Flexible Selection of Iron Sources by Marine Microbial Consortia Related to Temperature

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Effects various soluble iron(III) complexes: iron (III)chloride and nitrate salts, iron(III) - ethylene diamine tetraacetate complex (EDTA; synthetic ligand), and succinate, citrate and malate complexes (naturally occurring low molecular weight ligands) and temperature, on the growth and cell volume of a diatom Skeletonema marinoi Sarno and Zingone in iron limited laboratory conditions, were studied. Temperatures were ranging from 18 to 25°C. Phytoplankton growth and cell volume responded differently to various iron(III) complexes. The growth of S. marinoi was found to be significantly influenced by temperature in treatments with iron(III)-malate and iron(III)nitrate. In treatments with iron(III)-EDTA, -succinate and -citrate significant correlation between growth and temperature was not found indicating that in these three cases the influence of iron(III) speciation was the predominant limiting factor, especially in treatments with iron(III)-succinate and -citrate. S. marinoi growth curves, cell volumes and log cell volumes were analyzed on temperature with a second order polynomial regression analysis. Results denote significant correlation of cell volume and temperature in treatments with iron(III)-EDTA, iron(III)-citrate and iron(III)-malate. Our results indicate that constant extracellular release of metabolites (i.e. malate) by eucariotes and tricarboxylic acid cycle (TCA cycle, the Krebs cycle) metabolites excreted by prokaryotes can play a significant role in the pathways of iron in the ocean as an important iron solubilization mechanism.

Introduction

Chemical (nutrients, trace metals) and physical (temperature, light) factors limit phytoplankton growth. In some cases limitation is induced by one factor at a time. In natural conditions it is mostly the case of synergetic effects of various limiting factors. Numerous laboratory experiments have been conducted evaluating responses of marine phytoplankton to the variation of these factors.

Bioavailable iron as a soluble organic complex appears in very low concentrations in natural aquatic systems (Martin and Gordon, 1988) although iron is one of the most abundant metals on the Earth's crust (Crumbliss and Garrison, 1988). Eastern Mediterranean oligotrophic seawater is characterized with total dissolved iron in concentration of 3.7 nmol L^{-1} (Öztürk et al. 2003). Due to low solubility of its thermodynamically stable 3+ ionic form under oxidizing conditions, most of the dissolved iron in natural waters appears in the form of iron(III) organic complexes (Millero, 2001). However, it is low iron concentrations combined with strongly-bound Fe that limits phytoplankton growth (Coale et al., 2004). The origin, chemical identity and availability of iron complexes are largely unknown due to extremely complex chemical behaviour in seawater (Byrne, Luo and Young, 2000). Recent voltammetric and spectrophotometric studies on model aqueous solutions of iron(III) complexes with succinic, malic and citric acid (Cmuk, Piantanida and Mlakar, 2009; Vukosav, Tomšić and Mlakar, 2010; Vukosav, Mlakar and Tomišić, 2012) have shown high solubility potentials of these complexes in seawater. These results indicate that retention time of the investigated complexes under seawater conditions is long enough to be potentially utilized by phytoplankton to initiate biomass growth.

Constant excretion of malate by marine phytoplankton (Puskaric and Mortain-Bertrand, 2003) may be used by bacteria in a reverse TCA cycle allowing incorporation of CO_2 and synthesis of oxaloacetate, citrate, isocitrate, fumarate and succinate (Evans and Buchanan, 1965), which could themselves be excreted by heterotrophic bacteria and then used by phytoplankton. Hoare and Moore (1965) have shown that algae are capable of assimilating organic acids. Donderski, Mudryk and Walczak (1998) have reported that bacteria are also capable of assimilating low molecular weight organic compounds. Furthermore, Peters, Liu and Aleem (1970) showed an absence of a complete TCA cycle in most marine autotrophic organisms. The lack of NADH oxidase shows that the cells are incapable of coupling ATP synthesis with the respiration of organic acids. This is a basic obstacle to heterotrophic growth. In algae the TCA cycle is only necessary for the biosynthesis of essential carbon compounds. Since they lack major oxidative enzymes, phytoplankton can obtain TCA intermediate products for biosynthesis directly from bacteria, giving them something else in return: malate (Puskaric and Mortain-Bertrand, 2003). Given such partnership scenario and the ability of these compounds to strongly solubilise iron (Cmuk, Piantanida and Mlakar, 2009; Vukosav, Tomšić and Mlakar, 2010), it could be assumed that this mechanism could have a certain role in iron transport in marine microbial food webs. Previous work has shown that the strategy of iron uptake differs between eukaryotic and prokaryotic plankton and that prokaryotic algae have an advantage in obtaining iron at limiting conditions (Hutchins et al., 1999). Nevertheless, diatoms appear in significant fraction of the phytoplankton community in iron-limited oceans (de Baar and Boyd, 1999). We hypothesized that the disadvantage of diatoms might be balanced with their extracellular release of photosynthetic metabolites which in close partnership with heterotrophic bacteria increase bioavailability of dissolved iron. High solubility potentials of malic, succinic and citric acids and their constant release by algae and bacteria argue that these compounds can compete with the strong iron binding ligands found in seawater whose concentrations are low (1-3 nM) (Rue and Bruland, 1995).

Even though temperature has strong influence on algal growth and photosynthesis (e.g. Raven and Geider, 1988; Davison 1991), it does not appear as a variable in phytoplankton primary productivity models (Behrenfeld and Falkowski, 1997). It is generally accepted that the effects of temperature are overshadowed by other processes i.e. irradiance (Gibson and Foy, 1989) and the fact that phytoplankton possess strong ability to adapt to growth at different temperatures (Li, 1980; Smith et al., 1994; Suzuki and Takahashi, 1995).

The primary objective of the experiments was to assess the influence of organically bound Fe to the microbial community including phytoplankton and heterotrophic bacteria and understand their response to Fe amendment at different temperatures. We measured the changes in algal growth rates and autotrophic biomass in response to amendments of dissolved free Fe or Fe bound to different model low molecular weight ligands.

Method

Standard f/2 culture medium was prepared (Guillard and Ryther, 1962) absent trace metals, iron and EDTA. All standard solutions of iron(III) complexes were prepared using Milli-Q water. A separate solution of iron(III)-EDTA was prepared with $FeCl_3 \cdot 6H_2O$ and $Na_2EDTA \cdot 2H_2O$ according to Guillard and Ryther (1962). Standard solution of iron(III) nitrate (1.79 x 10^{-2} mol L^{-1}) (Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland) and stock solutions of malic acid (Sigma-Aldrich Chemie Gmbh, Steinheim, Germany), succinic acid (Fluka Chemie GmbH, Sigma-Aldrich, Buchs, Switzerland) and citric acid (Merck, Darmstadt, Germany) (2 mol L^{-1}), were prepared by adjusting pH at about 7 by adding diluted s.p. HCl or s.p. NaOH (Merck, Darmstadt, Germany). Iron(III) nitrate was added to the acid solution 24 hours before addition to cultures in purpose to avoid formation of highly insoluble $Fe(OH)_3$. Final concentration of iron(III) in all treatment tests was 500 pmol L^{-1}. Concentration ratio between iron(III) and ligands was $1:10^4$.

Tests on naturally occurring phytoplankton assemblages were conducted in spring 2012. Seawater was sampled from a natural body of water in Dubrovnik (Croatia) aquatorium from 7 m depth. Sampled seawater was screened through the plankton net (mesh diameter 20-µm) to remove larger grazers. 500 mL subsamples were placed in five acid washed and sterilized glass Erlenmeyer flasks. Culture medium as described above was added to each flask and samples were maintained in continuous light cycle (cool white fluorescent) at 18°C. The rest of the seawater sample was oxidized by ultraviolet light and filtered through Whatman GF/F glass microfibre filter and left in the dark (UVFSW). Major problem in studying phytoplankton growth in iron limiting conditions is the presence of iron other than added iron. Preparing artificial seawater leads to micromolar concentrations of iron from added chemicals even though they have the attribute of suprapur and if no other forms of iron are added. Chelating iron from seawater could disturb the balance of other important nutrient constituents.

Skeletonema marinoi Sarno and Zingone was isolated from the cultures after the tests on natural phytoplankton communities during April 2012 at 20°C as this temperature was previously described as optimal for the growth of this species (Davis, Harrison and Dugdale, 1973). This species was selected since it is common within phytoplankton community in the southern Adriatic (Bosak et al., 2012). Cells were grown in non-axenic 0.5 L batch cultures at temperatures ranging from 18 to 25°C under continuous mixing (air bubbling) and illumination (cool white fluorescent) with a photosynthetically active radiance (PAR) of 180 µmol m^{-2} s^{-1}. Five 0.5 L flasks were filled with 0.5 L UVFSW. Culture medium (as described above) was added to each flask and vigorously mixed. Each flask was inoculated with a different iron(III) complex: Fe(III)-EDTA, Fe(III)-succinate , Fe(III)-citrate, Fe(III)-malate and iron(III)nitrate (regardless the iron(III) specie, the final concentration of Fe(III) in culture was 500 pmol L^{-1}), respectively.

At the end of each cycle (as soon as the cells entered the "death phase"), 12000 cells (cell density counting method described below) from each flask were transferred to fresh medium as described above to start a new cycle. The cultures were sub-sampled for cell density counts and cell volume measurements were performed every twenty-four hours.

To investigate the influence (contribution) of iron existing in filtered seawater used in experiments to the growth of the investigated microbial community, 100 µL subsamples from natural phytoplankton cultures (diatoms *Nitzschia* sp., *Chaetoceros* sp., and *Skeletonema marinoi,* nonloricate protozoan ciliates, heterotrophic bacteria and cyanobacteria) with iron(III)-EDTA, -chloride, -succinate, -citrate, -malate, and –nitrate in exponential phase of growth were inoculated into separate flasks with fresh medium and different complexes of iron as denoted above, and into separate flasks with the fresh medium but without the addition of iron.

Subsamples taken for cell counts and volume measurements were first stained with proflavine (Haas, 1982) to have a better insight in the entire community structure within the culture. Cells in the cultures were counted by filtering 2 – 5 mL of sample onto Millipore 0.2 µm pore-size black polycarbonate filters (25 mm diameter); only the cells exhibiting autofluorescence of chlorophyll a (Chl a) were counted. At least one hundred individual cells were counted per sample. A Jenalumar Zeiss microscope with an epifluorescence attachment was used at 375 x and 1500 x magnifications. Immersion oil Cargille Type A was used. A standard blue excitation method was used (main wavelength 495 nm), consisting of Zeiss filter set 15 and Mercury lamp type HBO 200. To evaluate the effect of temperature and iron species on cell-volumes, at least 100 cells per sample were measured for cell length (l) and width (w). Linear cell dimensions were measured from digital micrographs photographed at 1500x magnification. Cell volume was calculated taking into account the capsular shape of *S. marinoi* cells: Volume = $\pi r^2((4/3)r + a)$ where radius (r) = w/2 and cylinder length (a) = l – w. Total biomass (B) was calculated from cell density and cell volume.

Calculated cell volumes were log-transformed to evaluate linear relationship between log volume and temperature. A second order polynomial regression analysis was performed on non-transformed values as data indicated curvilinear responses. Minitab 16 and Grapher 1.23 (Golden Software) software programs were used for statistical analyses and calculations.

Omega microprocessor thermometer model HH21 was used to monitor temperature in the flasks, salinity of the cultures was monitored with WTW Conductivity 3110 Set-2 incl. TetraCon® 325-3 and pH by Metrohm 826 pH-meter Mobile.

Results

Approximately 150 L seawater was sampled in March 2012 outside Dubrovnik city walls from 7 m depth after an early spring bloom. Temperature of the sample was 15.25°C, salinity 38.8, oxygen 5.43 mL L^{-1}, total chlorophyll 0.67 mg L^{-1}, NO_3 0.17 µmol L^{-1}, NO_2 0.03 µmol L^{-1}, NH_4 0.32 µmol L^{-1}, PO_4 0.04 µmol L^{-1}, total N 5.39 µmol L^{-1}, inorganic N 0.52 µmol L^{-1}, organic N 4.87 µmol L^{-1}, Si 0.2 µmol L^{-1}.

In all tests pH ranged from 7.94 ±0.01 at the beginning of the test (Time = 0 hours) to 8.39±0.03 at the end of each growth cycle (Time = 98, 121, 134 and 194 hours). According to the recent findings regarding iron(III) speciation in solutions with succinic, malic and citric acid (Cmuk et al., 2009; Vukosav et al. 2010 and Vukosav et al. 2012), in mentioned pH range iron(III) form complexes with two molecules of each acid attached to the Fe^{3+} ion. In case of EDTA as a ligand, in this pH range predominate FeEDTA and FeEDTAOH species (Smith and Martell, 1976). Described speciations were calculated for *c*(Fe) = 500 pmol L^{-1} and *c*(ligand) = 5 µmol L^{-1}.

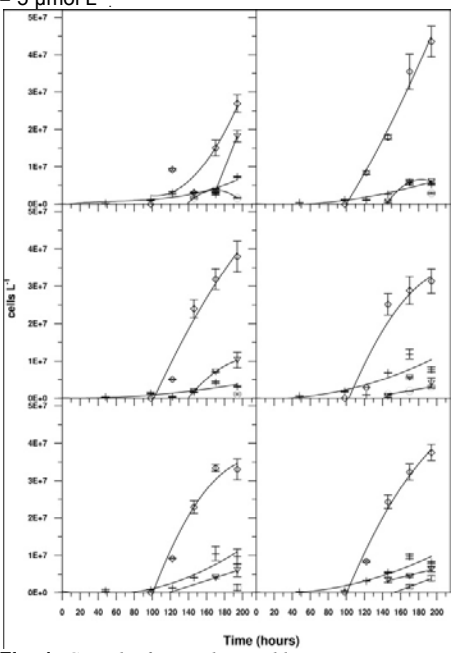

Fig. 1. *Growth of natural assemblages in preliminary experiments after nutrient treatments A) iron(III)-EDTA, B) iron(III)-succinate, C) iron(III)-citrate, D) iron(III)-malate, E) iron(III)nitrate, and F) iron(III)chloride. Means ± SD (n=3) are shown. Crosses represent Nitzschia sp., diamonds Chaetoceros sp., circles Skeletonema marinoi and triangles nonloricate protozoan ciliates. Means ± SD (n=3) are shown.*

The development and growth of natural plankton assemblage (preliminary tests, Methods) from the southern Adriatic at 18°C is shown in Fig. 1. The community was dominated by diatoms *Nitzschia* sp., *Chaetoceros* sp., and *Skeletonema marinoi* (Fig. 2). *Nitzschia* sp. was the first species that exhibited positive growth 48 hours after nutrient treatment, followed by *Chaetoceros* sp. 98 hours after nutrient treatment, then *Skeletonema marinoi* and a nonloricate protozoan ciliate after 146 hours and cyanobacteria after 194 hours (iron(III)-EDTA 2.1 x 10^8 cells L^{-1}; iron(III)-succinate 7.4 x 10^8 cells L^{-1}; iron(III)-citrate 8.81 x 10^8 cells L^{-1}; iron(III)-malate 8.6 x 10^8 cells L^{-1}; iron(III)nitrate 6.4 x 10^8 cells L^{-1}). Given conditions were the most favourable for *Chaetoceros* sp. Growth curves of *Chaetoceros* sp. were different at each different iron treatment (Fig. 1). *Chaetoceros* sp. reached the maximum cell density of 4.6 x 10^7 cells L^{-1} during iron(III)-succinate treatments; with iron(III)-citrate the maximum cell density was 4.11 x 10^7 cells L^{-1}; with iron(III)chloride 4.0 x 10^7 cells L^{-1}; in the casee of iron(III)nitrate 3.59 x 10^7 cells L^{-1}; with iron(III)-malate 3.45 x 10^7 cells L^{-1}; and with iron(III)-EDTA 2.9 x 10^7 cells L^{-1}. It out-competed all other species in all nutrient treatments.

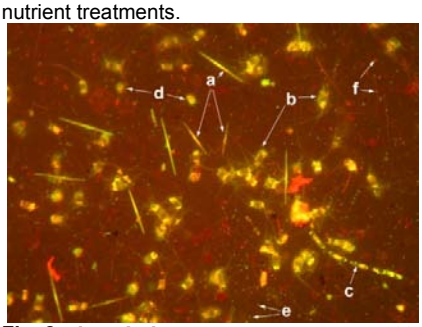

Fig. 2. Phytoplankton community comprising (a) *Nitzschia* sp., (b) *Chaetoceros* sp., (c) *Skeletonema marinoi,* (d) nonloricate protozoan ciliate, (e) heterotrophic bacteria (green) and (f) cyanobacteria (red).

Nitzschia sp. had similar growth curves in all nutrient treatments (Fig. 1) with maximum cell density of 1.33 x 10^7 cells L^{-1} observed in iron(III)-malate treatment. Iron(III)nitrate treatments peaked at 1.20 x 10^7 cells L^{-1}, iron(III)chloride at 1.03 x 10^7 cells L^{-1}, iron(III)-EDTA at 7.4 x 10^6 cells L^{-1}, iron(III)-succinate at 6.42 x 10^6 cells L^{-1}, and iron(III)-citrate at 4.66x 10^6 cells L^{-1}.

Skeletonema marinoi exhibited short growth patterns with maximum measured cell density 4.33 x 10^6 cells L^{-1} in iron(III)chloride treatments (Fig. 1). Iron(III)-EDTA treatments peaked at 4.0 x 10^6 cells L^{-1}, iron(III)-malate at 3.38x 10^6 cells L^{-1}, iron(III)-succinate at 3.0 x 10^6 cells L^{-1}, iron(III)-citrate at 1.3 x 10^6 cells L^{-1}.

Nonloricate protozoan ciliate showed maximum occurrences in iron(III)-EDTA (1.93 x 10^7 cells L^{-1}) and iron(III)-citrate (1.2 x 10^7 cells L^{-1}) treatments (Fig. 1). In iron(III)nitrate treatments the ciliate peaked at 7.03 x 10^6 cells L^{-1}; in iron(III)chloride treatments the maximum density was 6.86 x 10^6 cells L^{-1}, in iron(III)-succinate treatments the ciliate peaked at 6.2 x 10^6 cells L^{-1}, and in iron(III)-malate treatments the maximum density was 5.7 x 10^6 cells L^{-1}. It appears that the protozoan ciliate controlled the population size of heterotrophic bacteria throughout the test period. In the samples after 194 hours microscopic examination indicated that it also fed on cyanobacteria; up to seven cyanobacterial cells were observed within the ciliate cells.

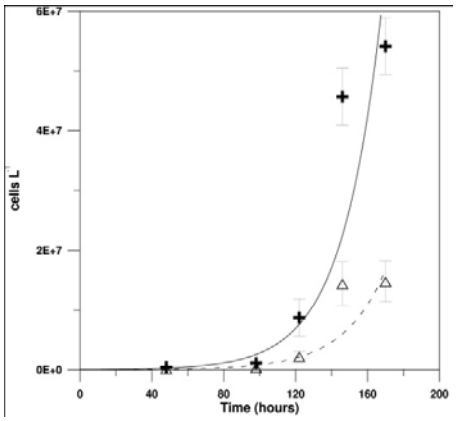

Fig. 3. *Growth curves of phytoplankton in treatments with iron (crosses) and in non treated tests (triangles).*

The results from the experiments designed to compare growth of the natural microbial communities (diatoms *Nitzschia* sp., *Chaetoceros* sp., and *Skeletonema marinoi,* nonloricate protozoan ciliates, heterotrophic bacteria and cyanobacteria)(Fig. 2) in medium enriched with Fe to conditions without iron enrichment (as described in Method) are shown in Fig. 3. In cultures with added iron the microbial community remained the same in all six flasks during the course of the experiment (diatoms *Nitzschia* sp., *Chaetoceros* sp., and *Skeletonema marinoi,* nonloricate protozoan ciliates, heterotrophic bacteria and cyanobacteria)(Fig. 2), while in the flasks without iron treatments the only phytoplankton species that remained alive was *Chaetoceros* sp. (Fig. 2b). Growth of phytoplankton in the flasks treated with iron was significantly different from the populations in the flasks without iron treatment (iron treatment growth curve, crosses, Fig. 3, log(y) = 0.045 · x + 10.3, R-squared = 0.92, n=6; non treated growth curve, triangles, Fig.3, log(y) = 0.043 · x + 9.32, R-squared = 0.88, n=6).

Observed behavioural characteristics of the investigated natural microbial community indicated different reaction to iron species variation. To thoroughly investigate these effects a detailed experimental protocol was designed in a simpler model. For such purposes we decided to focus on non-axenic batch culture of *Skeletonema marinoi*.

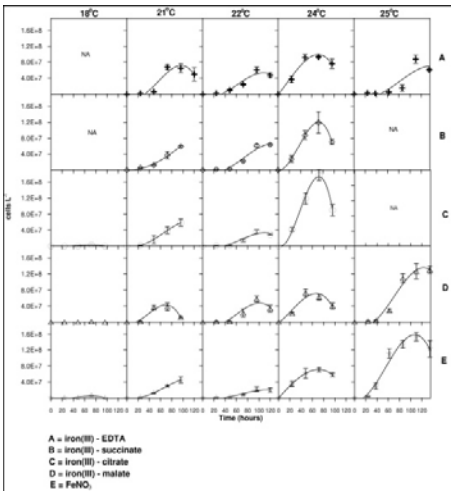

Fig. 4. *Growth patterns of Skeletonema marinoi at 18, 21, 22, 24 and 25°C treated with 0.5 nmol of:A) iron(III)-EDTA, B) iron(III)-succinate, C) iron(III)-citrate, D) iron(III)-malate, and E) iron(III)nitrate. Means ± SD (n=3) are shown.*

Growth of *Skeletonema marinoi* Sarno and Zingone batch culture on different iron(III) species was monitored at 18°C, 21°C, 22°C, 24°C and 25°C (each triplicated, Fig. 4). The culture did not show any signs of growth in treatments with iron(III)-EDTA and -succinate at 18°C as well with iron(III)-succinate and -citrate at 25°C. The flasks remained transparent throughout the study period. In other treatments *S. marinoi* growth cycles reached death phases within 98 hours at 18, 21 and 24°C, within 122 hours at 22°C while at 25°C growth cycles lasted 134 hours in treatments with iron(III)-EDTA, iron(III)-malate and iron(III)nitrate. The culture achieved highest densities at 24°C in treatments with iron(III)-succinate and iron (III)-citrate: 1.47 x 10^8 cells L^{-1} and 1.92 x 10^8 cells L^{-1}, respectively, and at 25°C in treatments with iron(III)-malate and iron(III)nitrate; 1.49 x 10^8 cells L^{-1} and 1.65 x 10^8 cells L^{-1}, respectively.

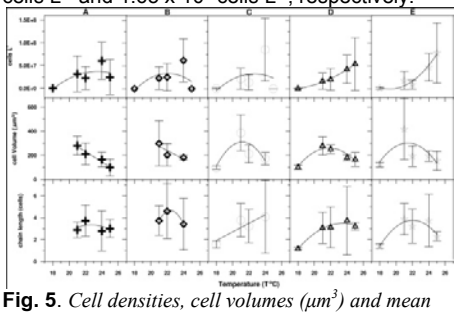

Fig. 5. *Cell densities, cell volumes (μm³) and mean chain lengths of Skeletonema marinoi treated with* ***(A) iron(III)-EDTA*** *(density polynomial regression, means ± SD (n=28), cell volume polynomial regression, means ± SD (n=18), mean chain lengths linear regression, means ± SD (n=18) are shown),* ***(B) iron(III)-succinate*** *(density polynomial regression, means ± SD (n=28), cell volume polynomial regression, means ± SD (n=12), mean chain lengths linear regression, means ± SD (n=12) are shown),* ***(C) iron(III)-citrate*** *(density polynomial regression, means ± SD (n=28), cell volume polynomial regression, means ± SD (n=15), mean chain lengths linear regression, means ± SD (n=15) are shown),* ***(D) iron(III)-malate*** *(density polynomial regression, means ± SD (n=28), cell volume polynomial regression, means ± SD (n=21), mean chain lengths linear regression, means ± SD (n=21) are shown), and* ***(E) iron(III)nitrate*** *(density polynomial regression, means ± SD (n=28), cell volume polynomial regression, means ± SD (n=21), mean chain lengths linear regression, means ± SD (n=21) are shown).*

S. marinoi growth patterns in treatments with a) iron(III)-EDTA (Fig. 5, A), b) iron(III)-succinate (Fig. 5, B) and c) iron(III)-citrate (Fig. 5, C) did not show significant responses to temperature:
a) density (cells L^{-1}) = 4.802 x 10^6·T – 7.93 x 10^7, R^2 = 0.11, p = 0.082
b) density (cells L^{-1}) = 2.284 x 10^6·T – 3.008 x 10^7, R^2 = 0.029, p = 0.384
c) density (cells L^{-1}) = 3.62 x 10^6·T – 5.81 x 10^7, R^2 = 0.045, p = 0.280

Positive relationship between growth patterns and temperature can be observed in treatments with a) iron(III)-malate (Fig. 5, D) and b) iron(III)nitrate (Fig. 5, E):
a) density (cells L^{-1}) = 7.82 x 10^6·T – 1.45 x 10^8, R^2 = 0.263, p = 0.005
b) density (cells L^{-1}) = 1.085 x 10^7·T – 2.08 x 10^8, R^2 = 0.34, p = 0.001
where T is the temperature (°C), analyzed by linear regression.

The only significant response of cell volume to temperature was observed in treatments with iron(III)-EDTA (Fig. 5, A):
V (μm³) = 1150 – 48.8·T, R^2 = 0.508, p = 0.001, decreasing trend
$\log^{10}$V (μm³) = 11.27 – 0.2692·T, R^2 = 0.501, p = 0.001, decreasing trend
where V is the cell volume (μm³) and T is temperature (°C), analyzed by linear regression. In all other treatments (Fig. 5, B-E), we could not find a significant response of size to temperature.

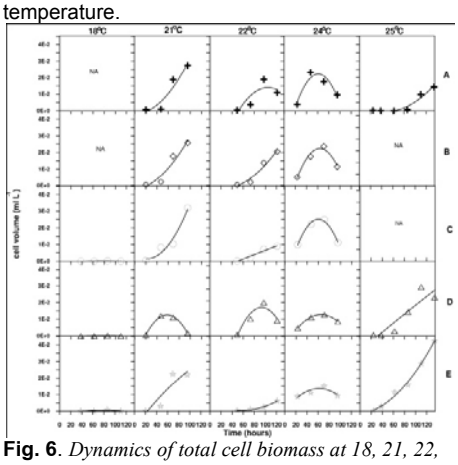

Fig. 6. *Dynamics of total cell biomass at 18, 21, 22, 24 and 25°C treated with A) iron(III)-EDTA, B)*

iron(III)-succinate, C) iron(III)-citrate, D) iron (III)-malate, and E) iron(III)nitrate.

We also looked for a relationship between the total biomass (mL L^{-1}) and temperature (Fig. 6). Visible tendency between total biomass and temperature in treatments with a) iron(III)-malate (Fig. 6, D) and b) iron(III)nitrate (Fig. 6, E) can be described by following equation analyzed by linear regression:
a) total biomass (mL L^{-1}) = -0.027 + 0.0016·T, R^2 = 0.22, p = 0.032
b) total biomass (mL L^{-1}) = -0.037 + 0.0021·T, R^2 = 0.196, p = 0.044
where T is the temperature (°C), analyzed by linear regression. In other treatments the relationship between biomass and temperature was not significant.

S. marinoi growth curves, cell volumes and log cell volumes were analyzed on temperature with a second order polynomial regression analysis. Results denote significant correlation of cell volume and temperature in treatments with iron(III)-EDTA, iron(III)-citrate and iron(III)-malate (Tab. 1).

Tab. 1 *Response of cell density and cell volume to temperature analyzed by sedond degree polynomial regression analysis.*

Treatment	Coefficient of determination (R^2)		
	Cells L^{-1}	Volume (μm^3)	Log^{10} Volume (μm^3)
Iron(III)-EDTA	0.150	0.508	0.518
Iron(III)-succinate	0.164	0.191	0.087
Iron(III)-citrate	0.094	0.432	0.588
Iron(III)-malate	0.275	0.578	0.632
Iron(III)nitrate	0.422	0.236	0.304

Mean chain lengths varied from 1.1 to 9 cells to chain (Fig. 5). No response of chain size to temperature and iron speciation was found (R^2 ≤0.163).

Bacterial abundances were relatively uniform (8.2 x 10^8 cells L^{-1} to 9.14 x 10^8 cells L^{-1}) with the exception at 18°C, 98 hours after treatments. In culture treated with iron(III)-citrate bacterial density was 4.1 x 10^{10} cells L^{-1}, in culture treated with iron(III)-malate bacterial density was 5.6 x 10^{10} cells L^{-1}, and in culture treated with iron(III)nitrate bacterial density was >1 x 10^{11} cells L^{-1}. It was practically impossible to count the bacterial cells being clustered both around *S. marinoi* cells and individually in irregular "snowball" aggregate forms each including roughly approximated >1000 cells (Fig. 7h).

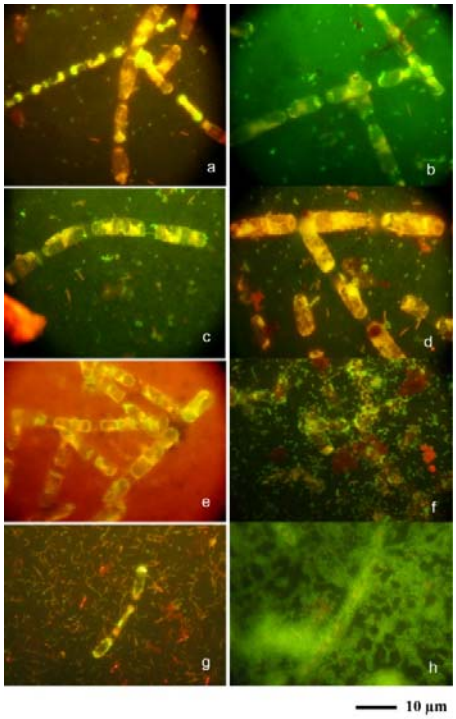

Fig. 7. *Examples of the differences of cell appearance properties in terms of cell fluorescence and size observed during testing with a) iron(III)-EDTA at 24°C, b) iron(III)-succinate at 24°C, c) iron(III)-citrate at 24°C, d) iron(III)-malate at 24°C, e) iron(III)-nitrate at 24°C, f) iron(III)- malate at 25°C, g) iron(III)-EDTA at 25°C, and h) iron(III)-malate at 18°C.*

Examination by epifluorescent microscopy also denoted the influence of temperature and iron speciation on cell appearance in terms of size and fluorescence (Fig. 7). Figure depicts visible differences between cell appearance at different iron(III) treatments and temperature. Iron(III) speciation had more pronounced influence on the size and fluorescence properties of cells than temperature.

Discussion

Experiments were conducted in UVFSW with approximated background concentrations of Fe 3.7 nmol L^{-1} (Öztürk et al. 2003). Our assumption was that very little of the iron in UVFSW was available to phytoplankton growth (Martin and Gordon, 1988; Millero, 2001). This assumption was tested by comparing growth of the same microbial assemblage in conditions with added 0.5 nmol L^{-1} iron(III) to the same assemblages without added iron (Figs. 2, 3). Two groups of tests showed significant difference in growth where the growth rates of natural phytoplankton assemblages in flasks with added 0.5 nmol L^{-1} iron(III) were significantly higher than in the flasks without added iron. Furthermore, assemblages growing on background amounts of iron in seawater exhibited significant decrease in the community structure. Only the diatom *Chaetoceros* sp. (Fig. 2b) actively growed in the flasks. Survival of *Chaetoceros* sp. could be attributed to its larger cell size and the ability to utilize iron (alone or in partnership with heterotrophic bacteria), which is not accessible to other members of the community. It shows that the community is capable of making at least part of the existing iron available for growth. Therefore, the addition of 0.5 nM Fe results in the difference in growth curves as shown in Fig. 3. Whatever the case, these tests have shown that the addition of iron(III) complexes used in this study can result in significant and observable effect on the phytoplankton growth or that the added ligand mobilized the existing iron in the medium.

Preliminary tests with natural assemblages indicated that iron speciation could be a limiting factor along with the effects of temperature (e.g. Berges, Varela and Harrison, 2002). Different algal species started their exponential phase of growth at different times (Fig. 1). While other species started growing exponentially in all treatments at the same time, the start of *S. marinoi* exponential phase did not occur at the same time in different iron treatments (Fig. 1). These results indicate possible adaptability of microplankton to temperature and iron speciation (and limitation) depends on the plankton species. Furthermore, the appearance of cyanobacterial bloom 194 hours after iron(III) treatments indicated possible competition between prokaryotes and eukaryotes for biologically available iron. It can be presumed that the bioavailability of iron changed during 194 hours of continuous culture resulting with niche separation thus allowing the rapid bloom of cyanobacteria. Hutchins et al. (1999) have demonstrated that *Synechococcus* does not possess a non-specific ferrireductase system as has been suggested for eukaryotic phytoplankton. Our results corroborate these suggestions.

Within the model experimental conditions in this study, the growth patterns of *S. marinoi* were influenced by both, temperature and iron(III) speciation. Temperature growth threshold was 18-25°C. This was very surprising since this species is known to have high adaptability to wide range of temperatures (0-8 °C, Lihua et al., 2010; 4-12°C, Rüger and Sommer, 2012; 15-30°C, Curl and McLeod, 1961; 5-30°C, Claquin, Probert, Lefebvre and Vernon, 2008). Cultures exhibited short and intense cycles (98-134 hours) with optimal growth temperature at 24°C. *S.marinoi* did not grow at 18°C in iron(III)-EDTA and iron(III)- succinate treatments neither at 25°C in iron(III)- succinate and iron(III)-citrate treatments (Fig. 4). These results showed that besides temperature, iron(III) speciation was the limiting factor as salinity and oxygen concentration did not change during the experiments. The growth of this diatom was significantly influenced by temperature in treatments with iron(III)-malate and iron(III)nitrate (p≤0.005). In treatments with iron(III)-EDTA, iron(III)-succinate and iron(III)-citrate, correlation between growth and temperature was not significant (p=0.082,

p=0.384, p=0.280, respectively) indicating that in these three cases the influence of iron(III) speciation was the main limiting factor, especially in treatments with iron(III)-succinate and iron(III)-citrate.

Further evidence for the significance of iron(III) speciation can be found in responses of cell volumes to temperature. Within five different tests analyzed with linear regression analysis, the cell volumes of four tests did not show response to temperature. Because the data indicated curvilinear responses, second order polynomial regression analysis was applied indicating that out of five different iron(III) treatments, two did not show response to temperature.

It has been shown that *S. marinoi* can reach cell density of >1.5 x 10^9 cells L^{-1} in batch cultures with initial iron(III) concentrations of 3 µmol L^{-1} (iron(III)-EDTA) at 18°C (Puskaric, Mortain-Bertrand, 2003) indicating that during this study other nutrients were still abundant when the cells reached their death phases. The maximum cell density within this study was measured at 24°C in cultures treated with iron(III)-succinate and iron(III)-citrate (Fig. 4), an order of magnitude lower than described for the particular species at higher concentrations of iron. This additionally suggested that the depletion of iron was the limiting factor and that the iron(III) complexes used in this study are capable of competing with the natural organic Fe(III) complexes present in the oceans.

High abundances of heterotrophic bacteria in tests at 18°C were the probable cause of growth limitation (Fig. 9h). It was presumed that phytoplankton exudates were the factor enabling pronounced bacterial growth, especially because the treatment with the maximum bacterial densities was observed in the treatment with iron(III)nitrate, an inorganic complex. However, such behaviour was not observed in preliminary tests with natural assemblages.

Results from the tests presented here are different from reports from earlier experiments performed on the same species and the same experimental system (Atkinson, 1994; Forster et al., 2011). The Temperature Size Rule (TSR) is visible from 21-25°C and not in the entire temperature spectrum of this species. Lihua et al. (2010) described that decreasing temperatures can only restrain the velocity of *S. marinoi* growth. In our study the restraining growth rates of *S. marinoi* could be described as bidirectional (Fig.5) (highest at 24°C, decreasing towards both 18 and 25°C).

Despite iron concentrations of <1 nmol L^{-1} in natural waters were taken as iron limited conditions (Johnson et al., 1997), and that iron deficiency limits phytoplankton growth (Martin and Fitzwater, 1988) our tests showed that iron(III) concentration of 0.5 nmol L^{-1} did not cause interrupted growth until the cultures reached cell densities of 1.5 x 10^8 cells L^{-1}. While investigating the development of a spring phytoplankton bloom King et al. (2012) reported decreasing intracellular Fe quotas with increasing chlorophyll. This notion suggested possible eukaryotic extracellular release of intracellular iron with metabolites. Iron can thus be complexed e.g. with malate intracellularly before it has been released. Such hypothesis could find confirmation in our studies; our results show that iron(III) in concentrations of only 0.5 nmol L^{-1} which is considered to be the concentration below iron limited conditions are sufficient to produce a phytoplankton bloom. This could be possible with the existence of microbial iron loop in the form of resource sharing, and/or that the existing insoluble iron in seawater used in the experiments slowly becomes more available being mobilized by added ligands. Further investigations should be done for better understanding of real conditions in the oceans due to the synergistic effects of other possible direct and indirect interactions, e.g. species specific iron-uptake strategies or chemical competition between iron(III) and other metals present in seawater for organic ligand.

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References

Atkinson, D. (1994) Temperature and organism size: a biological law for ecotherms? *Adv. Ecol. Res.*, **25**, 1--58.

Behrenfeld, M. and Falkowski, P. (1997) A consumer’s guide to phytoplankton primary productivity models. *Limnol. Oceanogr.*, **42**, 1479--1491.

Berges, J.A., Varela, D.E. and Harrison, P.J. (2002) Effects of temperature on growth rate, cell composition and nitrogen metabolism in the marine diatom *Thalassiosira pseudonana* (Bacillariophyceae). *Mar. Ecol. Prog. Ser.*, **225**, 139--146.

Bosak, S., Šilović, T., Ljubešić, Z., Kušpilić, G., Pestorić, B., Krivokapić, S. and Viličić, D. (2012) Phytoplankton size structure and species composition as an indicator of trophic status in transitional ecosystems: the case of a Mediterranean fjord-like karstic bay. *Oceanologia,* **54**, 255--286.

Byrne, R.H., Luo, Y.R. and Young, R.W. (2000) Iron hydrolysis and solubility revisited: observations and comments on iron hydrolysis characterizations. *Mar. Chem.*, **70**, 23--35.

Claquin, P., Probert, I., Lefebvre, S. and Veron, B. (2008) Effects of temperature on photosynthetic parameters and TEP production in eight species of marine microalgae. *Aquat. Microb. Ecol.*, **51**, 1--11.

Cmuk, P., Piantanida, I. and Mlakar, M. (2009) Iron(III)-complexes engaged in the biochemical processes in seawater. I. Voltammetry of Fe(III)-succinate complexes in model aqueous solution. *Electroanalysis*, **21**, 2527--2543.

Coale, K.H., Johnson, K.S., Chavez, F.P., Buesseler, K.O., Barber, R.T., Brzezinski, M.A., Cochlan, W.P., Millero, F.J., Falkowski, P.G., Bauer, J.E., Wanninkhof, R.H., Kudela, R.M., Altabet, M.A., Hales, B.E., Takahashi, T., Landry, M.R., Bidigare, R.R., Wang, X., Chase, Z., Strutton, P.G., Friedrich, G.E., Gorbunov, M.Y., Lance, V.P., Hilting, A.K., Hiscock, M.R., Demarest, M., Hiscock, W.T., Sullivan, K.F., Tanner, S.J., Gordon, R.M., Hunter, C.N., Elrod, V.A., Fitzwater, S.E., Jones, J.L., Tozzi, S., Koblizek, M., Roberts, A.E., Herndon, J., Brewster, J., Ladzinsky, N., Smith, G., Cooper, D., Timothy, D., Brown, S.L., Selph, K.E., Sheridan, C.C., Twining, B.S. and Johnson, Z.I. (2004) Southern Ocean iron enrichment experiment: Carbon cycling in high- and low-Si waters. *Science*, **304**, 408--414.

Crumbliss, A.L. and Garrison, J.M. (1988) A Comparison of Some Aspets of the Aqueous Coordination Chemistry of Aluminium(III) and Iron(III). *Comments Inorg. Chem.*, **8,** 1--26.

Curl, H.J. and McLeod, G.C. (1961) The physiological ecology of a marine diatom Skeletonema costatum (Grev.) Cleve. *J. Mar. Res.*, **19**, 70--88.

Davis, C.O., Harrison, P.J. and Dugdale, R.C. (1973) Continuous culture of marine diatoms under silicate limitation. I. Synchronized life cycle of *Skeletonema costatum*. *J. Phycol.*, **9**, 175--180.

Davison, I.R. (1991) Environmental effects on algal photosynthesis: temperature. J. *Phycol.*, **27**, 2--8.

de Baar, H.J.W. and Boyd, P.W. (1999) The role of iron in plankton ecology and carbon dioxide transfer of the global oceans. In Hanson, R.B., Ducklow, H.W., Field, J.G. (Eds.), *The Dynamic Ocean Carbon Cycle: a Midterm Synthesis of the Joint Global Ocean Flux Study*. Cambridge University Press, Cambridge, pp. 61--140.

Donderski, W., Mudryk, Z. and Walczak, M. (1998) Utilization of low molecular weight organic compounds by marine neustonic and planktonic bacteria. *Pol. J. Env. Sci*., **7**, 279--283.
Evans, M. C. W. and Buchanan, B. B. (1965) Photoreduction of ferredoxin and its use in carbon dioxide fixation by a subcellular system from a photosynthetic bacterium. *Proc. Natl Acad. Sci. USA*, **53**, 1420--1425.
Forster, J., Hirst, A.G. and Atkinson, D. (2011) How do organisms change size with temperature? The importance of reproductive mode and ontogenetic timing. *Funct. Ecol*., doi:10.1111/j. 1365-2435.2011.02852.x.
Guillard, R. R. L. and Ryther, J. H. (1962) Studies on marine planktonic diatoms. I. *Cyclotella nana* Hustedt and *Detonula confervacea* (Cleve) Gran. *Can. J. Microbiol*., **8**, 229--239.
Haas, L. W. (1982) Improved epifluorescence microscopy for observing planktonic microorganisms. *Annls Inst. oceanogr. Paris*, **58**, 261--26
Hoare, D. S. and Moore, R. B. (1965) Photoassimilation of organic compounds by autotrophic blue-green algae. *Biochim. Biophys. Acta*, **109**, 622--625.
Hutchins, D.A., Witter, A.E., Butler, A. and Luther III, G.W. (1999) Competition among marine phytoplankton for different chelated iron species. *Nature*, **400**, 858--861.
Johnson, K. S., Gordon R. M. and Coale K. H. (1997) What controls dissolved iron in the world ocean? *Mar. Chem*., **57**, 137--161.
King, A.L., Sañudo-Wilhelmy, S.A., Boyd, P.W., Twining, B.S., Wilhelm, S.W., Breene, C., Ellwood, M.J. and Hutchins, D.A. (2012) A comparison of biogenic iron quotas during a diatom spring bloom using multiple approaches. *Biogeosciences*, **9**, 667--687.
Li, W.K.W. (1980) Temperature adaptation in phytoplankton:cellular and photosynthetic characteristics. In Falkowski, P.G. (ed.) *Primary productivity in the sea*. Plenum Press, New York, pp. 259--279.
Lihua, X., Jianfeng, H., Fang, Z., Yan, G., Rumin, Z. and Shikai, C. (2010) The adaptability of three microalgae to different low temperatures. *Chinese J. Polar Sci*., **21**, 193-203.
Martin, J.H. and Fitzwater, S.E. (1988) Iron deficiency limits phytoplankton growth in the north-east Pacific subarctic. *Nature,* **331**, 341--343.
Martin, J.H. and Gordon, R.M. (1988) Northeast Pacific iron distributions in relation to phytoplankton productivity. *Deep Sea Res*., **35**, 177--196.
Millero, F.J. (2001) The speciation of metals in natural waters. *Geochem. Trans*., **8**, 1--26.
Öztürk, M., Bizsel, N. and Steinnes, E. (2003) Iron speciation in eutrophic and oligotrophic Mediterranean coastal waters; impact of phytoplankton and protozoan blooms on iron distribution. *Mar. Chem*., **81**, 19--36.
Peters, T. L., Liu, M. S. and Aleem, M. I. H. (1970) The tricarboxylic acid cycle in Thiobacillus dentrificans and Thiobacillus A-2. *J. Gen. Microbiol*., **64**, 29--35.
Puskaric, S. and Mortain-Bertrand, A. (2003) Physiology of diatom *Skeletonema costatum* (Grev.) Cleve photosynthetic extracellular release: evidence for a novel coupling between marine bacteria and phytoplankton. *J. Plankt. Res*., **25**, 1227--1235.
Raven, J.A. and Geider, R.J. (1988) Temperature and algal growth. *New Phytol*., **110**, 441--461.
Rue, E.L. and Bruland, K.W. (1995) Complexation of Fe(III) by natural organic ligands in the central North Pacific as determined by a new competitive ligand equilibration/adsorptive cathodic stripping voltammetric method. *Mar. Chem*., **50**, 117--138.
Rüger, T. and Sommer, U. (2012) Warming does not always benefit the small – Results from a plankton experiment. *Aquat. Bot*., **97**, 64--68.
Smith, R., Stapleford, L. and Ridings, R. (1994) The acclimated response of growth, photosynthesis, composition, and carbon balance to temperature in the psychrophilic ice diatom *Nitzschia seriata*. *J. Phycol*., **30**, 8--16.
Smith, R.M. and Martell, A.E. (1976) *Critical Stability Constants*. Plenum Press, Oxford
Suzuki, Y. and Takahashi, M. (1995) Growth responses of several diatom species isolated from various environments to temperature. *J. Phycol*., **31**, 880--888.
Vukosav, P., Mlakar, M. and Tomišić, V. (2012) Revision of iron(III)–citrate speciation in aqueous solution. Voltammetric and spectrophotometric studies. *Analytica Chimica Acta*, **745**, 85—91.
Vukosav, P., Tomšić, V. and Mlakar, M. (2010) Iron(III)-complexes engaged in the biochemical processes in seawater. II. Voltammetry of Fe(III)-malate complexes in model aqueous solution. *Electroanalysis*, **22**, 2179--2186.