# Effects of nutrients, salinity, pH and light:dark cycle on the production of reactive oxygen species in the alga *Chattonella marina*

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**Abstract**

Experiments were carried out to investigate the effects of nutrients, salinity, pH and light:dark cycle on growth rate and production of reactive oxygen species (ROS) by *Chattonella marina*, a harmful algal bloom (HAB) species that often causes fish kills. Different nitrogen forms (organic-N and inorganic-N), N:P ratios, light:dark cycles and salinity significantly influenced algal growth, but not ROS production. However, iron concentration and pH significantly affected both growth and ROS production in *C. marina*. KCN (an inhibitor of mitochondrial respiration) and 3-(3,4-dichlorophenyl)-1,1-dimethylurea (an inhibitor of photosynthesis) had no significant effects on ROS production. Vitamin $K_3$ (a plasma membrane electron shuttle) enhanced ROS production while its antagonist, dicumarol, decreased ROS production. Taken together, our results suggest that ROS production by *C. marina* is related to a plasma membrane enzyme system regulated by iron availability but is independent of growth, photosynthesis, availability of macronutrients, salinity and irradiance.

*Keywords*: *Chattonella marina*; reactive oxygen species; iron; nutrient; pH; physical parameters; plasma membrane; redox

*Abbreviations:* DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DIN, dissolved inorganic nitrogen; DON, dissolved organic nitrogen; Glu, glutamic acid; HAB,

harmful algal bloom; PHPA, *p*-hydroxyphenyl acetic acid; PSP, paralytic shellfish poisoning; ROS, reactive oxygen species; RUBISCO, ribulose-1-5-biphosphate carboxylase; SOD, superoxide dismutase

## Introduction

*Chattonella marina*, a harmful algal bloom (HAB) species, is able to produce reactive oxygen species (ROS, including superoxide anion radicals, $O_2^{-\bullet}$, hydrogen peroxide, $H_2O_2$, and hydroxyl radicals, $OH^{\cdot}$) (Oda et al., 1998) at levels 100 times higher than those produced by most algal species (Marshall et al. 2002). In photosynthetic organisms, ROS are continuously produced as byproducts through various metabolic pathways localized in mitochondria, chloroplasts, and peroxisomes (Apel and Hirt, 2004). Photosynthetic organisms can also generate ROS by activating various oxidases and peroxidases in response to environmental stresses such as pathogens (Peng and Kuc, 1992), drought (Moran et al., 1994), light intensity (Karpinski et al., 1997) and contaminants such as paraquat (Iturbe-Ormaetxe et al., 1998). In *C. marina*, production of ROS has been related to growth phase, and maximum production was found during the exponential growth phase (Kawano et al., 1996). Production of superoxide anion ($O_2^{-\bullet}$) was found to be suppressed by iron deficiency (Kawano et al., 1996), and irradiance also appears to play a role in $O_2^{-\bullet}$ production in *C. marina* (Marshall et al., 2001; 2002). In *Heterosigma akashiwo*, another raphidophycean flagellate, iron depletion and an increase in temperature (from 7°C to 30°C) enhanced ROS production (Twiner and Trick 2000). Nevertheless, factors affecting ROS production in raphidophycean flagellates remain unclear.

*C. marina* has caused mass mortalities of fish and great economic losses in many countries (Marshall et al., 2002), and ROS production has been implicated as one of the major factors leading to fish kills (Kawano et al., 1996; Oda et al., 1995). Although the toxic mechanism of this alga remains unclear, it is generally accepted that nutrient availability is one of the key factors in determining the toxicity of HAB species (e.g., Boyer et al., 1987). Furthermore, N:P ratio has also been shown to play an important role in algal toxicity (Hall, 1982; Boyer et al., 1987; Anderson et al., 1990). For example, phosphorus stress increases saxitoxin production and hence toxicity in *Alexandrium* spp. (Béchemin et al., 1999; John and Flynn, 2000), while toxicity in laboratory cultures of other microalgae (e.g. *Prymnesium parvum* and *Chrysochromulina polylepis*) has been shown to increase when nitrogen or phosphorus become limiting (Johansson and Granéli, 1999). Field studies also provide evidence that certain HAB species only occur within a certain range of N:P ratios. For example, *Phaeocystis* blooms were only found when N:P ratio decreased below the Redfield ratio of 1:16 (Riegman et al., 1992).

While the vast majority of previous studies on algal toxicity have focused on dissolved inorganic nitrogen (DIN), recent work has revealed that dissolved organic nitrogen (DON, including urea, dissolved free amino acids, and nucleic acids) may also serve as important nitrogen sources for many phytoplankton species, including

HAB-causative species (Anderson et al., 2002; Berman and Bronk, 2003). If and in what way DON affects growth and ROS production in *C. marina* remains unknown. Availability of micronutrients, especially iron, has also been implicated as an important factor in the bloom of some HAB species (Bruland et al., 2001; Maldonado et al., 2002), and an increase in toxicity in *Microcystis aeruginosa* was found when iron became limiting (Lukac and Aegerter, 1993).

Toxicity of HAB species (e.g. *Alexandrium catenella* and *Heterosigma akashiwo*) is also affected by physical and chemical factors such as irradiance (Ono et al., 2000), pH and salinity (Siu et al., 1997). Conceivably, toxicity and ROS production by *C. marina* may similarly be affected by these factors. Both laboratory and field evidence showed that toxicity of *C. marina* varies considerably under different culture/field conditions. Nevertheless, no systematic studies have been carried out thus far to determine how physical and chemical factors may affect ROS production in *C. marina*. Notably, environmental factors may also directly affect algal toxicity (Boyer et al., 1987; John and Flynn, 2000) or indirectly affect algal toxicity by affecting growth rate (Parkhill and Cembella, 1999).

It has been proposed that *C. marina* may generate ROS through an NADPH-dependent pathway, and an enzymatic system analogous to neutrophil NADPH oxidase was identified in the plasma membrane of *C. marina* (Kim et al.,

2000). Many redox enzymes located in the plant plasma membrane play a significant role in nitrate and ferric reduction (Berczi and Moller, 2000). In plants, ROS are mainly byproducts from the electron transport chains in chloroplasts (Asada, 1999), mitochondria and the plasma membrane (cytochrome *b*-mediated electron transfer) (Elstner, 1987). It is likely that these organelles are also sites for ROS production in *C. marina*, but this has yet to be demonstrated. .

The objective of this study is to carry out a systematic and comprehensive study to investigate the effects of macro- and micro-nutrient availability, nutrient ratio, nutrient forms, irradiance, pH and salinity on ROS production and related growth rate in *C. marina*. Attempts were also made to identify the site of ROS production in this HAB species.

## Materials and methods

### *Algae and culture conditions*

The *Chattonella marina* (Subrahmanyan) Hara et Chihara (NIES-3) stock culture was kindly provided by Prof. M. Watanabe of the National Institute of Environmental Studies (NIES), Japan. Seawater used in this study was collected from a clean site in Hong Kong and filtered through a 0.22 µm filter before being used for culture medium preparation. Unless otherwise stated, all cultures of *C. marina* were grown in seawater-K-medium (Keller and Guillard 1985; salinity: 30‰; pH: 7.8–8.2) at 22–24°C under a 12:12-h light:dark cycle with light intensity of ~42 µmol photons.$m^{-2}$.$s^{-1}$.

### *Algal growth rate*

Cell number was estimated using a hemacytometer every two days over a period of 8 days, and growth rate was calculated by the difference in cell number between two consecutive sampling intervals, using the equation given by Guillard (1973):

$$\text{Growth rate } K' = \ln (N_2 / N_1) / (t_2 - t_1) \quad (1)$$

Where: $N_1$ and $N_2$ = biomass at $t_1$ and $t_2$ respectively. Divisions per day were calculated as K' / ln2. The maximum growth rate during the entire study period was presented and compared between treatments in this study.

*Effects of nutrient availability (N, P and Fe) on growth rate and ROS production*

Three experiments were carried out to determine ROS production under different DON:DIN (atomic) ratio, N:P (atomic) ratio and iron concentrations. In the first experiment, glutamic acid (Glu) and urea were individually used as the source of DON, and nitrate plus ammonium (atomic ratio 18:1, identical to that in K-medium) was used as the source of DIN. The total nitrogen concentration (0.95 mM) was also kept identical to that in K-medium. Algal growth and ROS production were determined for 6 different combinations of medium DON:DIN (0:100, 20:80, 40:60, 60:40, 80:20 and 100:0). In the second experiment, algal growth and ROS production under 6 different N:P ratios (4:1, 8:1, 16:1, 32:1, 64:1 and 128:1) (Yin et al., 2000) were determined, with the total concentration of N+P kept constant at 0.95 mM N+0.03 mM P as in the original K-medium. In the third experiment, algal growth and ROS production were measured under five iron concentrations (0, $0.02x10^{-8}$, $0.2x10^{-8}$, $1x10^{-8}$ and $5x10^{-8}$ M, each with $Na_2$-EDTA 10 times that in K-medium to ensure bioavailabilty). In each treatment of the above three experiments, triplicate cultures of *C. marina* were grown in 5 mL medium in 12-well microplates with an initial cell density of ~500 cells.$mL^{-1}$. No stirring was provided since *C. marina* grows best under static conditions. Cell numbers were counted every 2 days (viz. day 2, 4, 6, 8,

10 and 12) using a hemacytometer. Samples were collected on days 4, 6 and 8 for ROS measurements. All samplings were non-repetitive and algal samples in each well were used only once to avoid possible disturbance to the algae.

*Effects of physical parameters on growth rate and ROS production*

Three sets of experiments were carried out to investigate effects of light:dark cycles, pH and salinity on growth rate and ROS production of *C. marina.* In each treatment, triplicates of *C. marina* were grown in 5 mL of K-medium in a 12-well microplate with an initial cell density of ~500 cells.mL$^{-1}$. In the first experiment, the *C. marina* culture was placed inside controlled environmental chambers with five different light:dark cycles (light hour:dark hour: 16:8, 14:10, 12:12, 10:14 and 8:16). In the second experiment, the initial pH of the culture was adjusted to 7.5, 8.0, 8.5, 9.0 and 9.5 with 1 M HCl or NaOH prior to the experiment. In the third experiment, *C. marina* was grown under 5 different salinities (30, 25, 20, 15 and 10‰) by diluting seawater with milli-Q water before nutritents were added. Cell density was measured at 2 d intervals from day 0, and ROS were measured at days 4, 6 and 8, as decribed below. Similar to the nutrient availability experiment, all samplings were non-repetitive and algal samples in each well were used only once to avoid possible disturbance to the algae.

*Measurement of reactive oxygen species*

Nutrient concentration, cell density and pH may change with growth of the alga, and these changes may affect ROS measurements. The results of our preliminary experiments indicated that production of ROS peaked during the log-phase (days 4 to 10) and generally not later than day 8 (unpublished data). As a result, ROS levels in control algal cultures and those subjected to various treatments were only measured at day 4, 6 and 8. Algal cells were centrifuged at 300 g for 3 min., and algal pellets were then washed with K-medium twice and resuspended at ~$10^4$ cells.$mL^{-1}$ in K-medium. The algal suspensions were left undisturbed for 20 min. before ROS measurements. $H_2O_2$ (and superoxide radicals, which were transformed to $H_2O_2$ by addition of superoxide dismutase (SOD) in the present study) generated by *C. marina* was measured following the method of Hyslop and Sklar (1984), which is based on the horseradish peroxidase-mediated oxidation of *p*-hydroxyphenyl acetic acid (PHPA) to the fluorescent PHPA dimmer, (PHPA)2, in the presence of hydrogen peroxide. The reaction system contained ~$10^4$ cells.$mL^{-1}$ in K-medium, 1 mM PHPA, 10 units·$ml^{-1}$ horseradish peroxidase and 30 units·$ml^{-1}$ SOD. An increase in fluorescence intensity during the first minute of incubation was measured with a fluorescence microplate reader (*f*Max S/N, Molecular Devices) (excitation wavelength 320 nm and emission

wavelength 405 nm) in the presence/absence of 1000 units.mL$^{-1}$ catalase. ROS production was estimated from the inhibition of fluorescence by catalase, and the concentration of ROS was estimated using a standard curve of $H_2O_2$ and expressed in nmol $H_2O_2$. $10^4$ cells$^{-1}$.min$^{-1}$. We have also measured the increase of fluorescence with and without the addition of SOD, and our result indicated that fluorescence production in samples with SOD addition was consistently about 3 times higher than that in corresponding samples without SOD, showing that the ratio of $O_2^{-\bullet}$ and $H_2O_2$ produced by *C. marina* was quite stable, and the increase in fluorescence provided a reliable estimate on both $H_2O_2$ and $O_2^{-\bullet}$ production by *C. marina*.

*ROS production site*

*C. marina* was cultured in 75 ml K-medium in a cell cuture flask, and cells were harvested on day 6. Algal cells were then pelleted by centrifuging at 300 x g for 3 min., washed twice and finally resuspended into ~$10^4$ cells.ml$^{-1}$ in K-medium. In the first experiment, KCN was added to block complex IV in the mitochondrial electron-transfer chain at a final concentration of 1 mM. ROS production in the KCN treatment and control (with K-medium only) were then compared to evaluate the role of mitochondria in ROS production.

In the second experiment, 10 μM 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was added to block linear photosynthetic electron transport and inhibit photosynthesis. ROS production in the DCMU treatment and control (with K-medium only) were then compared to evaluate the role of photosynthesis in ROS production. 2-Methyl-1,4-naphthoquinone (Vitamin $K_3$) is a promoter of electron transfer in the plasma membrane of different cells (Barr et al., 1990; Döring et al., 1992), while dicumarol is a competitive inhibitor and antagonist of vitamin $K_3$. To test whether the redox enzyme system in the plasma membrane is involved in ROS production, 20 μM vitamin $K_3$ and 20 μM of its antagonist, dicumarol, were added to the algal culture, and ROS production was then determined. ROS production was measured after incubating individual chemicals with the re-suspended algal pellet for 1 hour.

*Statistical analysis*

Algal growth rate and ROS production rate were first tested for homogeneity of variance using Bartlett's test. Data was arc sin square root transformed if they were not homogenous or did not meet the assumption of normality before comparing by one-way ANOVA. Where significant differences were detected, a pair-wise Tukey test was used to identify differences between each level of treatment and control (Zar, 1984). α for all above tests was set at 0.05.

## Results

### *Nutrients*

Although both DON (urea or Glu) and DIN ($NO_3^-$ + $NH_4^+$) can support the growth of *C. marina*, growth rate significantly decreased when DON constituted ≥ 80% of total nitrogen (TN) supplied in the medium, as compared to growth rate in medium with 100% DIN (Figs. 1 and 2). ROS production, however, was not affected by DON/DIN ratio (Figs. 1 and 2). Redfield (1958) reported that the average N:P ratio is 16:1 in open marine waters, and this ratio generally supports balanced phytoplankton growth. It is also generally accepted that N:P atomic ratio <16 indicates that nitrogen is the limiting nutrient, whereas N:P atomic ratio >16 indicates that phosphorous is limiting. In our experiments, growth rate of *C. marina* was high and similar in treatments with N:P atomic ratios of 8:1, 16:1 and 32:1. However, growth rate was significantly reduced under extreme values of N:P (i.e. 128:1, 64:1 and 4:1) with the lowest growth rate observable when N:P was high at 128:1 (Fig. 3A). Although medium N:P ratios had a significant effect on growth rate of *C. marina*, ROS production was not affected (Fig. 3B). Interestingly, both growth and ROS production of *C. marina* were significantly reduced at low and medium iron concentration (<0.02 x $10^{-8}$M), as compared to higher iron concentration treatments (0.2x, 1x and 5x $10^{-8}$ M) (Fig. 4).

*Physical parameters*

Salinity is known to be one of the key factors controlling *C. marina* blooms in coastal waters (Marshall and Hallegraeff, 1999), perhaps through influencing osmoregulation and hence cell growth (Cembella, 1998). Our results showed that optimal growth of *C. marina* occurred at 20-30 psu, and growth was significantly reduced when salinity fell below 15 psu (Fig. 5A). ROS production, however, was not affected by salinity (Fig. 5B). Growth of *C. marina* remained unchanged in the normal range of pH in seawater (pH 7.5 to 8.5), while a significant reduction in growth was observed when pH increased beyond 9.0 (Fig. 6A). Interestingly, ROS production was significantly enhanced at high pH (9.0 and 9.5) while growth was significantly reduced (Fig. 6B). Significant differences in growth rate were only found when *C. marina* was cultured under light duration of <10 h. (i.e. in 10L:14D and 8L:16D), whereas ROS production was not affected by light:dark cycle (Fig. 7).

*Source of ROS production*

Effects of KCN, DCMU, vitamin $K_3$ and dicumarol on ROS production are shown in Table 1. KCN had no effect on ROS production. However, ROS production was significantly enhanced upon addition of 20 μM vitamin $K_3$, which promotes electron

transfer in the plasma membrane, while ROS production was inhibited by 20 μM of its antagonist, dicumarol.

## Discussion

### *Nutrients and ROS production*

ROS production by *C. marina* was decreased by elevated iron concentration but not affected by nitrogen source (DON and DIN) and N:P ratio in the culture medium. The enhancement of ROS production by iron limitation observed in *C. marina* is consistent with results reported for another raphidophyte, *Heterosigma akashiwo,* in which a significant elevation of $H_2O_2$ production (from 2.7 to 5.6 pmol $min^{-1}$ $10^4$ $cells^{-1}$) was found upon an increase in concentration of desferal (a chelating agent that lowers the availability of free iron) (Twiner and Trick, 2000). In contrast, Kawano et al. (1996) reported that desferal strongly inhibited ROS generation in *C. marina* in a concentration-dependent manner. The discrepancy between these studies may be due to differences in the methods employed for ROS measurement. In our experiments as well as in the study of Twiner and Trick (2000), ROS production in cell resuspensions was measured, whereas Kawano et al. (1996) directly measured ROS levels in the algal culture. Notably, many parameters (e.g. macronutrients, micronutrients and pH) will change in the culture medium with growth of algae, which can directly affect

ROS measurements. For example, fluorescence of the $(PHPA)_2$ probe used in ROS measurement will increase with pH (Panus et al., 1993), while pH in the medium of *C. marina* culture can increase to ~9.0 at day 8-10, as measured in our preliminary experiments.

The photosynthetic electron transport chain is the principal site of ROS production in photosynthetic organisms, and ROS are formed when electrons are diverted to oxygen but not $CO_2$ when photosynthetic efficiency decreases (Apel and Hirt, 2004). Iron plays a key role in the synthesis and functioning of the photosynthetic apparatus in eukaryotic algae (Greene et al., 1992; Desquilbet et al., 2003), and Fe-limitation has been found to reduce the quantum efficiency of photosynthesis and thus the photosynthetic energy conversion in marine chlorophytes (*Dunaliella tertiolecta*) and diatoms (*Phaeodactylum tricornutum)* (Greene et al., 1992). The fact that blockage of linear photosynthetic electron transport by DCMU had no effect on ROS production (Table 1) does not support that enhancement of ROS by iron is mediated through photosynthetic processes. The mitochondrion is another major site of ROS production, and any reduction in electron transfer will enhance ROS production (Møller, 2001). However, the fact that no induction of ROS production was found in the KCN (an inhibitor of respiratory electron transfer) treatment (Table 1) suggests that that electron transfer in the mitochondrion is

unlikely to be an important source of ROS production in *C. marina*. The other major ROS sources in photosynthetic organisms are certain enzymatic systems (Halliwell and Gutteridge, 1999), and results of Western blotting using antibody raised against the human neutrophil cytochrome *b*558 large subunit (gp91phox, a sub-unit of NADPH oxidase) showed that *C. marina* generates ROS possibly through a plasma membrane NADPH-dependent enzymatic pathway (Kim et al., 2000). Our experiments showed that ROS production was significantly enhanced upon addition of vitamin $K_3$, while ROS production was inhibited by dicumarol (Table 1), and hence offer supporting evidence that ROS production by *C. marina* was related to a plasma membrane enzyme system.

In seawater, iron exists in two oxidation states, viz. Fe(II) and Fe(III), and the bioavailability of iron is predominately limited by the solubility of Fe(III) (Johnson et al., 1997). Consequently, marine algae have developed specialized mechanisms for iron uptake. Numerous studies have shown that enhanced Fe(III) reduction in many algal species when iron becomes limiting (Eckhardt and Buckhout, 1998; Xue et al., 1998). Recently, Rose et al. (2005) proposed that the marine cyanobacterium *Lyngbya majuscula* may use superoxide as an electron shuttle to reduce Fe(III) for iron uptake. Our present finding that ROS production was enhanced when iron concentration was low may also suggest that ROS production in *C. marina* is related to the uptake of

iron by this alga (e.g. playing a role by reducing Fe(III) to enhance iron uptake). Other possibilities may also exist. For example, ferric reduction by plasma membrane ferric reductase is much greater than iron uptake rate in *Chlorella kessleri* (Chlorophyceae), and aerobic oxidation of excess Fe(II) may lead to ROS production (Middlemiss et al., 2001). A similar process may also occur in *C. marina.* Further studies are required to clarify the relationship between ROS production and iron acquisition.

The availability of macronutrients, including different nitrogen forms (DON and DIN) and N:P ratios, had no effect on production of ROS by *C. marina* (Figs. 1, 2 and 3). It has been well established that environmental conditions can influence the cellular chemical composition of phytoplankton (Harrison et al., 1990), and nutrient limitation will generally cause a reduction in intracellular nutrients (Cembella et al., 1984). Because they are important elements for cell composition, nitrogen and phosphorus limitation is likely to affect normal cellular functions (e.g. photosynthesis, respiration and some enzyme activities in the case of algae). For example, increasing nitrogen deficiency has been found to decrease photosynthesis and respiration, and increase the activities of nitrate reductase, NADPH-glutamate dehydrogenase and glutamine synthetase in *Chlorella stigmatophora* (Everest et al., 1986). The fact that different nitrogen forms and N:P ratios did not affect ROS production may suggest that either photosynthesis, respiration are not involved in ROS production. or

ROS-producing enzymatic system are not directly or indirectly affected by macronutrient availability.

*Physical parameters and ROS*

Our results showed that ROS production in *C. marina* was not significantly affected by light:dark cycle and salinity, but was significantly enhanced at high pH values (9.0 and 9.5, above the normal range of pH in seawater) (Figs. 5, 6 and 7). High pH may affect cellular enzyme function and change the speciation of metals (McKnight et al., 2001). Since enzyme activities are pH-dependent, changes in extracellular pH are likely to affect intracellular pH and hence enzymatic activities (Smith and Raven, 1979) and enzyme structure in algae (Taraldsvik and Myklestad, 2000). The enhanced ROS production observed in *C. marina* under high pH (9.0 and 9.5) may possibly be attributable to an increase in the activities of enzymes regulating ROS production. The possibility also exists that elevated pH may affect metal speciation and reduce the bioavailability of iron (McKnight et al., 2001; Lundholm et al., 2004), which may in turn enhance ROS production.

ROS production in *C. marina* was not affected by the light:dark cycle (Fig. 7). It has been shown that ROS production in *C. marina* was significantly reduced with 24 h of darkness (Marshall et al., 2001), or 5 h after DCMU treatment (an inhibitor of

photosynthesis) (Marshall et al., 2002). However, measurements in both of these experiments were conducted not long after but not immediately following exposure, and no measurements were made of energy storage and energy metabolism (e.g. the efficiency of the pentose phosphate pathway for NAD(P)H production). In another study, Twiner and Trick (2000) found that irradiance (in the range of 0-150 µmol photons.$m^{-2}$.$s^{-1}$) had no significant effects on ROS production in another raphidophyte, *H. akashiwo*. Likewise, ROS production by another marine algal species, *Hymenomonas carterae*, was not influenced by irradiance (Palenik et al., 1987).

*Growth rate and ROS production*

Growth rate is closely related to cell cycle (especially G1) and toxicity of certain HAB-causing algae, e.g. *Alexandrium tamarense* (Parkhill and Cembella, 1999) and *A. fundyense* (Taroncher-Oldenburg et al., 1999). In this study, however, both nutrient availability (different nitrogen forms and N:P ratio, iron) and physical parameters (salinity, pH and light:dark cycle) affected growth rate under extreme conditions, while only low iron concentrations (0 and 0.02 x $10^{-8}$M) and high pH (9.0 and 9.5) had significant effects on ROS production. Previous studies have shown that the relationship between growth rate and toxicity of HAB algae may not be a simple one.

For example, high toxicity has been observed at very low growth rate, whereas in other cases *Prymnesium parvum* bloom in nature was not associated with any observable toxic effects (Shilo, 1967). Our results suggest that growth rate and ROS production in *C. marina* are independent and regulated by different factors.

*Source of ROS production*

KCN had no effect on ROS production, suggesting that mitochondrial respiration may be not involved. DCMU, an inhibitor of linear photosynthetic electron transport also did not affect ROS production, and this result does not support that ROS production by *C. marina* is related to photosynthetic processes. Another study by Oda et al. (1998) also demonstrated that superoxide anion production in *C. marina* (in re-suspended algal cells) was not affected by treatments with KCN and DCMU during the exponential growth phase, and further supports the notion that respiration and photosynthesis are not the primary pathways for ROS production in *C. marina*.

ROS production was significantly enhanced upon addition of vitamin $K_3$, but inhibited by dicumarol. Due to its high hydrophobicity, Vitamin $K_3$ can easily penetrate the plasma membrane and act as a mobile transmembrane electron shuttle (Döring and Lüthje, 1996). As an antagonist of vitamin $K_3$, dicumarol can inhibit the plasma membrane redox enzyme system (in *Zea mays* L. roots; Döring et al., 1992).

Our results therefore imply that a plasma membrane enzymatic system may be primarily related to ROS production in *C. marina*. The fact that instantaneous $H_2O_2$ production by *H. akashiwo* was directly affected by temperature (Twiner and Trick, 2000) also supports our contention that ROS production by *C. marina* is mediated through enzymatic processes.

## Conclusion

*C. marina* can not only grow in a wide range of N:P ratios, iron concentrations, salinity, pH and light:dark cycles, but is also able to utilize different forms of nitrogen (DON and DIN). In our experiments, growth was only affected by extreme and environmentally unrealistic levels of available macro-nutrients, light, pH, salinity and iron. Except for high pH and iron limitation, these factors had no significant effects on ROS production, suggesting that ROS is not directly related to growth rate in *C. marina*. It is possible that pH may affect ROS production through affecting speciation and availability of iron in seawater, and this offers additional, indirect evidence to support the notion that iron is important in regulating ROS production. Enhanced ROS production was observed in the presence of a plasma membrane electron shuttle (vitamin $K_3$) and decreased ROS production was found in the presence of its antagonist (dicumarol), suggesting that a plasma membrane enzyme system may be

the primary source of ROS production in *C. marina*. Taken together, the results of our study suggest that ROS in *C. marina* are related to a plasma membrane enzyme system which is regulated by iron availability but independent of growth rate, photosynthesis, macronutrient availability, salinity and light irradiance. This plasma membrane enzyme may directly produce ROS, which may in turn enhance iron uptake by reducing Fe(III) to the more bioavailable Fe (II). Alternatively, ROS may be produced during the iron uptake process if more reduced iron (Fe(II)) is produced than the amount that the alga can take up. Further studies are required to decipher the relationship between ROS production and iron uptake in *C. marina.*

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Tables

Table 1 Effects of KCN, DCMU, vitamin $K_3$ and dicumarol on ROS production by *C. marina.* Data are presented as mean $\pm$ SD (n=3). Data sets significantly different from each other are identified by different letters ($p<0.05$).

| **Treatment** | **ROS production (nmol $H_2O_2$. $10^4$ $cells^{-1}$)** |
|---|---|
| Control | $0.231 \pm 0.021^a$ |
| 1 mM KCN | $0.249 \pm 0.017^a$ |
| 10 μM DCMU | $0.227 \pm 0.023^a$ |
| 20 μM vitamin $K_3$ | $0.476 \pm 0.035^b$ |
| 20 μM Dicumarol | $0.118 \pm 0.008^c$ |

Figure Legends

Fig. 1. Effects of urea (in the medium) on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are identified by different letters ($p$<0.05). TN=total nitrogen.

Fig. 2 Effects of Glu (in the medium) on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are identified by different letters ($p$<0.05). TN=total nitrogen.

Fig. 3 Effects of N:P ratios (in the medium) on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are

identified by different letters ($p$<0.05).

Fig. 4 Effects of iron concentrations added into the medium on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are identified by different letters ($p$<0.05).

Fig. 5 Effects of salinity on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are identified by different letters ($p$<0.05).

Fig. 6 Effects of pH on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are identified by different letters ($p$<0.05).

Fig. 7 Effects of light:dark cycles on (A) growth and (B) ROS production by *C. marina* (Mean ± SD, n=3). Data sets significantly different from each other are identified by different letters ($p$<0.05).

Figure 1

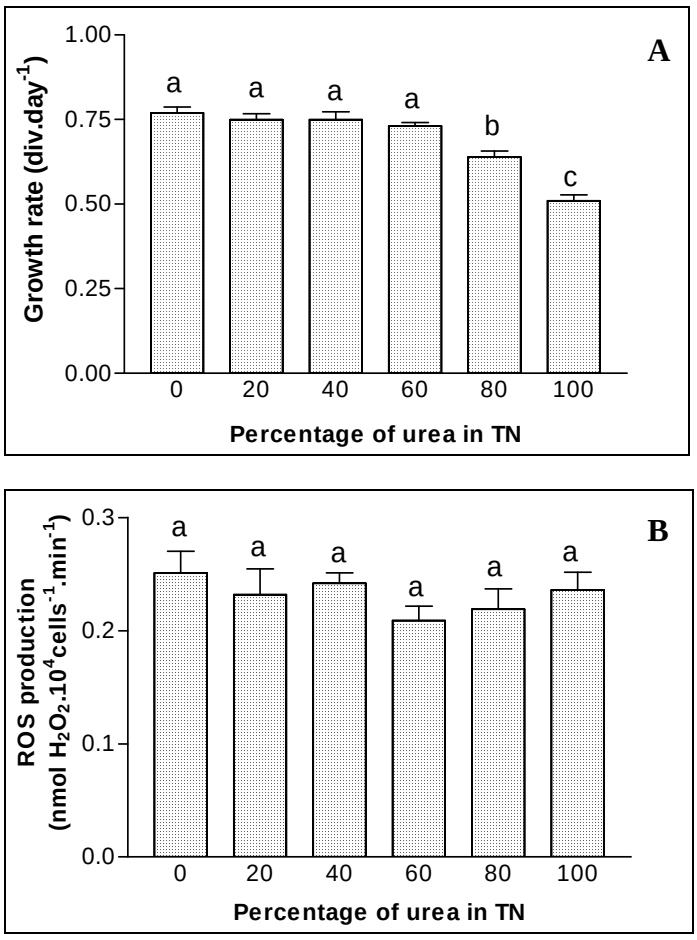
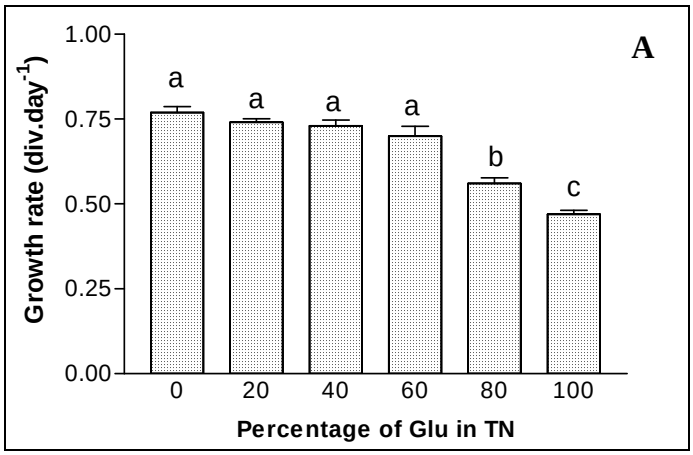

A
Growth rate (div.day-1)
1.00
0.75
0.50
0.25
0.00
a
a
a
a
b
c
0
20
40
60
80
100
Percentage of urea in TN
B
ROS production
(nmol H2O2.10^4cells-1.min-1)
0.3
0.2
0.1
0.0
a
a
a
a
a
a
0
20
40
60
80
100
Percentage of urea in TN

Figure 2


A
Growth rate (div.day-1)
1.00
0.75
0.50
0.25
0.00
a
a
a
a
b
c
0
20
40
60
80
100
Percentage of Glu in TN

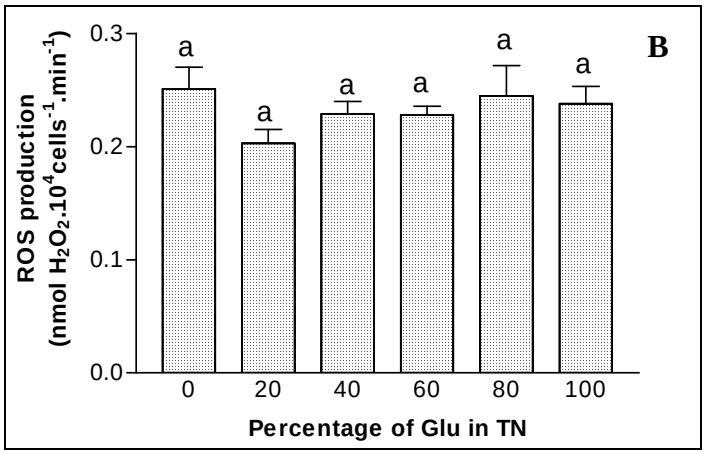

B
ROS production
(nmol H2O2.10^4cells-1.min-1)
0.3
0.2
0.1
0.0
a
a
a
a
a
a
0
20
40
60
80
100
Percentage of Glu in TN

Figure 3

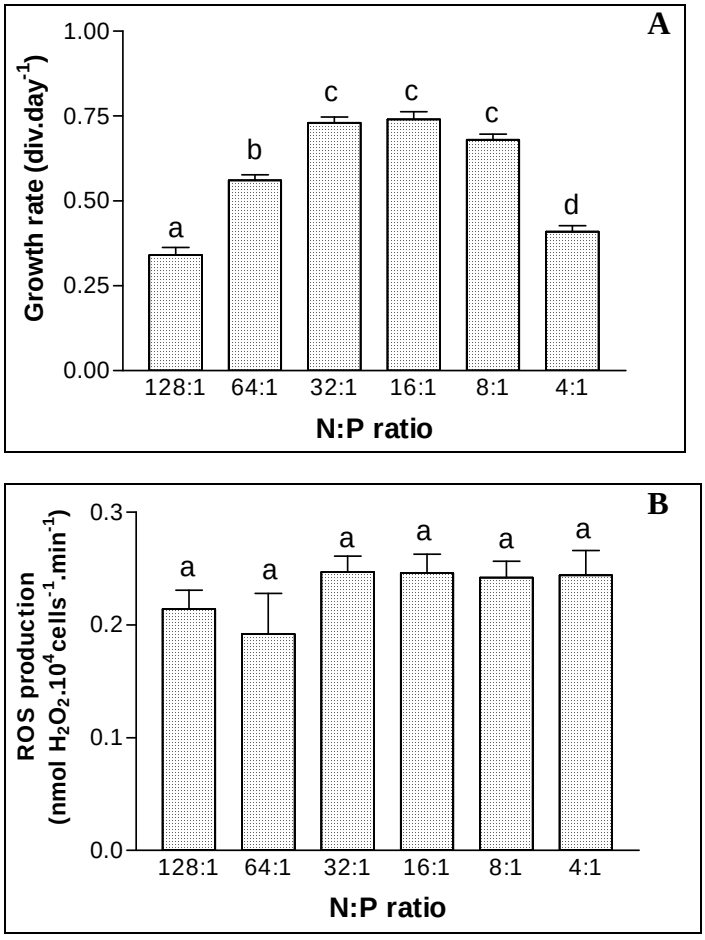

A
Growth rate (div.day-1)
1.00
0.75
0.50
0.25
0.00
a
b
c
c
c
d
128:1
64:1
32:1
16:1
8:1
4:1
N:P ratio
B
ROS production
(nmol H2O2.10^4cells-1.min-1)
0.3
0.2
0.1
0.0
a
a
a
a
a
a
128:1
64:1
32:1
16:1
8:1
4:1
N:P ratio

Figure 4

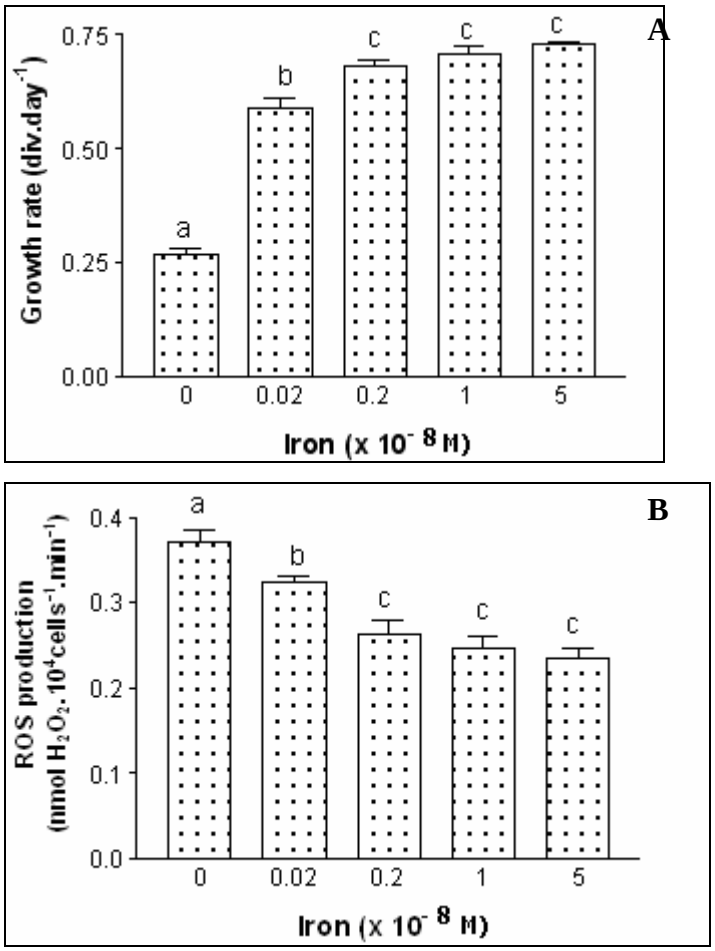

A
Growth rate (div.day$^{-1}$)
0.75
0.50
0.25
0.00
a
b
c
c
c
0
0.02
0.2
1
5
Iron (x 10$^{-8}$ M)
B
ROS production
(nmol $H_2O_2$.10$^4$cells$^{-1}$.min$^{-1}$)
0.4
0.3
0.2
0.1
0.0
a
b
c
c
c
0
0.02
0.2
1
5
Iron (x 10$^{-8}$ M)

Figure 5

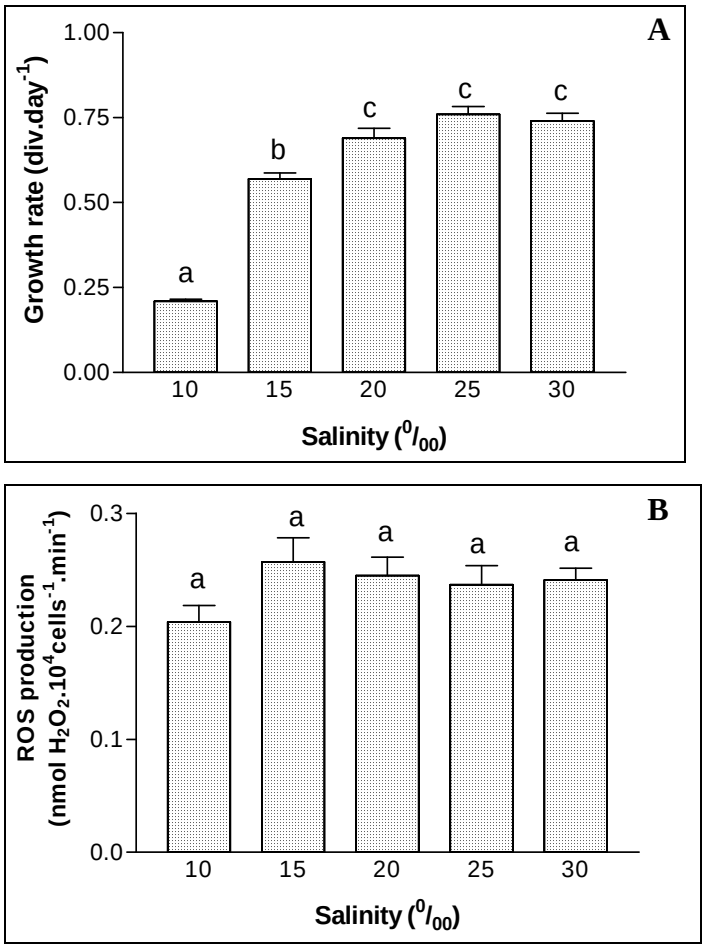

A
Growth rate (div.day-1)
1.00
0.75
0.50
0.25
0.00
a
b
c
c
c
10
15
20
25
30
Salinity (0/00)
B
ROS production
(nmol H2O2.10^4cells-1.min-1)
0.3
0.2
0.1
0.0
a
a
a
a
a
10
15
20
25
30
Salinity (0/00)

Figure 6

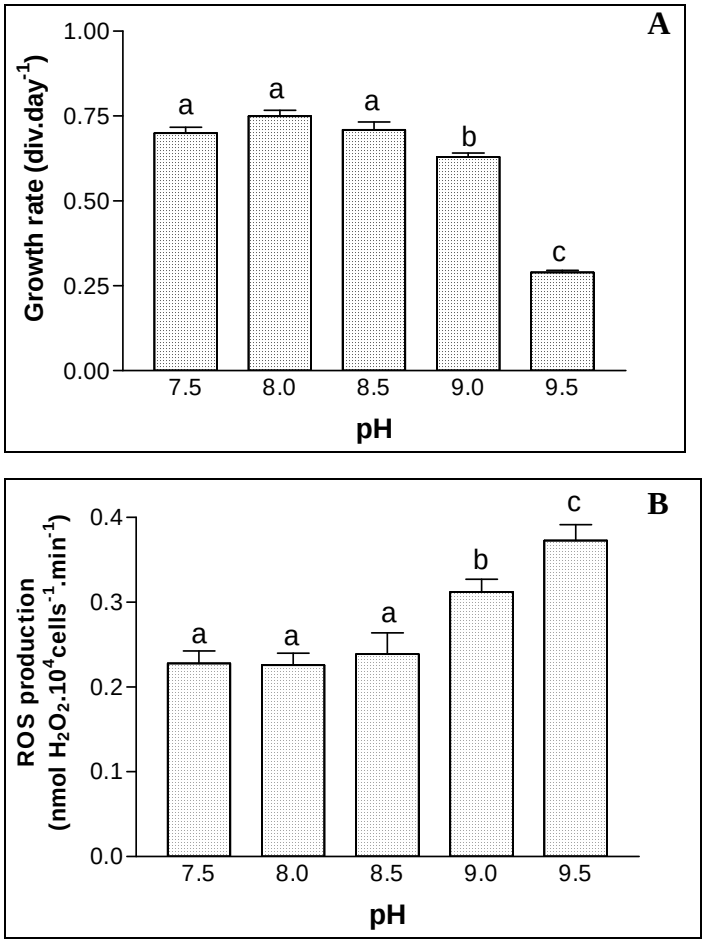
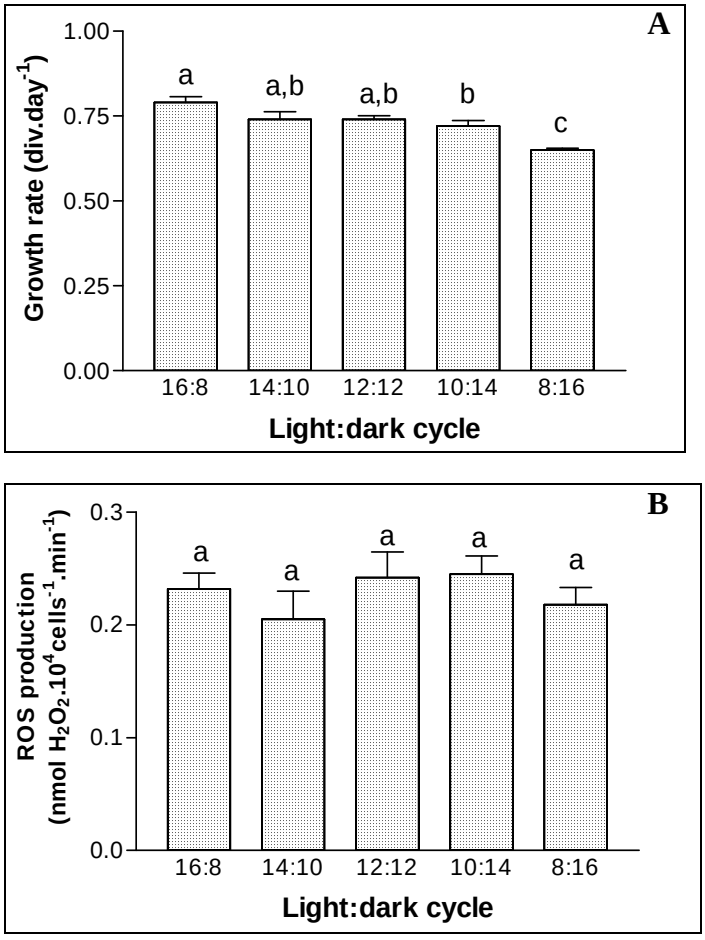

A
Growth rate (div.day-1)
1.00
0.75
0.50
0.25
0.00
a
a
a
b
c
7.5
8.0
8.5
9.0
9.5
pH
B
ROS production
(nmol H2O2.10^4cells-1.min-1)
0.4
0.3
0.2
0.1
0.0
a
a
a
b
c
7.5
8.0
8.5
9.0
9.5
pH

Figure 7


A
1.00
0.75
0.50
0.25
0.00
Growth rate (div.day-1)
a
a,b
a,b
b
c
16:8
14:10
12:12
10:14
8:16
Light:dark cycle
B
0.3
0.2
0.1
0.0
ROS production
(nmol H2O2.10^4cells-1.min-1)
a
a
a
a
a
16:8
14:10
12:12
10:14
8:16
Light:dark cycle