Environmental Pollution 157 (2009) 1933–1938

Contents lists available at ScienceDirect

Environmental Pollution

journal homepage: www.elsevier.com/locate/envpol

Effects of macronutrient additions on nickel uptake and distribution in the dinoflagellate *Prorocentrum donghaiense* Lu

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Macronutrient additions significantly affected nickel uptake and distribution in the subcellular substructures and components of the dinoflagellate.

ARTICLE INFO

Article history:
Received 27 May 2008
Received in revised form
4 December 2008
Accepted 9 January 2009

Keywords:
Dinoflagellate
Distribution
Nickel
Nutrient enrichment
Uptake

ABSTRACT

The influences of macronutrient additions on nickel (Ni) uptake and distribution in the subcellular structures and macromolecular components of the dinoflagellate *Prorocentrum donghaiense* Lu were examined using a radioisotope tracer method. The results showed that nitrate addition enhanced the uptake of Ni by *P. donghaiense*, whereas phosphate addition inhibited Ni uptake at high-Ni concentration. Nitrate or phosphate addition significantly affected Ni distribution in the subcellular structures and components. The majority of Ni was found in the soluble substances (>70%) and in the proteins (55.0–79.6%) of the algal cells. Urea reduced the Ni content in the amino acid-carbohydrate but elevated its content in proteins, and shown significantly correlated with the protein content of the algal cells. Thus, nutrient enrichment could influence both metal uptake and its distribution in the subcellular structures and components of the phytoplankton, as well as its subsequent transfer in marine food chains.

1. Introduction

In many estuarine and coastal waters, eutrophication has become a serious environmental problem (Jong, 2006). The input of macronutrients has not only changed the concentration and ratio of marine nutrients, but also affected phytoplankton structure (including species composition and abundance) in the coastal ecosystem (Smith et al., 1999), and sometimes it has influenced the structure and functioning of the entire marine ecosystem (Sanders et al., 1987; Vitousek et al., 1997). Therefore, nutrient enrichment might considerably affect the cycling of carbon, nitrogen and phosphorus in the sea (Smith et al., 1999; Vitousek et al., 1997). Little attention has been paid to the impacts of metal uptake on marine organisms. Many bioactive metals are essential nutrient elements to phytoplankton growth, and also play an important role in regulating succession and primary production in the phytoplankton community (Hudson, 1998; Sunda and Huntsman, 1998). However, due to metal pollution in coastal waters, high metal concentrations have had toxic effects on the phytoplankton and via them have accumulated at higher levels in the food chain. As a result, some attention has been paid to investigating the interactions between metals and phytoplankton (Currie et al., 1998; Rijstenbil et al., 1998; Lee and Luoma, 1998). Some studies show that a change in ambient macronutrient concentrations affects metal uptake by the phytoplankton and its subsequent transfer along the food chain (Lee and Wang, 2001; Wang and Dei, 2001a, b, c; Wang et al., 2007). However, few studies have shown that metal might have a different distribution in different structures and components of phytoplankton cells (Joux-Arab et al., 1998; Miao and Wang, 2006), and little effort has been devoted to the influence of macronutrients (e.g. N, P and Si) on metal distribution in the phytoplankton cell.

Nickel (Ni) is an essential trace metal for aquatic organisms but it is toxic at elevated concentrations. It is also indispensable to the functioning of urease, which is found in bacteria, fungi, phytoplankton and some invertebrates, and is a catalyst in the hydrolysis of urea to produce ammonia and carbamate (Smyj, 1997). However, there has been a particular lack of study on Ni uptake and its subcellular distribution in phytoplankton. Recently, a study demonstrates that higher nitrate or phosphate status increases the Ni uptake in phytoplankton and facilitates the transfer from phytoplankton to cpepods (Wang et al., 2007). Therefore, it is

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doi:10.1016/j.envpol.2009.01.009

essential to examine the interaction of Ni and nutrients in phytoplankton, and thus the functioning and fate of Ni in the context of marine biogeochemical cycles.

Prorocentrum donghaiense Lu is a key harmful algal bloom species in the coastal waters of China and, in the past few years, it has formed extensive blooms in the East China Sea, characterized by eutrophication which might significantly affect the biogeochemical cycle of trace metals in this area. However, no work has been done to investigate this possibility. In this study, we investigated the influence of the macronutrients N and P on the biological uptake of Ni by *P. donghaiense* and its distribution in the subcellular compartments (the cell wall, organelles and soluble substances) and the macromolecular components (amino acid-carbohydrate, lipid and protein) of the algal cells, using a radiotracer method, so as to reveal the impacts and mechanisms of different nutrients (and their concentrations) on metal uptake and distribution in the phytoplankton.

2. Materials and methods

2.1. The alga and its culture

The algal species *P. donghaiense* was isolated from natural phytoplankton assemblages collected in the East China Sea in 2005. *P. donghaiense* was maintained under axenic conditions in f/2 medium at 20 °C under a light illumination of 100 μmol photon m^{-2} s^{-1} with a 14:10 h light:dark cycle.

2.2. The uptake experiment

Seawater was collected from Xiamen Bay in China and was used in experiments after being held for half a year. The background concentrations of nitrate and phosphate in the collected seawater were <4 and <0.1 μmol L^{-1}. All seawater used was filtered through a 0.2 μm acetic fiber membrane to remove trace metals by passing it through a Chelex ion exchange, and finally sterilized.

To examine the influence of different macronutrients on Ni uptake in *P. donghaiense*, two nutrient treatments were designed. The experimental N addition included 20, 80 and 320 μmol L^{-1} nitrate additions as N source and 20 μmol L^{-1} urea as N source with a fixed phosphate concentration of 5 μmol L^{-1}. The experimental P treatments included 1 μmol L^{-1} P addition and 10 μmol L^{-1} P addition with a fixed nitrate concentration of 20 μmol L^{-1}. Other nutrients were added according to the f/2 medium in both treatments. After addition of nutrients (N and P), cells of *P. donghaiense* were inoculated into the medium at an initial density of 10^4 cell mL^{-1}. Then $^{63}NiCl_2$ (in 0.5 mol L^{-1} HCl, obtained from PerkinElmer Life and Analytical Sciences) was added into the culture media (400 mL) at a concentration of 1.0 μg L^{-1} Ni. For the high-Ni treatment, in addition to the radioactive Ni, stable Ni was added to the medium at a total concentration of 100 μg L^{-1}. NaOH was added to adjust the pH value to 8.0 in the medium. The uptake experiment was carried out under axenic conditions in f/2 medium at 20 °C, under an illumination of 100 μmol photon m^{-2} s^{-1}, and with a 14:10 h light:dark cycle, and lasted for 8 d.

After the experiment, the cell density in all treatments was counted. For each treatment, the alga of 10^7 cells was collected, filtered, and rinsed three times with 100 mL of sterilized seawater, and finally cleaned three times with 100 mL of 1 mmol L^{-1} EDTA (pH = 7.0) to remove Ni^{2+} bound to the exterior of the cells (Campbell and Smith, 1986). The cell pellets were prepared for further separation and for the analysis of Ni in different subcellular partitions.

To measure the dry weight of the cells, the algal cells were filtered onto a pre-weighed glass fiber filter, rinsed with 0.5 mol L^{-1} ammonium formate, and dried at 80 °C for 1 d.

2.3. The analysis methods

2.3.1. Measurement of Ni in the cells

In this experiment, the Ni content was calculated based on the radioactivity detected. For the high-Ni treatment where stable Ni was introduced, we recalculated the Ni radioactivity, using the conversion $M = S * (N + 1)$ (Shi and Wang, 2004; Yu and Wang, 2004), where M indicates the total theoretical radioactivity, S stands for the detected radioactivity and N represents the ratio of the stable Ni to the radioactive Ni content added. Based on the above conversion, we could then calculate the absolute content of Ni in the high-Ni treatment.

2.3.2. Distribution of Ni in subcellular compartments and macromolecular components

The radioactivity of each subcellular compartment or macromolecular component was regarded as P, representing the Ni content in each compartment. Meanwhile, the radioactivity of the whole cell was denoted as Q, and thus the ratio of P to Q was used to represent the Ni distribution in different compartments or macromolecular components.

2.3.3. Separation of the cell wall, organelles and soluble substances

The cells, collected in a 1.5 mL tube, were homogenized in freezing Tris–HCl buffer (10 mmol L^{-1}, pH = 7.4) using ultrasonics (cell disruption being >95%). The homogenate was centrifuged at 1000 × *g* for 30 min, and the resulting pellet was regarded as the cell wall. The supernatant was further centrifuged at 65,000 × *g* for 30 min, and this pellet and the supernatant represented the organelles and the soluble substances, respectively (Weigel and Jäger, 1980; He et al., 2008; Wang et al., 2008).

2.3.4. Separation of the macromolecular components

The macromolecular components of cells including the lipids, proteins and amino acid-carbohydrate were prepared based on the methods of Bligh and Dyer (1959) and Kochert et al., (1978). After using prechilled perchloric acid (0.2 mol L^{-1}) to remove inorganic materials and some small molecules from the cells, the mixture was centrifuged and the supernatant was carefully removed. The pellet was broken in a mixture of chloroform-methanol (V/V, 1/1) with the help of a sonicator and extracted three times. The supernatants were pooled as the extracted lipid and air-dried at low temperature. Meanwhile, the pellet was extracted with 6% prechilled TCA twice, and the supernatants of the two centrifugations were pooled to represent the amino acid-carbohydrate with the remaining pellet being proteins.

Radioactivity in the sample was counted in 10 mL of a liquid scintillation cocktail for aqueous and non-aqueous samples (obtained from Sigma), using a Packard Tricarb 4640 liquid scintillation counter with a "wide ^{63}Ni" window. The counting process was routinely conducted for a counting error <5%.

2.4. Statistical tests

The statistical differences between two or three nutrient treatments were processed using an independent-samples *t*-test or one-way ANOVA using SPSS 11.5 software. Significant difference was described at $p < 0.05$.

3. Results

3.1. Influence of N or P concentrations on the uptake of Ni by *P. donghaiense*

The Ni content in algal cells was affected by the Ni concentration in the medium, with higher Ni treatment elevating Ni bioaccumulation in all treatments (Figs. 1–3A). For both high- and low-Ni treatments, the total cellular Ni content increased with the increase of nitrate concentration in the medium (Fig. 1A). However, in the low-Ni treatment, the increase of phosphate concentration had an insignificant influence on the total cellular Ni content, and instead exerted an inhibitory effect in the high-Ni treatment (Fig. 2A). Meanwhile, the cellular Ni content showed some relationship with the nutrient source in the medium in that, for example, at the same N concentration, the Ni content in the cells grown with urea was higher than that with nitrate (Fig. 3A).

Additionally, at the same nutrient concentration, the growth of *P. donghaiense* was inhibited in the high-Ni treatment (with an inhibiting percentage of 5.1–11.7%), by comparison with that in the low-Ni treatment (data not shown). However, based on microscopic observations, the morphology of *P. donghaiense* cells showed no obvious differences between the high- and low-Ni treatments (data not shown).

3.2. Ni distribution in the substructures of algal cells under different nutrient treatments

Regardless of the different nutrient treatments, the majority of Ni was distributed in the soluble substances (>70.0%) after its uptake, but the Ni content in each of the subcellular compartments was dependent on the nutrient concentration in the medium (Figs. 1–3B). Thus, statistical analysis indicated that the percentage of Ni in the substructures correlated with the nitrate concentration, i.e. the percentage of Ni in the cell wall and soluble substances decreased from 11.4 to 7.8%, and from 82.8 to 75.3%, while the

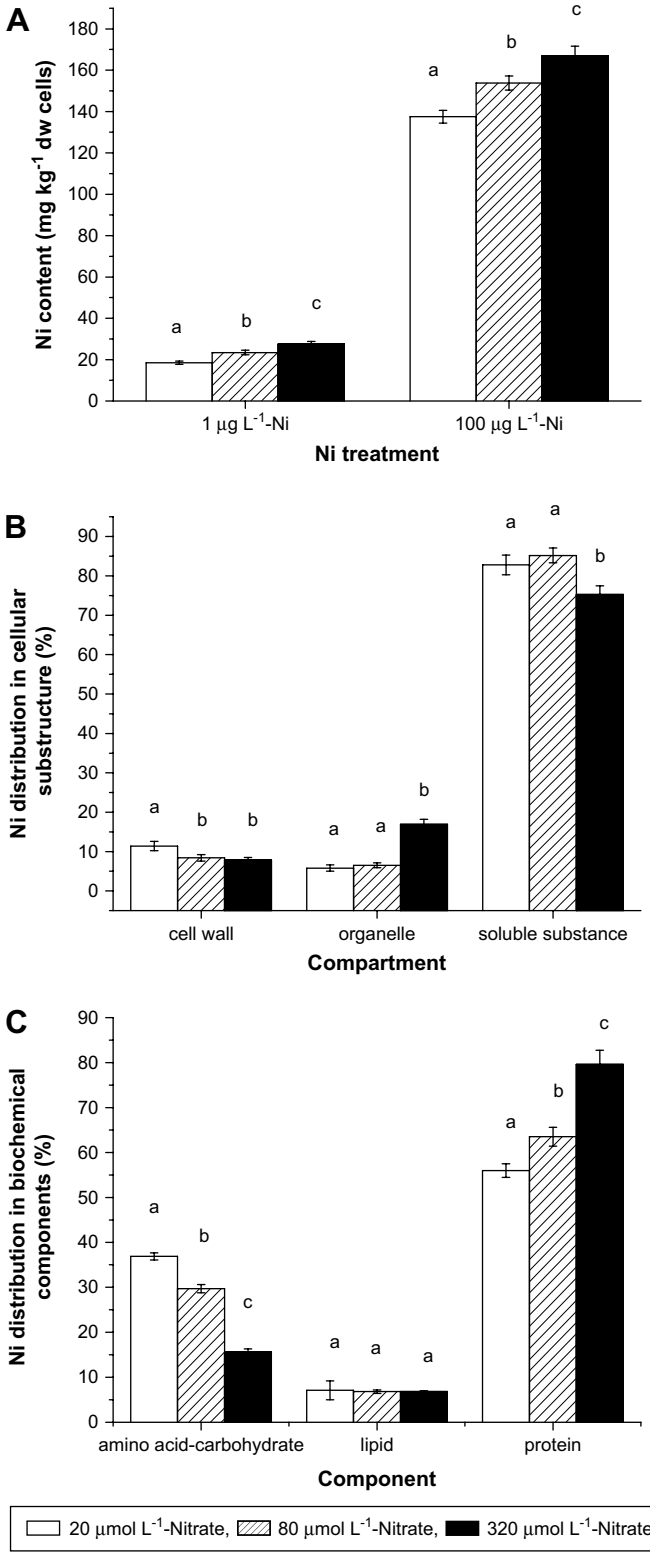

Fig. 1. Influence of different nitrate concentrations (20, 80 and 320 μmol L^{-1}) on Ni uptake by, and its subcellular distribution in, *Prorocentrum donghaiense* Lu under high- and low-Ni treatments. A: Total Ni uptake; B: Ni distribution in different substructures; and C: Ni distribution in different macromolecular components of the algal cells. Data are presented as means ± SD ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

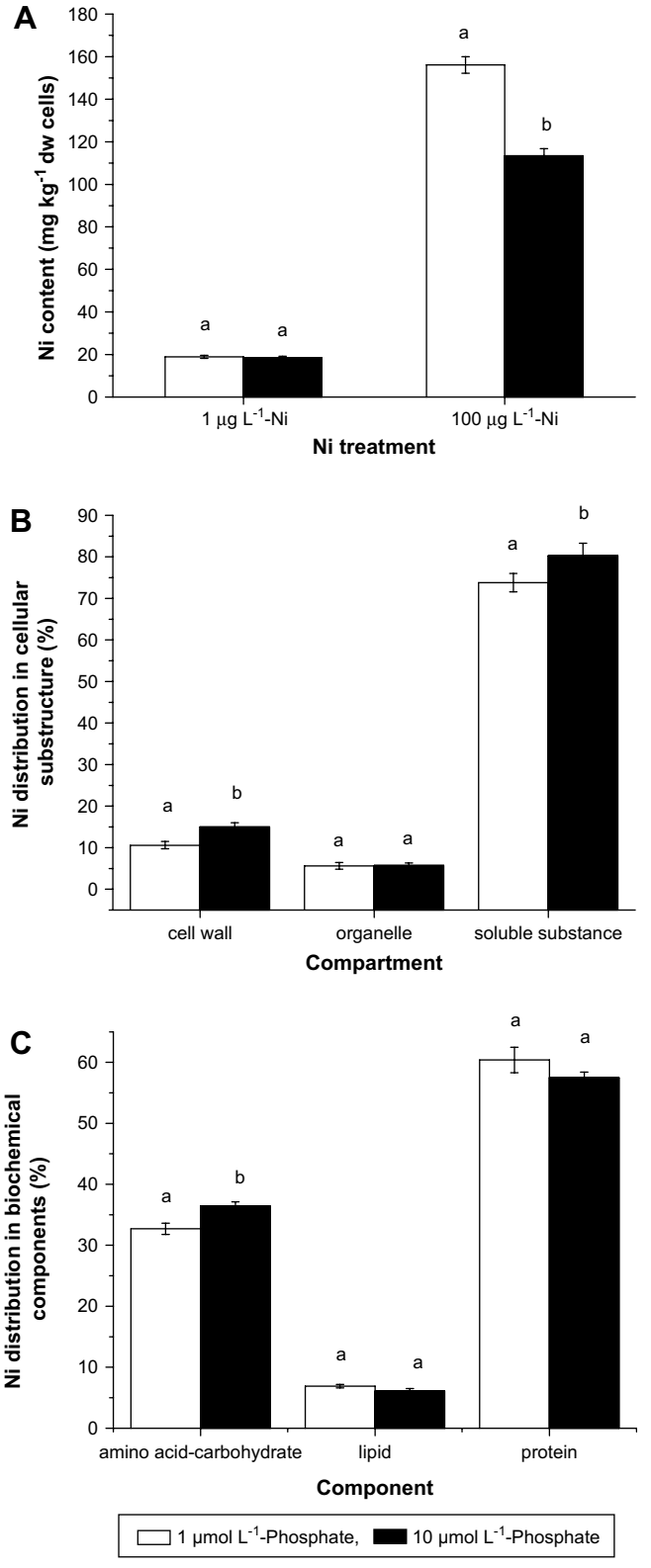

Fig. 2. Influence of different phosphate concentrations (1 and 10 μmol L^{-1}) on Ni uptake by, and its subcellular distribution in, *Prorocentrum donghaiense* Lu under high- and low-Ni treatments. A: Total Ni uptake; B: Ni distribution in different substructures; and C: Ni distribution in different macromolecular components of the algal cells. Data are presented as means ± SD ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

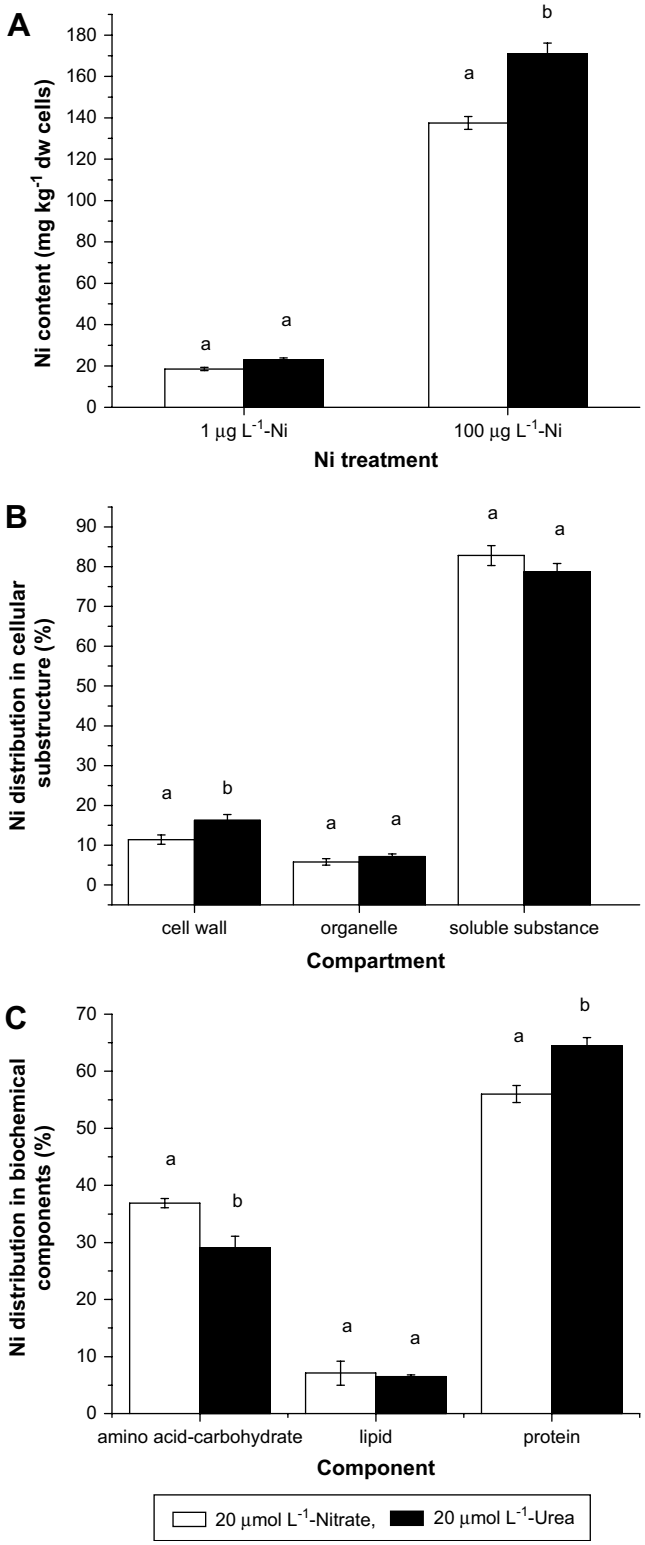

Fig. 3. Influence of different N sources (nitrate and urea) on Ni uptake by, and its subcellular distribution in, *Prorocentrum donghaiense* Lu under high- and low-Ni treatments. A: Total Ni uptake; B: Ni distribution in different substructures; and C: Ni distribution in different macromolecular components of the algal cells. Data are presented as means ± SD ($n = 3$). Different letters indicate significant difference ($p < 0.05$).

organelle-Ni content increased from 5.8 to 16.9% with the increase of the nitrate concentration from 20 μmol L^{-1} to 320 μmol L^{-1} (Fig. 1B). The phosphate concentration in the medium also affected the Ni content in the cell wall and soluble substances, which increased from 10.6 to 15.0% and from 73.8 to 80.3%, with the increase of the P concentration from 1 to 10 μmol L^{-1} (Fig. 2B). Additionally, the Ni content in the cell wall of the cells grown with urea increased 1.3 times, in contrast to that with nitrate at the same N concentration (Fig. 3B), but no significant relationship was observed between the Ni content in the organelles or the soluble substances and the N source (nitrate or urea).

3.3. Ni distribution in the macromolecular components of algal cells under different nutrient treatments

The study showed that the maximum Ni in cells was detected in proteins (55.0–79.6%), and the minimum Ni in lipids, being 6–7% (Figs. 1–3C). Different nutrient treatments influenced Ni distribution in the macromolecular components of the algal cells. For example, the content of protein-Ni increased markedly from 56.0 to 79.6% with an increase of nitrate concentration from 20 to 320 μmol L^{-1} (Fig. 1C), but the Ni content in amino acid-carbohydrate significantly decreased with the increase of nitrate concentration (Fig. 1C). Conversely, increased phosphate addition enhanced the content of Ni in the amino acid-carbohydrate fraction, though the lipid- and protein-Ni content were both unrelated to the P concentration (Fig. 2C). Furthermore, the content of protein-Ni was correlated with the N source, and the protein-Ni content was 14.3% higher when grown with urea than when grown with nitrate at the same concentration (Fig. 3C). However, urea decreased the Ni content in the amino acid-carbohydrate fraction compared to nitrate (Fig. 3C). The content of Ni in lipids was independent of the three different nutrient treatments (Figs. 1–3C). Interestingly, the Ni content of protein was correlated well with the content of proteins in the cells (Fig. 4).

4. Discussion

This study clearly demonstrated that the ambient nitrate concentration significantly affected the intracellular bioaccumulation of Ni by *P. donghaiense*, with high nitrate concentrations enhancing the uptake of Ni (Fig. 1A). This result is consistent with the previous studies which show that a high nitrate

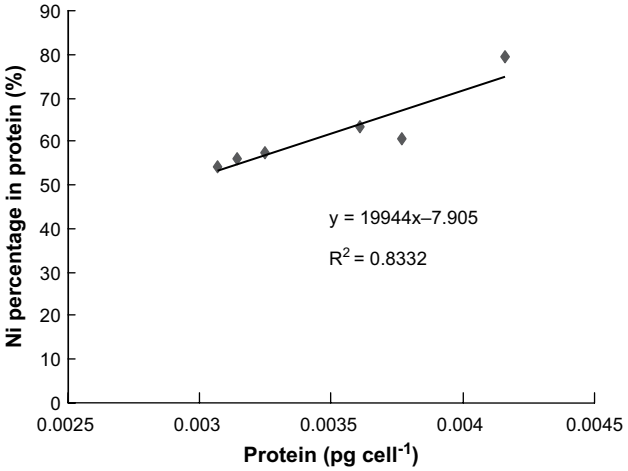

Fig. 4. Relationship between the Ni content in the protein fraction and the protein content of *Prorocentrum donghaiense* Lu cells.

concentration increases the uptake of Fe, Zn, Cd and Ni by phytoplankton (Wang and Dei, 2001a, b, c; Wang et al., 2007). In particular, Wang et al. (2007) illustrate that higher nitrate or phosphate concentration increases the intracellular bioaccumulation of Ni by two phytoplankton species, and facilitates its assimilation by copepods, hence improving its transfer from phytoplankton to copepods. Therefore, high nitrate concentration might improve the production of proteins, especially those transporting proteins in the cell wall, thereby elevating the Ni uptake. In fact, a previous study suggests that N-enrichment stimulates the synthesis of glutathione, which might respond to metal detoxification, and it also shows that there is more cellular Cu, Zn and Mn in diatom cells after 14 days of batch culture with N-addition (Rijstenbil et al., 1998). Meanwhile, we found that high phosphate concentration exerted little effect on Ni uptake by *P. donghaiense* under low-Ni treatment, but Ni uptake was unexpectedly inhibited under high-Ni treatment (Fig. 2A). However, other studies show that an increase in ambient phosphate concentrations significantly facilitates the uptake of Cd, Zn, Cr and Ni in algae (Yu and Wang, 2004; Lee and Wang, 2001; Wang et al., 2007). Skaar et al. (1974) also reveal that phosphate addition significantly improves the Ni-binding capability of phosphate-starved diatom cells, due to phosphate being involved in the synthesis of the Ni-binding system of the cells. In this study, higher phosphate concentration reduced Ni uptake by the cells under high-Ni treatment, and the possible reason might be that phosphate is responsible for metal detoxification in the phytoplankton. A previous study on maize mitochondria reveals that the addition of Pb followed by phosphate results in fewer enzymatic inhibitions than if Pb alone is introduced to mitochondrial suspensions (Koeppe and Miller, 1970). They then speculate that the reduction of Pb toxicity is attributable to phosphate precipitation by Pb. However, Sicko-Goad and Stoermer (1979) report that phosphate may protect the algal cells to some extent from Pb toxicity by reducing the amount of Pb available to intracellular sites through incorporation into polyphosphate in an energy-requiring reaction, which is different from Pb-phosphate precipitation. A further challenge lies in investigating the exact interaction between phosphate and trace metals in phytoplankton at the biochemical and cellular level, i.e. metal transport systems.

Interestingly, our study revealed that urea facilitated the Ni uptake by *P. donghaiense* compared to nitrate at the same N concentration (especially under high-Ni treatment). The possible reason might be that, because the sole N source was urea, the alga had to synthesize more urease for the utilization of urea, and consequently this facilitated the uptake of Ni by the cells due to urease being a Ni-dependent enzyme. Gerendás and Sattelmacher (1999) show that Ni addition markedly enhances the dry matter production of urea-grown plants, but its deficiency significantly reduces the urease activity in the leaves and roots of plants. A previous study demonstrates that an addition of the urease inhibitor phenylphosphoro–diamidate to foliar-applied urea elevates leaf tip necrosis and increases the urea content, but decreases ammonia levels and urease activity in soybean leaves (Krogmeier et al., 1989). Furthermore, the increased urea levels in necrotic areas suggests that the leaf tip necrosis results from accumulation of excess urea rather than from the formation of excess ammonia (Krogmeier et al., 1989). Thus, *P. donghaiense* might synthesize more urease because of the accumulation of excess urea, which in turn might elevate the Ni uptake by cells. In addition, it should be pointed out that although the effects of nitrate concentration on Ni uptake were significant, they seemed relatively small in comparison with the effect of urea versus nitrate on Ni uptake under high-Ni treatment.

The present study indicated that Ni distribution in the subcellular structures of *P. donghaiense* cells was affected by different nutrient status. Thus, with an increase in nitrate concentration, the Ni content in the cell wall and soluble substances tended to decrease, but that in the organelles showed a tendency to increase (Fig. 1B). However, the increase of phosphate level increased the Ni content in the cell wall and soluble substances, while showing insignificant impact in the organelles (Fig. 1B). Thus, the increasing nitrate concentration might cause the algal cells to suffer more from metal toxicity, but phosphate addition could show a protecting effect, since metal internalization in the cell wall could act on the protective mechanism and the organelle is usually sensitive to metal attack. Neumann et al. (1995) show that the cell wall can accumulate heavy metals and prevent metal attack of sensitive sites (e.g. the cytosol), hence displaying a protective effect. Zenk (1996) and Hall (2002) also suggest that besides the portion of Cd being present in the cell wall, most accumulates in the vacuole to protect the cells from toxicity, which also exemplifies the function of the cell wall in protecting the protoplast from metal attack. In this study, despite the Ni content in the cell wall being 7.8–14.2%, the cell wall could, to some extent, protect the algal cells from metal contamination. Additionally, different N sources affected the Ni distribution in the cell wall, but showed no relationship with that in the organelles and soluble substances, i.e. the algal cells grown with urea accumulated more Ni than that grown with nitrate at the same concentration (Fig. 3B). It is well known that urease is commonly located in the algal cell wall (Molina et al., 1993; Millanes et al., 2004), although few studies have shown that this happens in phytoplankton. Thus, more Ni distribution in the cell wall under urea treatment versus nitrate treatment might seem acceptable, due to the Ni-dependent urease being responsible for urea utilization.

Similarly, the results indicated that Ni distribution in the macromolecular components of the cells correlated with the ambient nutrients (Figs. 1–3C). With the increase of nitrate concentration, the Ni content in amino acid-carbohydrate decreased markedly, but the protein-Ni content increased significantly. Thus, nitrate enrichment might have stimulated the synthesis of proteins, especially the detoxifying ligands (e.g. glutathione and phytochelatin), and consequently facilitated Ni accumulation in proteins, hence reducing the Ni concentration in amino acid-carbohydrate accordingly. Previous studies show that photoautotrophs have universally developed an internal detoxifying mechanism, i.e. the protection function is undertaken by the chelation of metal to proteins with abundant sulphydryls (e.g. GSH, MT and phytochelatin) (Kotrba et al., 1999; Cobbett, 2000; Mendoza-Cózatl et al., 2005). The fact that the cells with urea obviously improved Ni accumulation in proteins but had a reduction of the Ni content in amino acid-carbohydrate by contrast to nitrate (Fig. 3C), testified again that the algal cells might have to accumulate more Ni to yield urease for the hydrolysis of urea. However, phosphate addition only affected the Ni distribution in the amino acid-carbohydrate fraction. Simultaneously, the content of Ni in lipids ($<$7.5% in all treatments) was independent of different nutrient treatments, hinting that lipids might not be involved in the detoxifying process of Ni by the cells (Figs. 1–3C).

The present study clearly showed that, regardless of different nutrient treatments, the majority of Ni was distributed in the soluble substances or the protein fraction. Furthermore, the Ni content of the proteins was correlated with the protein concentration in the algal cells (Fig. 4). Consequently, the alga might have induced the production of the protein-SH (e.g. GSH and phytochelatin) to resist Ni toxicity. A previous study on copper distribution in the diatom *Haslea ostrearia* Simonsen (Joux-Arab et al., 1998) reveals that, in controls, insoluble compounds are predominant (63%) versus soluble compounds, whereas in contaminated cells soluble compounds become predominant (66%). Moreover, the same authors show that in the cytosol of both control and contaminated cells, copper is mainly associated with

thermoresistant compounds (about 80%), which might correlate with the detoxifying mechanism. In addition, the fact that most of the Ni is distributed in the soluble substance or proteins of the algal cells might imply that this algal-bound Ni could be easily assimilated by the predator (e.g. copepods) and subsequently tends to be transferred along the food chain. Several researchers find that copepods only assimilate the cytoplasmic pool of intracellular metal storage in the algal cells (Reinfelder and Fisher, 1991; Hutchins et al., 1995). A previous study, investigating the bioavailability of Cd sequestered within the oligochaete *Limnodrilus hoffmeisteri* by feeding ^{109}Cd contaminated oligochaetes to the shrimps, also reveals that the shrimp can assimilate 57% of the total cadmium in the oligochaetes and the majority of the assimilated metal comes from the fraction containing protein (Wallace and Lopez, 1997). Therefore, it could be supposed that the dietary Ni in *P. donghaiense* might easily show bioavailability to predators (e.g. copepods). In fact, Wang et al. (2007) indicate that the content of intracellular Ni in the algal cells is evidently correlated with the metal assimilation efficiency by copepods.

5. Conclusions

Our novel results demonstrated that the ambient nutrients (e.g. nitrate and phosphate) affected the uptake of Ni by *P. donghaiense*. Meanwhile, different nutrient treatments exerted notable effects on Ni distribution in the substructures and macromolecular components of the algal cells. In particular, urea in the medium notably enhanced the Ni uptake and its content in the protein fraction of the cells compared to nitrate at the same N concentration. Furthermore, the fact that the majority of Ni occurred in the soluble substances and proteins suggested that this could be the transfer-available fraction along the food chain. Thus, a further subject of investigation would be to examine in detail the interaction between the subcellular Ni distribution in the algal cells and its transference along the food chain. Also, more attention should be given to studying the exact role of urea in Ni uptake by the phytoplankton and its subsequent impact on the food chain.

Acknowledgements

The authors thank Prof. John Hodgkiss for helping to revise the manuscript. The work was funded by the Ministry of Science and Technology (No. 2005DFA20430), the National Natural Science Foundation Committee and State Bureau of Hong Kong (No. 40131160735), and Fujian Provincial Department of Science and Technology (No. 200691).

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