxic, or if the concentration at which the kairomones are used in experiments match those in the field.

A common question that arises when presenting results from such experiments is whether the unnatural laboratory environment, together with the addition of a mixture of unknown substances in an unknown dose produces reactions that are the product of these circumstances. One reaction of *Daphnia* to fish chemicals is smaller body size at first reproduction. This effect may represent more than a facultative life-history shift in response to the possibility of higher predation risk sensed by fish kairomones. Two other explanations are possible. First, animals exposed to fish substances have higher carbon losses through respiration than do control animals. Such losses might be influenced by the chemicals that are added with fish-inhabited water and(or) by the fact that other predator-induced reactions, such as behavioral defense mechanisms (diel vertical migration), cannot be expressed in the laboratory. Both factors can lead to stress with high respiration rates. Second, smaller body size at first reproduction may occur because the animals have lower carbon incorporation. This can be due to the same reasons mentioned above, which could also result in lower filtration rates in animals exposed to fish-treated water.

To determine whether life-history changes due to predator stimuli are not side-products of experimental setups, we need more data regarding the physiological conditions of prey organisms when these reactions occur.

Cladocerans of the genus *Daphnia* modify their life-his-