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ALGAE AND CYANOBACTERIA RELEASE ORGANIC CHELATORS IN THE PRESENCE OF INORGANIC Fe(III) THUS KEEPING IRON DISSOLVED

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Summary. The cells of the green unicellular alga *Scenedesmus incrassatulus* Böhl, strain R-83 released organic chelators for Fe(III) in inorganic nutrient medium both in iron-deficient and in iron-replete conditions. Iron-deficient cells released chelators capable to bind 11 nmol Fe. mg (cell DW)$^{-1}$.h^{-1}, while the chelators released from iron-sufficient cells after contact with Fe(III) were sufficient to bind 1 nmol Fe. (mg cell DW)$^{-1}$.s^{-1}. A perfect correlation existed between the capacity of humic complexes of Fe(III) to catalyze lipid peroxidation in lipid-containing extracts *in vitro*, and their capacity to trigger the release of organic chelators from cells *in vivo.*

Chelator release was stimulated by oxygen and was blocked by DCMU in the dark. Inorganic Fe(II) induced fivefold lower chelator release than inorganic Fe(III). Fe(III)-induced release of chelators was also registered in seven algal strains *(Chlorella, Scenedesmus, Porphyridium)* and in two cyanobacterial strains: *Arthronema (Plectonema)* and *Arthrospira (Spirulina).*

Key words: *Arthronema*, *Arthrospira*, *Chlorella*, Iron chelators, *Porphyridium*, *Scenedesmus*

Abbreviations: BHT – butylated hydroxytoluene, DCMU – 3-(3,4-dichlorophenyl)-1,1-dimethylurea, HA – hydroxylamine, TBARS – thiobarbituric acid reacting substances

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Introduction

Iron is an essential nutritious element for most living cells but it can be both noxious (because this metal can efficiently oxidate organic molecules) and not readily available (because in oxigenated environments it is practically insoluble at neutral and alkaline pH). The iron-limitation inducible suite of physiological and biochemical mechanisms for accessing extracellular iron by organisms have been classified into two distinct strategies. Dicots, non-graminaceous monocots and yeast, utilize a strategy that involves solubilization of Fe(III) by extracellular acidification, reduction of Fe(III) to Fe(II) by a plasma-membrane redox system, followed by uptake of Fe(II) by a specific transporter (Guierinot and Yi, 1994). This mechanism involves the obligatory reduction of extracellular ferric chelates leading to chelate splitting and the subsequent uptake of the released Fe(II) (Chaney et al., 1972; Römheld and Marschner, 1983). Graminaceous plants, bacteria, and some fungi, utilize a strategy that involves the release of iron-chelating organic compounds (siderophores or phytosiderophores), followed by uptake of Fe(III)-chelates across the plasma membrane. In higher plants these two strategies have been designated as Strategy I and Strategy II respectively. Neither of these two strategies involves a release of organic chelators in the presence of Fe(III). Fe-limited cyanobacteria utilize siderophores, in a manner similar to Strategy II (Wilhelm and Trick, 1994; Wilhelm, 1995; Trick and Wilhelm, 1995; Wilhelm et al., 1996). The mechanism(s) by which eukaryotic algae access extracellular iron is less clear (Hutchins, 1995). Iron-limited cells of the marine diatom *Thalassiosira weisflogii* reduce Fe(III)-EDTA to Fe(II) (Anderson and Morel, 1980), while the marine diatom *Phaeodactylum tricornutum* utilizes iron from ferrioxamine B by mechanism which resembles higher plant Strategy I, while uptake from ferrioxamine E resembles Strategy II (Soria-Dengg and Hortsmann, 1995). Among the green algae (Chlorophyta) *Scenedesmus incrassatulus* (Benderliev and Ivanova, 1994) utilizes iron-limitation-inducible Strategy II mechanism which involves a release of chelators and uptake of chelates with no reductive splitting of the latter in the medium. This alga releases low and high-MW organic chelators in the presence of Fe(III) (Benderliev and Ivanova, 1996) and preferably takes up Fe from hydroxylamine-stable organic complexes which are formed in the medium. Siderophores, bound to the plasma membrane are absent both in iron-replete and in iron-deficient cells of this alga (Benderliev and Ivanova, 1997).

Apart from the green alga *Scenedesmus incrassatulus* other algae and cyanobacteria have not been investigated for their capability to release organic Fe-binding chelators in the medium in the presence of Fe(III). The present report shows that this response is characteristic for other green algae, red algae and cyanobacteria.

One aspect of the metabolism of unicellular algae that attracted attention is their ability to liberate dissolved organic carbon in the environment. The release of dissolved organic carbon has been considered a process of major physiological and ecolog-

ical importance (Fogg, 1983, 1991). From 10 to 30% of the primary production in natural waters that appeared in the dissolved phase is due to release of organic molecules from phytoplankton (Sundh, 1992). In some cases up to 70% of the photosynthetically-fixed carbon is released as dissolved organic carbon (see the discussion in Hino, 1988). The release of dissolved organic carbon is not tightly linked to photosynthesis (Giordano et al., 1994), and the underlying mechanism for it is unknown. It was established (Benderliev and Ivanova, 1996) that the release of organic Fe chelators is higher at higher Fe/cell ratios. The present report shows that both inorganic Fe(III) in the presence of oxygen, and humic Fe(III) which stimulates lipid peroxydation, trigger or stimulate the release of chelators from green algae, red algae, and cyanobacteria.

Materials and Methods

Strains, nutrient media and cultivation

In all experiments *Scenedesmus incrassatulus* Böhl, R-83 was used, unless otherwise stated. The freshwater green algae *Scenedesmus incrassatulus* Böhl R-83, *Scenedesmus acutus* Mayen 10-2, *Chlorella regularis* S-50, *Chlorella* sp. H-23 and *Chlorella* sp. K-1 were received from the Algal Culture Collection of the Plovdiv University. The marine red algae *Porphyridium cruentum* Vischer 126 and *Porphyridium sordidum* Geitler/Ott 114.79 as well as the cyanobacteria *Arthrospira* sp. *("platensis")* Compere/86.79 and *Leptolyngbya boryana* (Gomont) Anagnastidis et Komarek 594 were granted by Dr. J. Lukavsky from the Culture Collection of Autotrophic Organisms, Trebon, CZ. The inorganic nutrient media for the cultivation of green algae, red algae, and cyanobacteria were prepared according Benderliev and Ivanova (1997, threefold diluted), Brody and Emerson (1959) and Allen (Vonshak, 1986) respectively. EDTA was not added to these media, and $FeCl_3$; was used as a single Fe source. In one experiment aqueous ferrous sulphate was used to register the effect of inorganic Fe(II) on the release of chelators from cells. In some experiments humic complexes of iron were used as a single Fe source. These complexes were prepared after Benderliev and Ivanova (1997), but were stored at 4°C in the dark for 0, 24, 48 and 72 h prior to use. The final concentration of humic iron in these media was 54 $nmol.ml^{-1}$, and of humic acid – 8 $mg.1^{-1}$.

Measurement of iron-binding chelators in the medium and intracellular iron

The concentration of the released chelators was assessed by their Fe-binding capacity. Chelator release in the presence of Fe(III) was measured as follows: cells were inoculated in inorganic medium in the dark at pH 6.9, then the suspension was gently mixed for 5 s and the cells were removed by centrifugation at 8000×g for 10 min. The supernatant was decanted in a glass vessel and aerated 80 min in darkness with 2% CO_2 in

the air to encourage the formation of stable chelates of iron. Then two equal samples were taken for determination of hydroxylamine-labile (HA-labile) and total Fe. The difference between total Fe and HA-labile Fe was reported as HA-stable Fe.

The release rate of chelators from cells immediately after contact with inorganic Fe(III) in the dark was measured as follows: Iron sufficient cells which contained 6 nmol Fe.mg (cell DW)$^{-1}$ were used as inoculum. At time intervals after cell inoculation, the samples were treated with 10^{-4} M DCMU and then the cells were removed. DCMU-free samples were also taken. This procedure was necessary because the time for the release of chelators is shorter than the time needed for removal of cells. Chelator release had to be stopped at time intervals with DCMU. DCMU completely inhibits the release of chelators from cells in the dark, thus inhibiting the uptake of non-chelated inorganic Fe(III), but it does not prevent the uptake of organic Fe complexes by cells of this alga (Benderliev and Ivanova, 1997). The total of released chelators was determined as HA-stable Fe in the DCMU-free suspension samples, while the rate of chelator release was determined by measurement of HA-stable Fe in supernatants of the DCMU-treated suspensions.

HA-labile Fe was determined after Anonymous (1975) as follows: to 5 ml cell-free supernatant were added to 0.2 ml solution of 5.9 M CH_3COONH_4 in 3 M CH_3COOH. The resulting pH was 4.5. Then 0.1 ml 2.87 M $NH_4OH.HCl$ and 0.2 ml 1,10-phenanthrolinum chloride solution (25.22 mM) were added and the absorbance of the Fe(II)-phenanthroline complex was red after 15 min at 492 nm.

Total Fe was determined by the same procedure, but instead of hydroxylamine to the 5 ml cell-free supernatant were added 0.1 ml of fresh daily-prepared 10% solution of ascorbic acid in order to reduce iron from its complexes. As known, some organic molecules bind iron in complexes, which cannot be reduced by ascorbic acid and hence prior to addition of ascorbic acid, ashing of samples is necessary (Anonymous, 1971). Ascorbic acid reduced all organic complexes of iron with chelators released by green algae, because the reduction of Fe in cell-free supernatants with ascorbic acid prior to, and after ashing of samples gave identical results. In contrast ashing of samples was necessary prior to addition of ascorbic acid in supernatants from red algae and cyanobacteria.

The concentration of chelators released by cells in iron-deficient medium (containing 6 nmol Fe.ml^{-1}) was measured in cell-free supernatants of samples taken at 24 h intervals upon cultivation in the light. To measure the Fe(III)-binding capacity of released Fe-free chelators excess (20 µM) $FeCl_3$ was added in the cell-free supernatants prior to HA-stable Fe determination.

Fe(II) in the $FeCl_3$-media and in the humic-Fe media was measured with the same method, but without addition of reducing agents.

The intracellular Fe was measured as total Fe after ashing of cells which had been previously washed with titanium reagent. 5 ml aliquots containing up to 1 mg cells.ml^{-1} were pelleted by centrifugation and resuspended in 5 ml titanium reagent (Hudson and Morel, 1989), as modified for use with freshwater algae (Benderliev and Ivanova,

1996). The samples were stored in the dark at room temperature for 20 min. Then the cells were washed twice with glass-distilled water and ashed. Water, ascorbic acid, and *o*-phenanthroline solution to a final volume of 5 ml were added and total Fe was determined. The result can be computed either per cell DW or per suspension volume. In all experiments the DW of cells was directly determined.

Preparation of lipid-containing extracts

Hot methanol (200 ml) was poured over 2 g washed algal cells. 10 ml of $CHCl_3$ and 2 ml of 0.2 N NaCl in water were added and the cells were removed by filtration. 3 ml of Na_2SO_4 were added, and the filtration was repeated. The solvent was evaporated at 40 °C in a rotary vacuum evaporator. The sample was tempered at room temperature, weighed and solubilized in methanol to achieve 2 mg extract per ml.

Determination of the catalytic activity of Fe

The Fe-catalyzed accumulation of lipid peroxidation products (TBARS) in lipid-containing extracts was used as an index for the catalytic activity of iron *in vitro.* Formation of TBARS in fresh lipid extracts was accomplished as follows: to 1ml extract 4 ml CH_3OH and 5 ml nutrient medium (which contains 0.246 µM humic Fe) were added. To 2 ml of this sample 2 ml TBA-reagent and 0.1 ml 2% (w/v) BHT in ethanol were added and the sample was boiled in a water bath for 10 min. The TBA-reagent contained 15% TCA, 0.375% TBA, and 0.254 N HCl. A standard curve was prepared using malonaldehyde bismethylacetal according Esterbauer and Cheeseman (1990). After cooling the absorbance was red at 532 nm in malonaldehyde equivalents and the result was reported as TBARS. Humic-Fe-catalyzed TBARS production was computed as the difference between TBARS content of the extract after mixing with humic-Fe medium and TBARS content of the extract after mixing with medium in which humic Fe had not been added.

Oxygen concentrations were measured with a Clark-type electrode DW1 (Hansatech, England) in 2 ml of reaction medium.

Results

Release of chelators in Fe-replete and in Fe-deficient conditions

The release of chelators after the contact of Fe-sufficient cells with non-chelated inorganic Fe(III) is shown at Fig. 1. The control (DCMU-free) samples, taken at time intervals contained equal HA-stable Fe concentrations, showing that the time for the release of chelators is shorter than the time needed for removal of cells by centrifugation. Analysis of the DCMU-treated samples showed that both chelator release and Fe uptake terminated within 120 s, but some Fe had been removed from the supernatant

during these 120 s. The computed values for the released chelators immediately after cell inoculation are plotted on Fig. 1.

The release of chelators in Fe-deficient conditions was measured after cultivation in light. The intracellular Fe (6 nmol.mg (cell DW)$^{-1}$) fell down to 2 nmol.mg (cell DW)$^{-1}$ after 120 h. Results concerning the released chelators are plotted in Fig. 2.

Results in Fig. 1 and 2 show that the chelator release rate in the presence of $FeCl_3$ is 350 times higher than the maximal rate for release of chelators in iron-deficient conditions. The chelators released from 1 mg DW of algal cells in the dark immediately after contact with Fe(III) are sufficient to bind 1 nmol Fe.s^{-1}. Within 2 min all iron in the nutrient medium was bound by algal extracellular chelators, as evidenced by the 100% solubility of Fe at pH 6.9 (after 15 min centrifugation at 8000 × g).

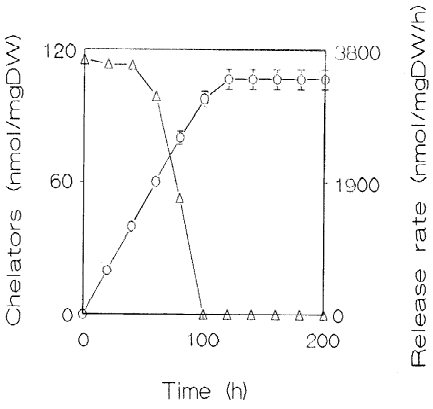

Fig. 1. Release of chelators from cells of *Sc. incrassatulus* in response to 60 µM $FeCl_3$ in the dark. Iron-sufficient cells containing 6 nmol Fe.mg (cell DW)$^{-1}$ were inoculated in inorganic medium. Chelator concentration (o) assessed by their capacity to bind Fe(III) in hydroxylamine-stable complexes; chelator release rate ( ). Values represent means ±SD (n=4).

Effect of oxygen concentration on the Fe-induced chelator release

Samples of inorganic medium were mixed with nitrogen for 0, 5, 10 and 15 min prior to cell inoculation. This procedure sharply diminished the content of oxygen, partially oxydized iron, and kept the concentration of total Fe constant. Inoculation of cells in media with lower oxygen content resulted in inhibited chelator release (Fig. 3). Since in this experiment the result cannot be attributed only to the effect of oxygen, we directly determined the effect of the oxidative state of iron on the release of chelators – the inoculation of cells in medium which contained non-chelated inorganic Fe(II) resulted in a fivefold lower chelator release than in medium with inorganic Fe(III) (data not shown). These results suggest that

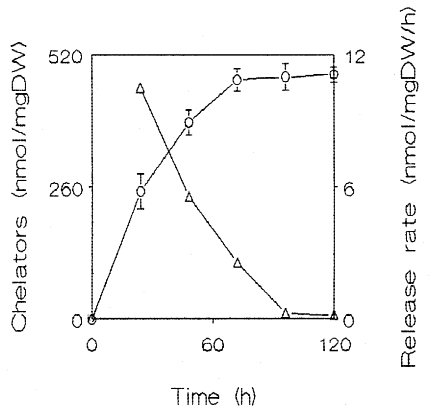

Fig. 2. Release of chelators from cells of *Sc. incrassatulus* in Fe-deficient conditions in light. Iron-sufficient cells containing 6 nmol Fe.mg (cell DW)$^{-1}$ were inoculated in inorganic medium containing 6 µM Fe. Concentration of chelators (o) assessed by capacity for binding of Fe(III) in hydroxylamine-stable complexes; chelator release rate ( ). Values are means ±SD (n=4).

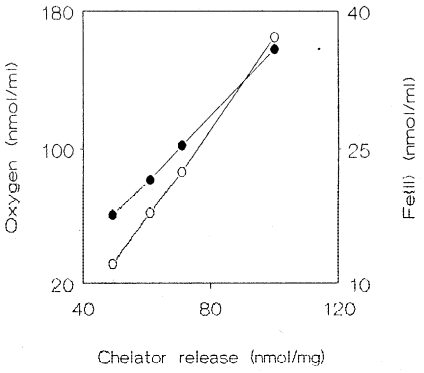

Fig. 3. Release of chelators from cells of *Scenedesmus incrassatulus* in dark as affected by oxygen. Inorganic nutrient media containing 54 μM $FeCl_3$ were mixed for 0, 5, 10, and 15 min with nitrogen prior to inoculation of cells. Oxygen (○) and Fe(II) (●) concenrtation in the medium prior to cell inoculation. The data are typical from three experiments.

Fe(III)-induced release of chelators from cells is oxygen-dependent. The effect might be attributed either to oxygen-dependent metabolic reactions in the cell, or to reactions of Fe(III) in the presence of oxygen.

Correlation between the capability of humic iron to catalyze lipid peroxidation *in vitro* and its capability to trigger a release of chelators from cells *in vivo.*

Our preliminary experiments showed that ageing of humic chelates of iron during storage at 4°C was accompanied by partial reduction of iron and a diminished capacity of humic iron to trigger a release of chelators from algal cells. So it was interesting to understand to which extent the capacity of humic Fe complexes to stimulate lipid peroxidation *in vitro* correlates with the capacity of these complexes to stimulate the release of chelators from cells. Fe-induced generation of TBARS *in vitro* was measured by addition of samples from humic-Fe media to lipid-containing extracts. These lipid-containing extracts from algal cells are convenient for measuring the catalytic activity of iron, because of the unsaturated fatty acids, which slowly form malonaldehyde (as part of TBARS) through autooxidation, and immediately – through iron-catalyzed peroxidation.

We compared the capacity of humic complexes of iron to catalyze lipid peroxidation *in vitro,* and the capability of the same complexes to trigger a release of chelators from cells *in vivo.* In the first set of experiments the extracts were mixed with nutrient media containing humic complexes of iron of different age and the Fe-catalyzed generation of TBARS was measured. In the second set of experiments algal cells were inoculated in the same humic-Fe media and the iron-induced release of chelators from cells was measured. The freshly prepared humic-Fe media contained equal oxygen concentrations (86 ± 4 nmol.ml^{-1}). Storage of humic complexes of iron in the dark was accompanied with partial reduction of the metal. Chelator release was higher at higher humic-Fe(III) concentrations (Fig. 4). Both the capacity of humic-Fe to stimulate lipid peroxidation *in vitro,* and its capacity to stimulate the release of chelators from cells *in vivo* diminished sharply upon storage. Iron which did not affect the autooxidation in extracts did not cause the release of chelators from cells *in vivo.* Since the initial oxygen content in all media was the same, the experiment demonstrates mainly the effect of humic catalytic Fe(III). Contrary to expectations humic Fe(III) caused generation of more TBARS than humic Fe(II).

Release of chelators from other algae and cyanobacteria in the presence of inorganic non-chelated Fe(III)

Both iron and oxygen are common in natural waters, and both algae (Fogg, 1983) and cyanobacteria (Hino, 1988) are capable to release high percentage of the photoassimilated carbon in the environment. So it is reasonable to assume that Fe(III)-induced release of chelators in the presence of oxygen might be characteristic for many planktonic species. Seven algal and two cyanobacterial strains were tested for their capability to release organic chelators in the medium in the presence of inorganic Fe in the dark (Table 1). All these strains released chelators capable to bind extracellular Fe in HA-stable complexes keeping the metal dissolved in the medium. Characteristically the chelators which were released from green algae formed HA-stable, ascorbate-labile complexes with iron, while cyanobacteria and red algae formed more stable complexes, which were resistant to the reducing action of ascorbic acid. The result suggests that the Fe(III)-induced release of organic chelators from cells in the dark in oxigenated environments might be characteristic for other algae and cyanobacteria as well.

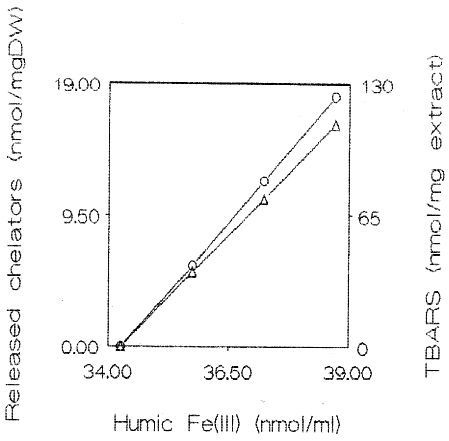

Fig 4. Effect of capacity of humic complexes of iron to catalyze lipid peroxidation in lipid-containing extracts on the capacity of the same complexes to induce a release of chelators from cells of *Sc. incrassatulus* in dark. Iron-sufficient cells were inoculated in nutrient media containing differently aged humic chelates of Fe (final concentration 54 µM Fe + 8 mg.l^{-1} humic acid) and equal oxygen content (86 ± nmol.ml^{-1}). The release of chelators was measured in cell-free supernatants. Samples from the same humic-Fe media were mixed with lipid-containing extracts from *Scenedesmus incrassatulus* at ratio of 0.123 µM Fe.mg extract^{-1} and the Fe-catalyzed accumulation of TBARS was measured. Released chelators by cells (o); TBARS catalytically generated by humic Fe in lipid-containing extracts ( ). The data are means of three replicates with single batches of humic Fe. Bars are smaller than symbols.

Discussion

In this paper we report that inorganic ($FeCl_3$) Fe(III) in the presence of oxygen, and humic Fe(III) (which is capable to stimulate lipid peroxidation *in vitro*) triggered or stimulated the release of organic chelators from cells of *Sc. incrassatulis.* Most probably the same factors caused a release of organic Fe chelators from other green algae (*Scenedesmus, Chlorella*), red algae (*Porphyridium*) and cyanobacteria (*Arthronema, Arthrospira*). All these chelators kept iron dissolved in the corresponding me-

Table 1. Fe(III)-induced release of organic chelators from green algae, red algae and cyanobacteria

Organism	Release response	Reducibility of organic Fe by:	
		Hydroxylamine	Vit. C
Green algae			
Scenedesmus incrassatulus Bohl, R-83	yes	no	yes
Scenedesmus acutus Mayen, 10-2	yes	no	yes
Chlorella regularis S-50	yes	no	yes
Chlorella sp. H-23	yes	no	yes
Chlorella sp. K-1	yes	no	yes
Red algae			
Porphyridium cruentum Visher 126	yes	no	no
Porphyridium sordidum Geitler/Ott 114.79	yes	no	no
Cyanobacteria			
Arthrospira sp. ("*platensis*") Compere 86.79	yes	no	no
Leptolyngbya boriana (Gomont) Anagnastidis et Komarek 594	yes	no	no

dia. The chelator release dependence on Fe(III) might explain why the dissolved organic carbon released from cells of *Dunaliella salina* (Giordano et al., 1994) was inversely proportional to cell density in the assay medium, while photosynthesis per cell basis was not.

The fact that some cyanobacteria and red algae release chelators which form ascorbate-stable chelates with Fe(III) (present results) while desferrioxamine B forms ascorbate-labile complexes with Fe(III) (Benderliev and Ivanova, 1996) deserves attention. Desferrioxamine B is a natural chelator used in medicine because Fe(III)-FOB does not catalytically generate the deleterious hydroxyl radical. (Marx and Asbeck, 1996). This property is attributed to the high stability constant of Fe(III)-FOB. Our results show that ferric chelates with higher stability constants than that of Fe(III)-FOB are produced by some red algae and cyanobacteria.

The rates of chelator release which we report here are much lower than these previously reported for release of proteins (0.87 mg.mg chl-a^{-1}.h^{-1}) and sugars (4.53 mg.mg chl-a^{-1}.h^{-1}) from supposedly dead or dying cyanobacterial cells (Hino, 1988). We report here release of organics from healthy cells, because at the same Fe(III)/cell ratios the growth lag phase was only 10–15 min (Benderliev and Ivanova, 1996). The release of chelators for Fe(III) seems to be an advantageous adaptation for organisms living in oxygenated environments at neutral or alkaline pH with sporadic and irregular Fe supply – the chelator release results in a quick enhancement of Fe solubility (present report) and Fe availability (Benderliev and Ivanova, 1997).

Diminishment of the ferric chelate reductase and ferricyanide reductase activities of *Chlamydomonas reinhardtii* upon severe Fe limitation (Lynnes et al., 1998),

and the fast drop of the reduction of non-chelated ($FeCl_3$) Fe(III) by Fe-limited cells of this alga (Weger, 1999) suggest that *C. reinhardtii* also might release organic Fe(III)-chelating molecules into the surroundings, similarly to Strategy II for Fe supply.

The nature and the sequence of the Fe-catalyzed reactions which stimulate the lipid peroxidation *in vitro* might be different from the reactions which trigger the release of chelators from cells *in vivo.* These results show that inorganic Fe(III) and humic Fe(III) rather than inorganic Fe(II) and humic Fe(II) stimulate chelator release from algal and probably from cyanobacterial cells. Fe(II) and its organic complexes usually catalyze more efficiently the oxidation of lipids than Fe(III) and its organic complexes (Halliwell and Gutteridge, 1984), probably in part because alkoxy radicals are more reactive than peroxy radicals in initiating peroxidation. A second reason is that reduced iron chelates in the presence of air generate oxygen radicals including superoxide and hydroxyl radical. Our results show that this does not apply for humic complexes of Fe. Our results suggest also that the contact of inorganic Fe(II) and humic Fe(II) to the cell surface might result in generation of strong oxidants such as superoxide radical and hydroxyl radical which tend to inhibit the release of chelators from cells. The contact of Fe(III) with the cell's surface triggers another set of reactions which stimulate the release of chelators from cells.

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