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Effects of cobalt and vitamin B_{12} additions on the growth of two phytoplankton species

Ana Z. SEGATTO*, Edna GRANÉLI* and Conny HARALDSSON**

Abstract: The effect of cobalt on the growth rate/biomass accumulation of a diatom, *Ditylum brightwellii* Bailey and a dinoflagellate, *Prorocentrum minimum* (Pavillard) Schiller was investigated. Experiments were performed under laboratory conditions in polycarbonate flasks with diluted (26 ‰), sterilized, and nutrient enriched sub-surface Atlantic water as growth media. Cobalt was added in concentrations between 0.5 and 3 nM, which corresponds to Co concentrations found in oligotrophic oceans and coastal waters of the Kattegat and the Skagerrak. Cell densities and chlorophyll *a* concentrations were monitored daily during the course of the experiment. *P. minimum* growth rate and biomass accumulation were not affected by cobalt additions, while *D. brightwellii* had both its growth rate and biomass accumulation inhibited by cobalt additions.

1. Introduction

As essential cofactors in metalloenzymes trace metals can influence the metabolism of algal cells (SUNDA, 1988 – 1989). Different phytoplankton species have different requirements for, and sensitivities to, trace metals (BRAND *et al.*, 1983; BRAND, 1991). Laboratory studies (SUNDA *et al.*, 1981; RUETER and MOREL, 1981) and field studies (MARTIN *et al.*, 1989; MARTIN and GORDON, 1988) have indicated that trace metals may affect not only algal growth but also the primary production, species composition and the trophic structure of phytoplankton communities.

Cobalt is one of the most important limiting trace metals for algal growth, since it is needed for the synthesis of vitamin B_{12}. It has been suggested that the synthesis of this vitamin can be limited by the amount of cobalt in the environment (SANGFORS, 1988; SWIFT, 1980).

Cobalt can stimulate nitrogen fixation and growth rates of blue-green algae (HOLM-HANSEN *et al.*, 1954; SAUBERT and STRIJDOM, 1968). Recently it has been found that cobalt and cadmium can substitute for zinc, another limiting trace element, promoving the growth of the diatom *Thalassiosira weissfloggii* (PRICE and MOREL, 1990). GRANÉLI and RISINGER (1994) have recently shown that cobalt (as cobaltacetate or vitamin B_{12}) favours the biomass accumulation of the toxic flagellate *C. polylepis.*

The aim of this experimental study was to test the effect of different additions of cobalt and vitamin B_{12} on two coastal phytoplankton species : the diatom *Ditylum brightwellii* and the dinoflagellate *Prorocentrum minimum.*

2. Material and methods

The experiments were performed at laboratory conditions (temperature of 15 ℃ ; 16 : 8 hours light : dark cycle) with monocultures of the dinoflagellate *P. minimum* and of the diatom *D. brightwellii.*

Light intensity was measured with a QSL-100 spherical quantameter (Biospherical Instruments Inc.) and was approximately 120 $\mu E\ m^{-2}\ s^{-1}$ (Philips cool-white fluorescent tubes) for the diatom and 90 $\mu E\ m^{-2} s^{-1}$ for the dinoflagellate.

Surface water (≈ 35.0 ‰) was collected in the Northern Atlantic (61° 11′ 14″N – 45° 00′ W), and contained low cobalt concentration (≈ 0.02 nM). In the laboratory the water was prefiltered through a GF/C filter and diluted to 26.0

* Department of Marine Ecology, Ecology Building, University of Lund, 223 62, Lund, Sweden
** Department of Analitical and Marine Chemistry, University of Gothenburg, 412 96, Gothenburg, Sweden

‰ with ultraclean water (obtained from a Milli-Q purification system) in order to maintain the same salinity as that at which these algae had been kept in cultures. The initial concentrations of dissolved inorganic nutrients in the Atlantic water were 0.11 μM of nitrate; 0.1 μM of phosphate; 0.28 μM of ammonia and 2.74 μM of silicate. After autoclaving in 20 *l* Teflon bottles, the water was transferred to 1 l polycarbonate flasks, which had been pretreated for one week with a 1 : 1 mixture of 1.0 M HCl and 1.0 M HNO_3 (Merck, Suprapure), followed by two weeks soaking in a weaker acid mixture.

D. brightwellii and *P. minimum* were first cultured in full f/2 medium (GUILLARD and RYTHER, 1962), including cobalt (at concentrations of 5×10^{-8} M). Nutrient stock solutions were pretreated in a chelating ion exchange resin (Chelex 100) column in order to eliminate possible contaminations by metals (MOREL *et al.*, 1979; PRICE *et al.*, 1988–1989). During the exponential phase of growth, we made sequential transfers of algae to a cobalt-free medium and phosphorus (0.5 μM as Na_2HPO_4), and nitrogen (5 μM as $NaNO_3$) were added to all bottles while silicon (5 μM as Na_2SiO_3, $5H_2O$) was added only to the diatom *D. brightwellii* cultures.

Different cobalt treatments were tested : additions of cobalt (as cobaltacetate, $(CH_3COO)_2Co.4H_2O$) alone or together with 500 nM of the chelator EDTA (added as the disodium salt) and vitamin B_{12} (as $C_{63}H_{88}CoN_{14}O_{15}P$), both in the range 0.5 to 3.0 nM. The different added vitamin B_{12} concentrations were calculated in order to have the same amount of inorganic cobalt added as for the treatment with cobaltacetate. Four replicates were made for each treatment, making a total of 64 bottles. The reason to choose this range of cobalt concentrations was that we would like to compare our results with the results found by GRANÉLI and RISINGER (1994) on the effects of cobalt on the toxic blooming flagellate *C. polylepis*.

Phytoplankton biomass in bottles was estimated as *in vivo* chlorophyll flurescence using a Turner 112 filter fluorometer and transformed to absolute chlorophyll *a* values (as μg l^{-1}) after spectrophotometric analysis of extracted chlorophyll *a* (according to JEFFREY and HUMPHREY, 1975) during certain days of the experiment. Daily phytoplankton samples were preserved with acidified Lugol's solution and cell counting was performed using Palmer Maloney Chambers in an inverted Nikon TMD Diaphot microscope.

Growth rates based on cell densities ware calculated for every bottle during the exponential phase of growth. Growth rates (div. day^{-1}) were calculated according to the formula: div. $day^{-1} = (\log_2 N - \log_2 N_0)/t$, where N_0 = cell numbers on Day 3; N = cell numbers on Day 9 and t = 7 days.

Samples for cobalt analyses in the growth media were collected at the beggining of the experiment (Days 1 and 4 for the dinoflagellate and the diatom, respectively); during the exponential growth phase (Days 11 and 16 for the dinoflagellate and Days 6 and 8 for the diatom) and at the end of the experiments (Days 48 and 17 for the dinoflagellate and the diatom, respectively). One hundred ml of water was then filtered (0.45 μm acid-washed Whatmann GF/C filter) through an acid-cleaned filtration system, in a laminar-flow sterile bench in order to avoid bacterial and/or metal contamination. Measurements of the total dissolved cobalt concentrations in the medium were carried out using the method of DANIELSSON *et al.* (1982). The method consists of complexing the total amount of cobalt present in the sample with a complex agent, such as carbamate. Afterwards the Co-dithiocarbamate complexes are then extracted with an organic solvent (Freon-TF) and finally back-extracted, i.e. extracted from the organic solvent with nitric acid, to an aqueous solution. The total amount of Co present in the solution is then measured.

3. Results

Cobalt concentrations in the initial medium and during the exponential growth phase for the treatments with additions of Co (as cobaltacetate) are shown in Table 1. The results indicate that there was an uptake of cobalt by *D. brightwellii* during the exponential growth phase (on Days 6 and 8 for the control and the treatments with 0.5 and 3.0 nM of added Co) and by *P. minimum* (on Days 11 and

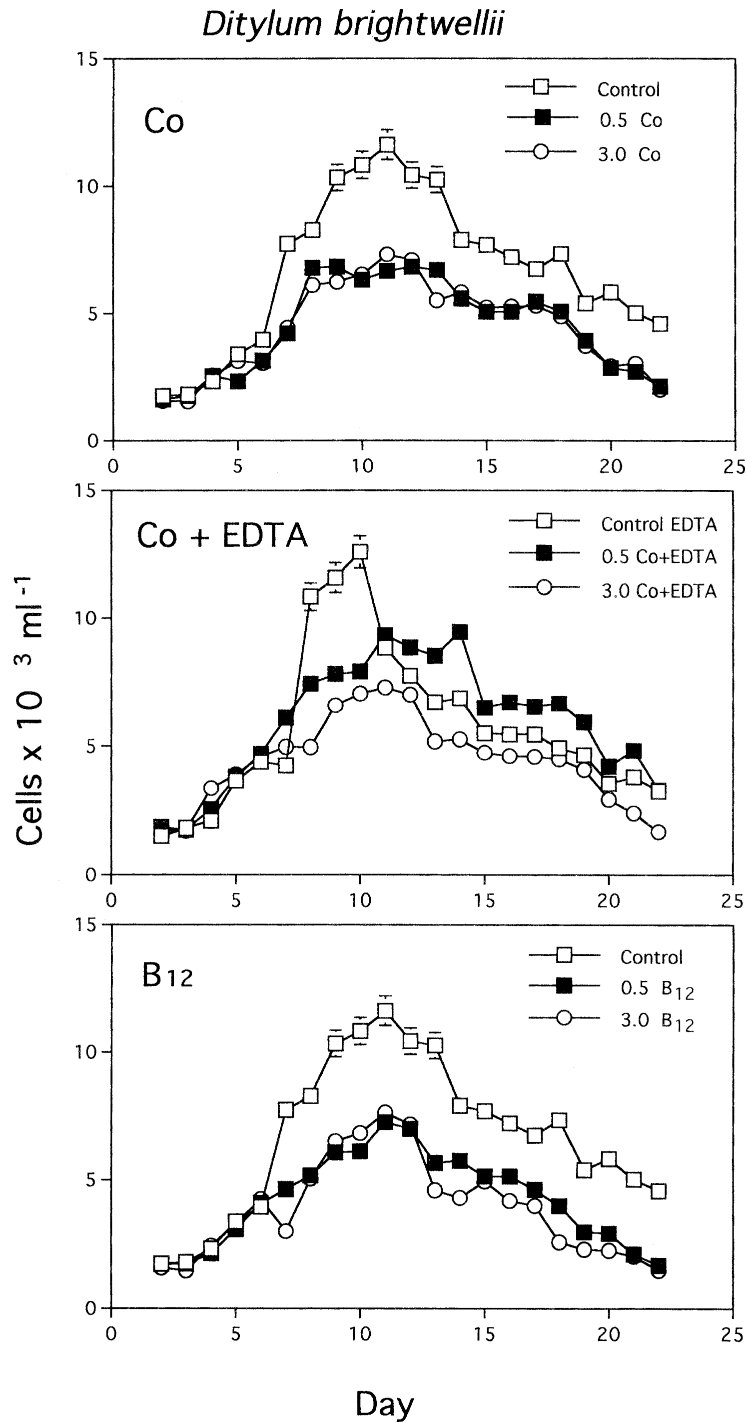

Fig. 1. Effects of different cobalt additions (as Co; Co+EDTA and vitamin B_{12}) on the cell densities of *Ditylum brightwellii*. Control: no Co or vitamin B_{12} additions.

Table 1. Specific growth rates (doublings per day, μ) from day 3 to day 9.
Values are mean of 4 replicates ± standard deviation.
The Mean-Whitney U test was used in order to compare the growth rates in the controls with the different treatments.

Treatment	*D. brightwellii*	*P. minimum*
Control	0.25±0.05	0.15±0.00
0.5 nM Co	0.19±0.01*	0.20±0.01*
3.0 nM Co	0.18±0.03*	0.19±0.01*
Con+EDTA	0.24±0.02	0.18±0.02
0.5 Co+EDTA	0.20±0.02	0.19±0.00
3.0 Co+EDTA	0.17±0.01*	0.22±0.02*
Control B12	0.25±0.05	0.16±0.01
0.5 nM B12	0.17±0.00*	0.19±0.02
3.0 nM B12	0.19±0.02*	0.20±0.01*

*$p<0.05$

16 for treatments with 3.0 nM Co additions and in the control flasks).

The growth response of the two phytoplankton species to cobalt additions were different. Maximal cell numbers of the diatom *D. brightwellii* (Fig. 1) were reached for the controls (no Co or B_{12} added) of cobalt and vitamin B_{12} additions, and also the controls with additions of only EDTA. There was a significant ($p<0.05$, Mann-Whitney U-test) increase of cells in the control treatments of *D. brightwellii* compared with the treatments with 0.5 and 3.0 nM of added cobalt (as cobaltacetate or as vitamin B_{12}). *D. brightwellii* reached the maximal biomass accumulation (12 $\mu g\ l^{-1}$ of chlorophyll *a*) during the stationary growth phase, for the controls of Co and vitamin B_{12} additions. For the other treatments, maximal chlorophyll *a* values were less than 9 $\mu g\ l^{-1}$. Maximal chlorophyll *a* concentrations were significantly higher in the controls than in the treatments with additions of 0.5 and 3 nM of Co (Fig. 2; $p<0.05$, Mann-Whitney U-test). The chlorophyll accumulation of the diatom in the treatment with additions of 0.5 and 3.0 nM of cobalt together with EDTA was significantly higher compared with additions of 0.5 and 3.0 nM of cobalt alone (as a salt or as vitamin B_{12}).

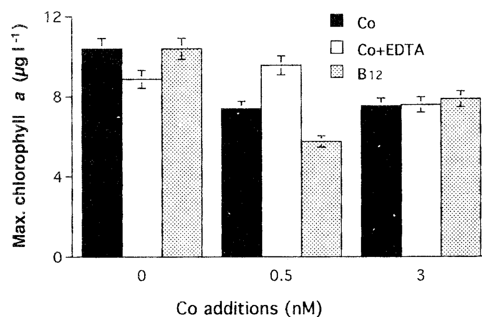

Fig. 2. Maximal biomass accumulation (expressed as chlorophyll *a* concentrations) of *Ditylum brightwellii* grown at different additions of Co, Co+EDTA and vitamin B_{12}. The initial chlorophyll *a* concentration just after the inoculation (Day 1) was 2 $\mu g\ l^{-1}$.

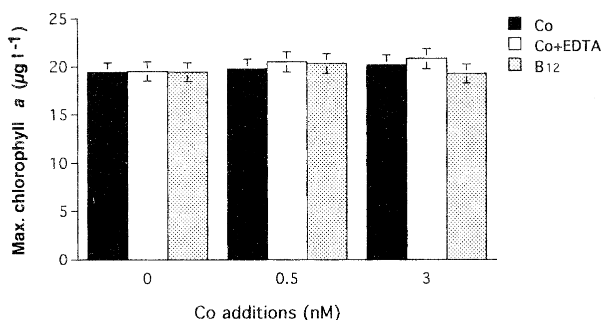

Fig. 4. Maximal biomass accumulation (expressed as chlorophyll *a* concentrations) of *Prorocentrum minimum* grown at different additions of Co, Co+EDTA and vitamin B_{12}. The initial chlorophyll *a* concentration just after the inoculation (Day 1) was 4 $\mu g\ l^{-1}$.

For *P. minimum* (Fig. 3) the effect of adding Co as a salt, with EDTA or as vitamin B_{12} were similar. There was a lag phase of approximately 6 days, whereafter exponential growth started. Maximal cell densities were reached after approximately 16 days. The dinoflagellate was maintained for a long time in the stationary growth phase (almost 30 days), and maximal cell densities reached approximately 20×10^3 cells ml^{-1}. No significant differences in cell numbers were observed between the different treatments ($p<0.05$, Mann-Whitney U-test). Just after the stationary phase, cell numbers of the dinoflagellate were significantly higher in the treatments with Co+EDTA additions compared with the treatments to which we have added Co (as cobaltacetate or as vitamin B_{12}). The maximal chlorophyll *a* accumulation (20

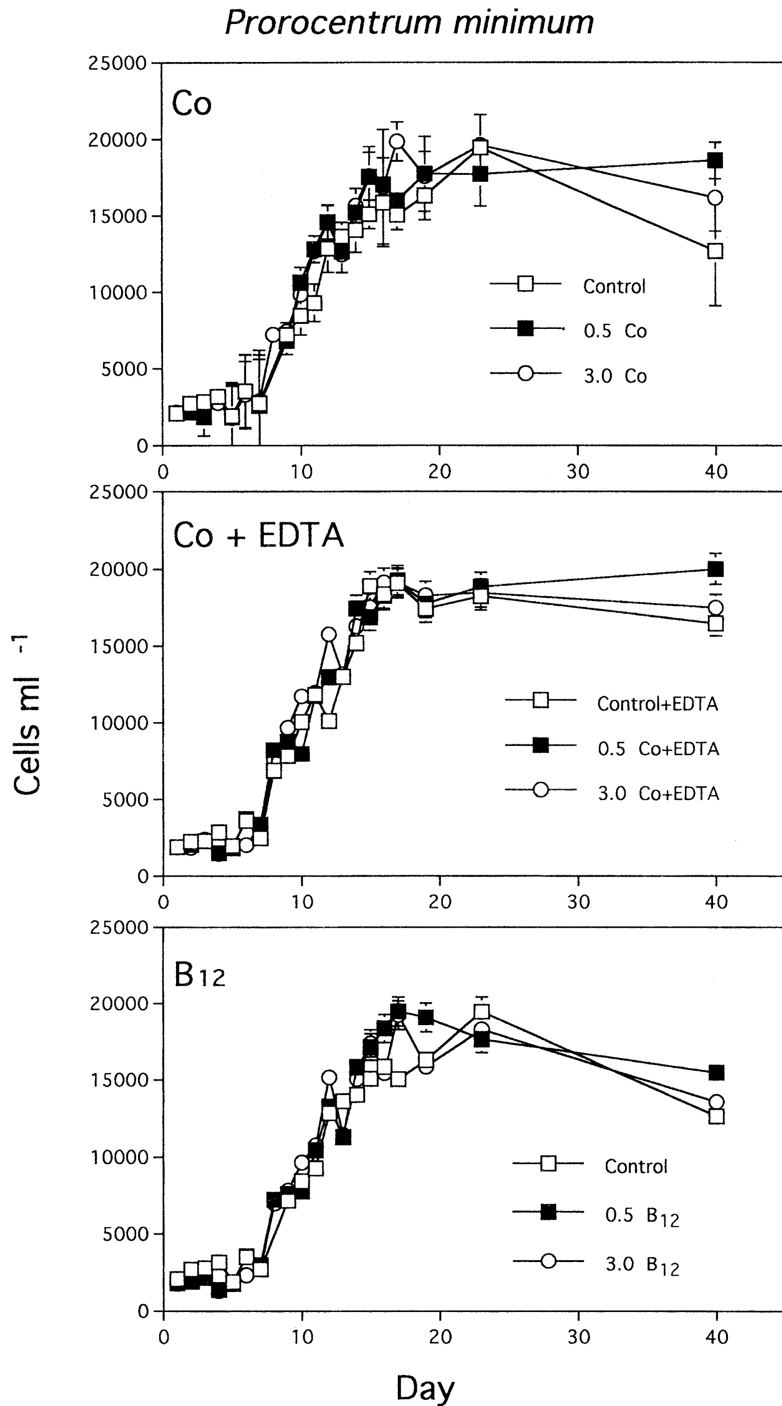

Fig. 3. Effects of different cobalt additions (as Co; Co+EDTA and vitamin B_{12}) on the cell densities of *Prorocentrum minimum*. Control: no Co or B_{12} additions.

Table 2. Cobalt concentrations (in nM) in the medium for the treatment with no Co additions (Control) and cobalt added at concentrations of 0.5 and 3.0 nM. The initial concentration of Co in Atlantic water was 0.02 nM.

Days	Control	0.5 Co	3.0 Co
D. brightwellii			
4	0.07	0.41	2.72
6	0.04	0.43	1.72
8	0.02	0.24	0.73
P. minimum			
4	0.08	0.32	1.72
11	0.05	0.45	1.33
16	0.04	0.47	0.54

$\mu g\ l^{-1}$) was not affected by the different additions and no significant differences in chlorophyll *a* concentrations were observed among the treatments (Fig. 4).

Growth rates based on cell numbers were calculated for the dinoflagellate and for the diatom during the exponential phase of growth according to the following formula: $\mu = (\log_2 N - \log_2 N_0)/t$, where N_0 represents the total number of cells on Day 3, N is the cell number on Day 9 and t=6 days.

Growth rates for the diatom *D. brightwellii* (Table 2) were significantly higher ($p < 0.05$, Mann-Whitney U-test) in the controls than in the treatments where cobalt (as a salt) was added in concentrations of 0.5 nM and 3.0 nM. The same tendency was observed for the treatment with additions of cobalt as vitamin B_{12}. The controls of the treatment with additions of cobalt together with EDTA were only significantly higher than that treatment with 3.0 nM of cobalt added together with EDTA.

Growth rates for the dinoflagellate *P. minimum* (Table 1) were significantly lower ($p < 0.05$, Mann-Whitney U-test) in the controls than in the treatments with 0.5 and 3.0 nM of cobalt additions. For the different treatments of cobalt+EDTA and vitamin B_{12} additions, the growth rates were only significantly higher ($p < 0.05$, Mann-Whitney U-test) when 3.0 nM of cobalt was added together with EDTA and 3.0 nM added vitamin B_{12} treatment.

GRANÉLI and RISINGER (1994) have studied the effects of additions of cobalt (alone or

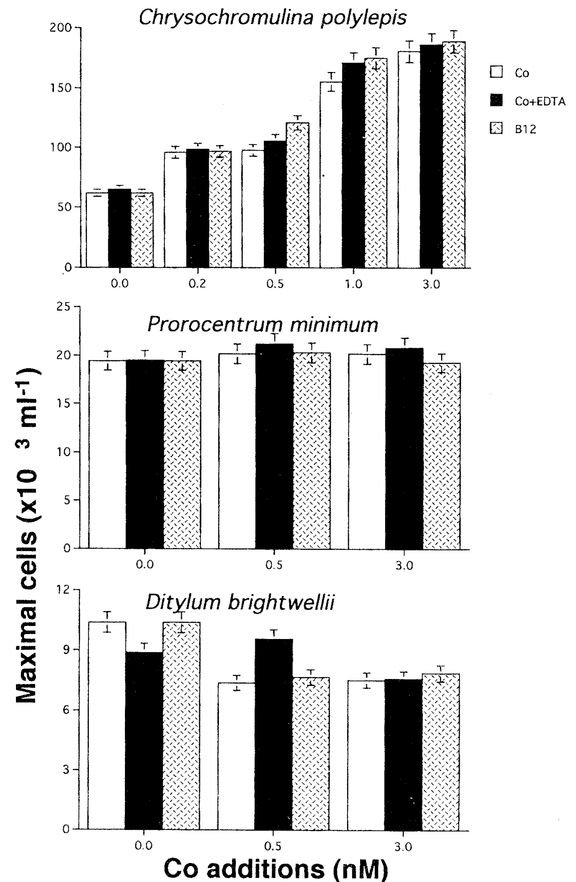

Fig. 5. Maximal cell densities for the cultures of *C. polylepis*, *P. minimum* and *D. brightwellii* grown at different additions of Co, Co+EDTA and vitamin B_{12}. C. polylepis figure is redrawn from GRANÉLI and HARALDSSON (1993).

together with EDTA or as B_{12}) on the toxic prymnesiophycean flagellate *Chrysochromulina polylepis*. The authors have shown that cell numbers of *C. polylepis* increased more or less linearly with additions of cobalt increased from 0 to 1.0 nM. An addition of 3.0 nM of cobalt only increased cell number accumulation slightly over the cell numbers produced at the 1.0 nM addition.

Comparing to our study (Fig. 5), the response of the three algae to the different cobalt additions was not similar. It was observed that not only cell densities were higher compared to the other two algae, but also their chlorophyll accumulation and growth rates as well (see also GRANÉLI and HARALDSSON, 1993).

4. Discussion

Cobalt (as cobaltacetate or vitamin B_{12}) favours the biomass accumulation of the prymnesiophycean flagellate *C. polylepis* (GRANÉLI and RISINGER, 1994). In that experiment cobalt concentrations up to 1.0 nM increased significantly the cell yields of the flagellate. In addition, the produced maximal cell numbers of *C. polylepis* increased proportionally to increasing cobalt (added as cobaltacetate or as vitamin B_{12}) levels.

Our results in the present study show that the two tested species were affected in different ways by cobalt additions. The cell yield and chlorophyll accumulation of the diatom *D. brightwellii* were inhibited by the three different forms of added cobalt, while the cell yield and chlorophyll accumulation of the dinoflagellate *P. minimum* were not affected by any of the three different forms of cobalt additions.

For both tested species, cobalt (as cobaltacetate) concentrations were lower in water filtered from samples in the middle or at the end of the experiments compared to samples taken at the beginning (Table 1), indicating that both algal species were able to take up cobalt from the media, independently of the treatment. Cobalt concentrations in the treatments where originally 0.5 and 3.0 nM of cobalt was added, decreased respectively 42 % and 73 % from Day 4 to Day 8 for the diatom. For *P. minimum*, a slight increase in cobalt concentrations was observed in the filtrates from Day 1 to Day 16 in treatments where 0.5 nM cobalt was originally added. However, a decrease of app. 69 % was observed in treatments with original additions of 3.0 nM of cobalt. We do not have data on the uptake rates for the two tested species, but some studies have shown that the uptake of two radioactive forms of cobalt, ($^{60}CoCl_2$ and ^{57}Co-cobalamine) by diatoms is very and essentially complete after 30 hours (NOLAN *et al.*, 1992). According to FISHER *et al.* (1983) this is supposed to be a typical pattern of accumulation of metal ions by phytoplankton. PRICE and MOREL (1990) have shown that cobalt stimulated the growth rates of the diatom *Thalassiosira weissflogii* after the third day of incubation. In addition, some authors (ZHOU and WANGERSKY, 1989) have also considered the importance of the dissolved organic matter (DOM) released by phytoplankton during the exponential and stationaly phases of growth in cultures. DOM is able to chelate trace metals, including cobalt (ZHANG *et al.*, 1990), and form complexes which can be retained by 0.45 μm membrane filters. In our experiments, there is also a possibility that cobalt may have been bound to cells or to DOM released by cells, during the exponential and stationary phases. Thus, the total cobalt concentrations in the filtrates could have been understimated. But, to what extent these extracellular metabolites exuded by phytoplankton may influence the medium chemistry and trace elements concentration, is still uncertain.

A chelating agent, in general, has two main functions in algal culture medium. It either keeps trace metals soluble and thus available to algae, or it reduces the toxicity of some metals, such as copper. EDTA is known as the most widely used organic synthetic chelator in algal cultures, and was used in our experiments as a chelator for cobalt. We added 5×10^{-7} M of EDTA together with different cobalt concentrations. This EDTA concentration is in the range of that suggested by JOHNSTON (1964), who recommended a use of at least 10^{-7} M of EDTA to support algal growth. In our studies, as expecting, the biomass accumulation (expressed as cell yield or as chlorophyll *a* concentrations) of the diatom was significantly higher in the treatments to which cobalt was added with EDTA than in the treatment with only cobalt (added as cobaltacetate or vitamin B_{12}). We believe that the added concentrations of EDTA was enough to alleviate the toxic effect of cobalt on the growth of the diatom. *P. minimum* did not have its maximal cell yield significantly affected neither by increasing inorganic cobalt or vitamin B_{12} additions, nor by adding cobalt together with EDTA.

It has been shown that additions of high concentrations (up to 10^{-5} M) of strong chelating agents, such as EDTA, may decrease phytoplankton growth rates and reduce the concentrations of free metal ions in solutions to very low levels (JACKSON and MORGAN, 1978). Trace metal bioavailability to phytoplankton may be

determined not only by the concentration of the different chemical species of the metal but also by the relative concentration of cells and chelators in the medium (PRICE *et al.*, 1988 1989). The relatively low growth rates for the two tested species during our experiments (less than 0.25 doublings day^{-1}, for all the treatments), can not entirely have been due to a possible toxic effect of cobalt or EDTA when they were added alone.

Problems concerning sampling procedures in the field and in laboratory studies, as well as contamination of samples, are known to have introduced significant errors in studies of phytoplankton-trace metal interactions. We have chosen to use North Atlantic offshore water in our experiments, as it contained only 0.02 nM of cobalt. Due to the great volume of water required for the experiments we have autoclaved the Atlantic water using a standard laboratory autoclave, which is known as a source of contamination for some trace metals (PRICE *et al.*, 1988–1989). In addition, the cobalt concentrations in the control flasks were at the beginning (Day 1) of both experiments 0.08 nM, suggesting that a relative low contamination of about 0.06 nM must have occurred.

Effects of vitamin B_{12} additions on phytoplankton growth

The growth rates and cell densities of the diatom *D. brightwellii* were negatively affected by increasing levels of vitamin B_{12} during the experiment. However, this inhibition is contradictory to the findings of GUILLARD and CASSIE (1968), who stated that *D. brightwellii* is a B_{12}-requiring species. In general, 80 to 90 % of the dinoflagellates are vitamin B_{12}-requirers. *P. micans*, e.g. requires B_{12} and biotin for growth. ACHIHA and IWASAKI (1990) have studied the growth characteristics of *Alexandrium tamarense* and observed that this dinoflagellate needed 0.005 nM of vitamin B_{12} to be able to grow in cultures.

As stated by some authors (see e.g. DROOP, 1968; PINTNER and ALTMEYER, 1979; SWIFT, 1980), media of algal monocultures in stationary phase can contain a substance that is able to bind vitamin B_{12}. This substance is found in greater concentrations in monoalgal cultures that reach very high densities, such as diatoms. Once bound, vitamin B_{12} can not be taken up by the algal cells, even in such small amounts that the cells require. This inhibition can be overcome by adding an excess of vitamin B_{12} (SWIFT, 1980). However, should the concentrations of vitamin B_{12} reach too high levels, the algal growth rates might once again be inhibited.

Vitamin B_{12} is found in surface oceanic waters only in very low concentrations (from app. 10^{-5} to 10^{-3} nM). Inshore waters and estuaries have higher concentrations of vitamin B_{12}. In some areas such as the Gulf of Maine (SWIFT, 1981), concentrations range from 0.1 to 1.9 ng l^{-1} ($\approx 7.3 \times 10^{-5}$ to 1.4×10^{-3} nM). In other coastal regions such as Long Island Sound, concentrations may reach as high levels as 16 ng l^{-1} (1.1×10^{-2} nM). Our additions of vitamin B_{12} by far exceed the concentrations found in natural waters. Thus, the added high B_{12} concentrations could explain the inhibition in growth rates and cell yields observed for the diatom *D. brightwellii.* We accordingly believe that high additions of vitamin B_{12} could lead to a decrease in the growth of *D. brightwellii* in offshore waters, since this type of water was the one we used in the experiments. However, the dinoflagellate *P. minimum* did not show any significant negative response to the increasing additions of this vitamin. In contrary, it seems that the dinoflagellate was able of using cobalt (in any of the three different forms added) to maintain theirselves in the long stationary phase (almost 40 days).

In this experiment, the cell yields and chlorophyll *a* concentrations of *D. brightwellii* were inhibited at cobalt and vitamin B_{12} additions of up to 0.5 nM. However, if a synthetic chelator is added together with cobalt, then the biomass accumulation of the diatom is significantly increased compared with the controls. The biomass of the dinoflagellate *P. minimum* was not significantly affected by any of the three forms of Co additions.

These findings agree with the hypothesis suggested by GRANÉLI and HARALDSSON (1993) that cobalt may affect phytoplankton growth in coastal waters in concentrations between 0.1 and 1 nM, which are the concentrations found

in Kattegat. Thus, phytoplankton growth in these waters may be affected both positively and negatively by cobalt additions. The same authors have discussed the possibility that atmospheric deposition of acidifying substances, through the inputs of trace metals, including cobalt, by riverine waters, can alter the balance between different phytoplankton spesies in coastal waters, thus promoving the formation of harmful phytoplanton blooms. A large part of the catchment area of the Kattegat/Skagerrak basin has been severely acidified during the last decades and it can therefore be pointed out that the transport of cobalt to this basin has been increased during the last 20 years (BORG, 1988).

The mechanisms that led to the *C. polylepis* bloom in Kattegat/Skagerrak in 1988 are, in general, poorly understood. According to MAESTRINI and GRANÉLI (1991), *C. polylepis* may have found favorable growth conditions: a shortage of silica, suficient amounts of inorganic nitrogen and cobalt. GRANÉLI and RISINGER (1994) have shown that *C. polylepis* has a cell quota of 0.6 fg Co. Thus, the concentrations of cobalt found in the Kattegat would be sufficient to promove cell densities of up to 20×10^6 cells 1^{-1}. Considering that during the bloom in 1988, *C. polylepis* reached densities of 200×10^6 cells 1^{-1}, the hypothesis that cobalt may control the phytoplankton speciation in Kattegat/Skagerrak can not be excluded.

It has been shown that cobalt can enter into different geochemical and biological interactions (BRULAND *et al.*, 1991) depending on its chemical speciation, i.e. in which chemical form cobalt could be found in natural waters. The determination of the chemical speciation of trace metals in natural waters and in laboratory studies has recently advanced substantially. Further research on chemical speciation will be necessary in order to try to understand the role of cobalt in phytoplankton growth in laboratory as well as in natural conditions.

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