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Effects of cobalt and vitamin B_{12} on the growth of *Chrysochromulina polylepis* (Prymnesiophyceae)

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ABSTRACT: Atlantic sea water with a low cobalt (Co) concentration (0.02 nM) was enriched with Co additions (0.2, 0.5, 1.0 and 3.0 nM), inoculated with a monoculture of *Chrysochromulina polylepis* Manton & Parke and incubated under laboratory conditions. Co additions (as a salt) increased the yield (number of cells) of *C. polylepis*. Biomass production was not markedly different if a chelator (EDTA) was added together with Co or if Co was added as vitamin B_{12} (cyanocobalamin). The maximal growth rate of *C. polylepis* was 0.8 d^{-1}. Growth rate during the exponential phase was not influenced by Co concentrations, possibly due to Co contamination. The cell quota of Co for *C. polylepis* in the stationary growth phase was estimated at 0.55 to 0.69 fg Co $cell^{-1}$ based on cell yield in relation to Co uptake and 0.55 to 0.70 fg Co $cell^{-1}$ based on analysis of cells. In the 1988 *C. polylepis* bloom in the Kattegat and Skagerrak, cell concentrations reached levels of 100×10^6 cells l^{-1}, requiring a Co supply of at least 1 nM. Concentrations of Co in the Kattegat are below 0.5 nM, implying possible Co control of *C. polylepis* biomass accumulation.

KEY WORDS: Cobalt · *Chrysochromulina polylepis* · Vitamin B_{12} · Phytoplankton · Kattegat

INTRODUCTION

Trace metals can enhance or inhibit the growth of marine phytoplankton in a species-specific way (Anderson & Morel 1978, Anderson et al. 1978, Brand et al. 1983, Bruland et al. 1991). Co is an essential trace metal for enzyme function and in particular for the production of cobalamin (vitamin B_{12}). Recent studies have shown that the total concentration of Co in lakes and rivers increases with decreasing pH (Borg 1988, Sangfors 1988, Granéli & Haraldsson 1993). Over the last 15 yr blooms of dinoflagellates have become common in Scandinavian coastal waters (Granéli et al. 1989, 1990). A coupling between the availability of trace metals essential for phytoplankton growth and the initiation of red tides in coastal waters has been suggested by e.g. Doucette & Harrison (1990) and Wells et al. (1991). Maestrini & Graneli (1991) recently advanced the hypothesis that the *Chrysochromulina polylepis* bloom in Scandinavian waters in spring 1988 was an effect of several concurring factors, one of which may have been increased Co leaching into coastal waters due to soil acidification (Granéli & Haraldsson 1993).

In this paper we tested the hypothesis that chemical form and concentration of Co affects the growth rate and yield of *Chrysochromulina polylepis*.

MATERIAL AND METHODS

To remove Co and other trace metals from the walls of the flasks used as growth chambers, the flasks were filled with 1 M HCl/1 M HNO_3 (p.a.), stored with this solution for 1 wk and then washed with Millipore™ Q-water. The flasks were refilled with 0.1 M HNO_3 (Suprapur), stored 1 further week and then washed again with Milli-Q water. All material used during the experiments was plastic or glass and was cleaned as described above (plastic forceps, filtration holders, etc.).

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All culture flasks were sterilised by autoclaving the bottles half filled with Milli-Q water. The culture media were sterilised by filtration through 0.2 µm mesh size Nuclepore polycarbonate filters.

Chrysochromulina polylepis cells isolated from the Kattegat, SE Sweden, were grown in polycarbonate bottles (600 ml) at 16°C, under a 16:8 h light:dark cycle at 100 µE m^{-2} s^{-1} (Osram 'cool-white' fluorescent tubes).

The algae were grown in media prepared with low-Co-level Atlantic sea water (61.11° 14' N, 45° 0' W) sampled at 20 m depth with a non-metallic water sampler. Two experiments failed when we tried to cultivate *Chrysochromulina polylepis* in the metal-free and totally artificial medium Aquil (Morel et al. 1979).

Water used for the experiments initially contained 0.02 nM Co, 0.11 µM NO_3, 0.28 µM NH_4 and 0.01 µM PO_4 (before adjusting the salinity to 26‰). The salinity, originally 35‰, was adjusted to 26‰ with Milli-Q water in order to allow *Chrysochromulina polylepis* to grow at its optimum salinity (Lindahl & Rosenberg 1989).

Chrysochromulina polylepis was first grown in f/2 medium containing 50 nM Co, prepared with Atlantic sea water (Guillard & Ryther 1962). Thereafter 100 ml of inoculum was transferred to 1 l f/2 medium (except for the omission of Co and vitamin B_{12}). When the algae had attained a high biomass, but were still growing actively, we transferred them to f/10 and thereafter to f/20 media, in order to decrease the amount of macro- and micronutrients in the medium and in the algal cells. An inoculum of 2.16×10^6 *C. polylepis* cells (contained in 15 ml medium) was then added to the bottles. Phosphorus and nitrogen (as K_2HPO_4 and $NaNO_3$) were added to polycarbonate flasks containing 600 ml of Atlantic sea water equivalent to final concentrations of 1.0 and 10 µM, respectively. Co [as Co acetate, $(CH_3COO)_2Co{\cdot}4H_2O$], Co + EDTA (as the disodium salt) and vitamin B_{12} (cyanocobalamin = $C_{63}H_{88}CoN_{14}O_{15}P$) were added in different concentrations. Four replicates were used for each treatment (Table 1).

Phytoplankton growth in bottles was measured as *in vivo* chlorophyll *a* fluorescence using a FM3 filter fluorometer. Daily measurements of *in vivo* fluorescence (except for Days 10 and 11) were transformed to chlorophyll *a* after analysis of extracted chlorophyll (performed on some bottles during the different growth phases of the algal cultures) following the method of Jeffrey & Humphrey (1975).

Samples from the cultures (20 ml, which had been used to measure *in vivo* chlorophyll) were fixed with Lugol's solution and the cells were counted in a Palmer-Maloney chamber for Days 1, 3, 5, 6, 7, 8, 9 and 12.

Table 1. Co, EDTA and B_{12} additions to the experimental bottles. Note that the initial 0.02 nM Co found in the Atlantic sea water has to be added to the Co and B_{12} additions. The controls for the Co treatments and B_{12} did not receive any addition of either Co or B_{12}. The controls for Co + EDTA received only EDTA

	Co (nM)	EDTA (nM)	B_{12} (nM)
Atlantic sea water	0.02		
Additions:			
Series 1	0 (Control)		
	0.2		
	0.5		
	1.0		
	3.0		
Series 2	0 (Control)	50 + 450[a]	
	0.2	50 + 450[a]	
	0.5	50 + 450[a]	
	1.0	50 + 450[a]	
	3.0	50 + 450[a]	
Series 3			0
			0.2
			0.5
			1.0
			3.0

[a]50 nM EDTA was added on the first and 450 nM EDTA on the seventh day of the experiment

On Day 6, 10 ml of each culture was filtered through 0.2 µm mesh size polycarbonate Nuclepore black filters and checked for bacterial contamination with a Zeiss microscope equipped with epifluorescence light, following the method of Porter & Feig (1980). Inspection of cultures on Day 6 revealed no bacterial contamination.

Co concentration in the Atlantic water was analysed following the method of Danielsson et al. (1982), using a Plasma Source Mass Spectrometer (ICP-MS), VG model PQ1. Co concentrations were also analysed in the cells (triplicates) and in the filtrates (water was pooled from 3 cultures) on the last day of the experiment for the Co addition series.

Growth rate as a mean for Days 1 to 6 was calculated by fitting straight lines to the logarithm of cell numbers versus time (individually for each culture flask). Growth rate is given as μ (d^{-1}).

RESULTS

Co concentrations in the medium of the Co series at the end of the experiment were: controls and 0.2 and 0.5 nM Co additions = 0.04 nM; 1.0 nM Co addition = 0.05 nM; and 3.0 nM Co addition = 1.2 nM. Co in the *Chrysochromulina polylepis* cells varied between

Table 2. *Chrysochromulina polylepis*. Co cell quotas on the last day of the experiment (Day 12) in the controls and Co additions

Co addition (nM)	fg Co cell^{-1}	
	Mean (n = 3)	SD
0.0 (control)	0.55	0.10
0.2	Values missing	
0.5	0.61	0.11
1.0	0.55	0.03
3.0	0.70	0.03

0.55 and 0.70 fg cell^{-1}, with the highest value for the 3.0 nM Co addition (Table 2).

Growth of *Chrysochromulina polylepis* was logarithmic until Day 6 without a lag phase (Figs. 1 & 2). This conclusion is based on a linear relation between log cell numbers and time in individual cultures with r^2 usually equal to 0.99 (minimum value 0.97). Chlorophyll accumulation ceased after Day 8 or Day 9, except in the B_{12} additions, where slight chlorophyll accumulation continued until the end of the experiment (Day 12). Maximal chlorophyll *a* accumulation (Fig. 1) did not differ greatly between cultures with 0.2, 0.5, 1.0 and 3.0 nM Co, although there was a clear difference between these cultures and the control bottles (without added Co), except for the Co series (Fig. 1; $p < 0.001$, Mann-Whitney *U*-test).

In contrast to chlorophyll accumulation, cell accumulation differed markedly between the different additions, especially between controls and 0.2/0.5 nM additions, and between 0.2/0.5 and 1.0/3.0 nM additions (Fig. 2; $p < 0.001$, Mann-Whitney *U*-test). Thus chlorophyll cell quotas varied between treatments (Fig. 3).

Chrysochromulina polylepis cell number increased more or less linearly when additions of Co (or Co + EDTA and B_{12}) were increased from 0 to 1.0 nM (Fig. 4). An addition of 3.0 nM Co only increased cell number accumulation slightly over the cell numbers produced at the 1.0 nM addition. Even without any addition of Co, around 50×10^6 cells l^{-1} were produced, indicating either that Co was initially present or that Co contamination could have occurred during the sampling period. An extrapolation to zero yield gives a Co value of 0.63 to 0.78 nM for the 3 series, indicating the amount of Co contamination. The maximum yield of *C. polylepis* produced was similar irrespective of type of Co addition (inorganic Co alone, Co + EDTA or vitamin B_{12}) except for the 0.5 nM B_{12} additions that increased maximal cell numbers significantly compared to the Co or Co + EDTA additions (Fig. 4; $p < 0.001$, 1-way ANOVA with Fisher's PLSD *t*-test).

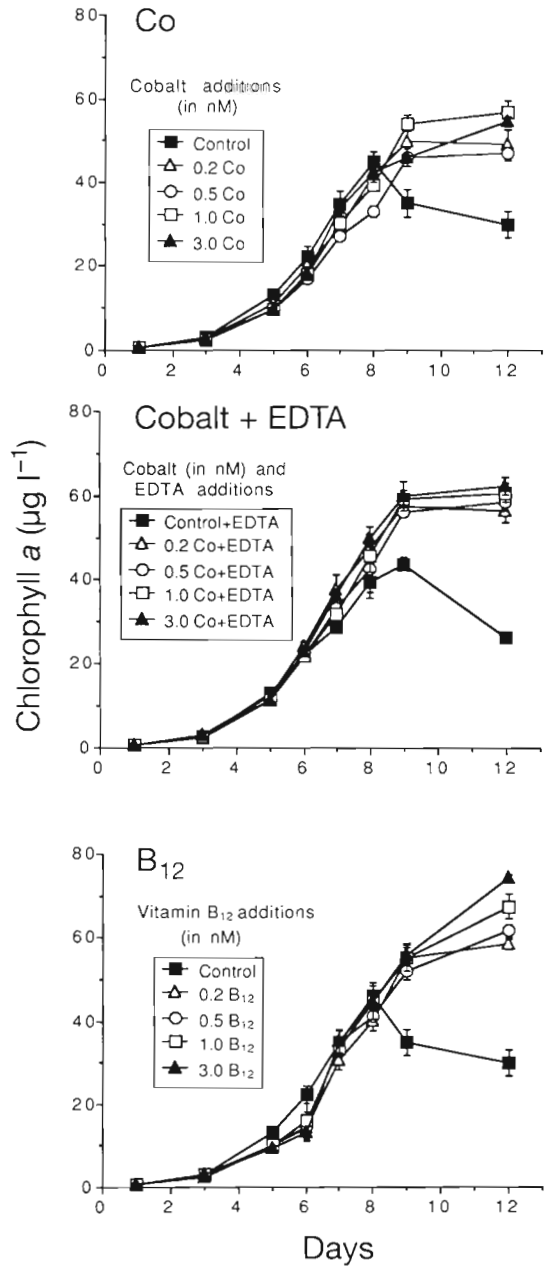

Fig. 1. *Chrysochromulina polylepis*. Chlorophyll *a* development of cells growing in 26‰ diluted Atlantic sea water containing additions of nitrogen and phosphorus, with or without different Co additions. Control: no extra Co added. Bottles containing EDTA were used as controls for these additions. Means ± SD, n = 4 bottles for each treatment

The growth rate of *Chrysochromulina polylepis* (Days 1 to 6) was not affected by additions of Co or Co + EDTA (Fig. 5), while growth rate was slightly higher for B_{12} additions equivalent to 1.0 and 3.0 nM Co compared to additions of 0, 0.2 and 0.5 nM (Fig. 5; $p < 0.001$, 1-way ANOVA with Fisher's PLSD *t*-test). Growth rates were 0.7 to 0.8 d^{-1}.

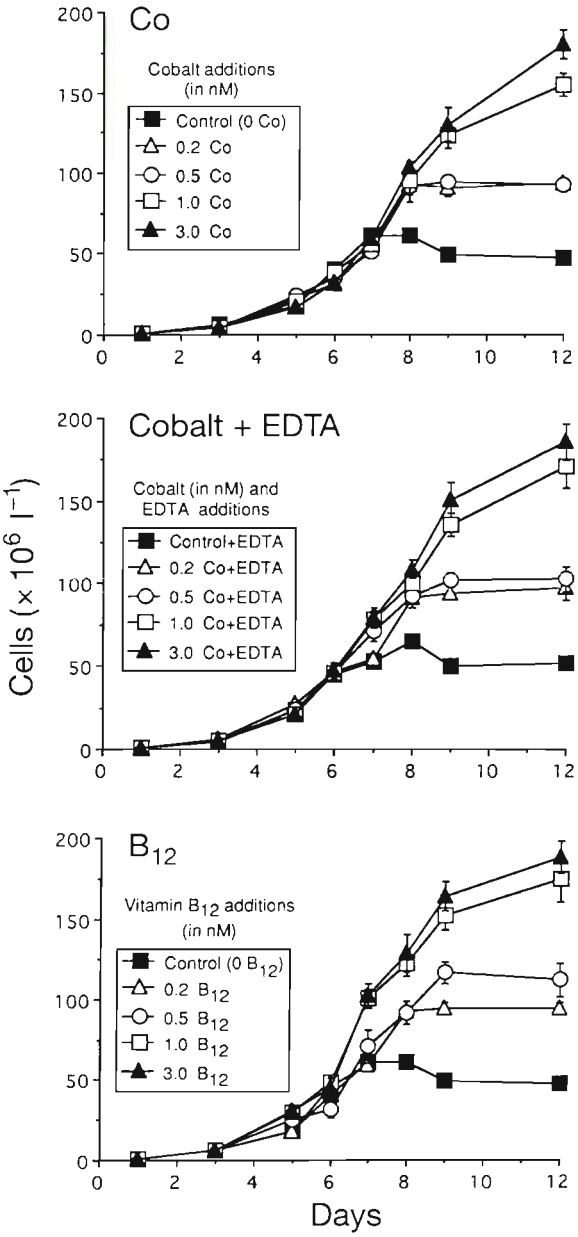

Fig. 2. *Chrysochromulina polylepis*. Development of cells growing in 26‰ diluted Atlantic sea water containing additions of nitrogen and phosphorus, with or without different Co additions. Other details as in Fig. 1

DISCUSSION

A large percentage of phytoplankton species are auxotrophic for vitamin B_{12} (Bonin et al. 1981). According to Droop (1962) 80% of the species which have been examined for their vitamin B_{12} requirement needed this vitamin. Nishijima & Hata (1989), studying the kinetics of production and uptake of vitamin B_{12} in natural sea water, found that there were no significant differences in the rates of uptake of the vitamin between light and dark periods, either for phytoplankton, or for bacteria. However, net production of B_{12} by the phytoplankton was negative, while for the bacteria it was slightly positive. These findings thus corroborate the idea that bacteria are the main producers of vitamin B_{12}, while phytoplankton are mainly consumers.

To what extent *Chrysochromulina polylepis* can synthesise vitamin B_{12} is not known. In our study, the growth of *C. polylepis* was almost the same when Co was added as a salt, as when the same amount of Co was added as vitamin B_{12}, which indicates that *C. polylepis* can produce vitamin B_{12}. Cultures were visually bacteria-free on Day 6, but it cannot be excluded that some bacterial growth occurred during the later phase of the experimental period.

Cadmium and Co can substitute for zinc (Zn) in certain macromolecules that are essential for the growth of *Thalassiosira weissflogii* (Price & Morel 1990). Zn is a micronutrient which is usually depleted in oceanic surface water. In experiments by Price & Morel (1990), additions of Co increased the growth rate of *T. weissflogii* cells that had been growing under Zn depletion to 60% of the maximum value. These results indicate that there is another important role for Co in the metabolism of marine phytoplankton besides that in the vitamin B_{12} molecule. The positive effect of Co additions on *Chrysochromulina polylepis* cell numbers and chlorophyll *a* production was only found after several days of incubation. The results presented by Price & Morel (1990) also indicate that the stimulation of *T. weissflogii* by Co additions only occurred on the third day of the experiment.

The growth rate of *Chrysochromulina polylepis* was not affected by Co or vitamin B_{12} additions, except for a slight stimulation at the highest B_{12} levels. However,

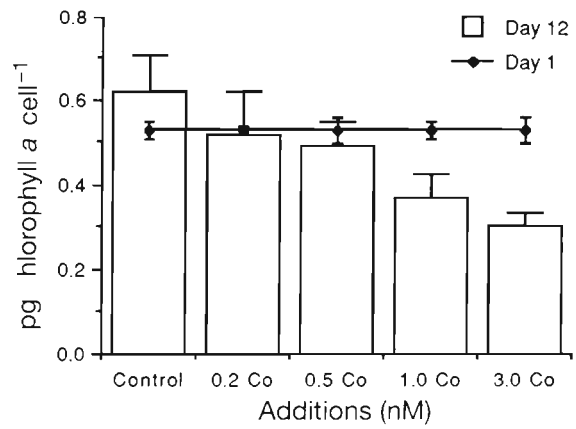

Fig. 3. *Chrysochromulina polylepis*. Chlorophyll *a* cell quotas immediately after inoculation of the cultures (Day 1) and at the termination of the experiment (Day 12). Error bars = ±1 SD (n = 4) in the controls and Co additions

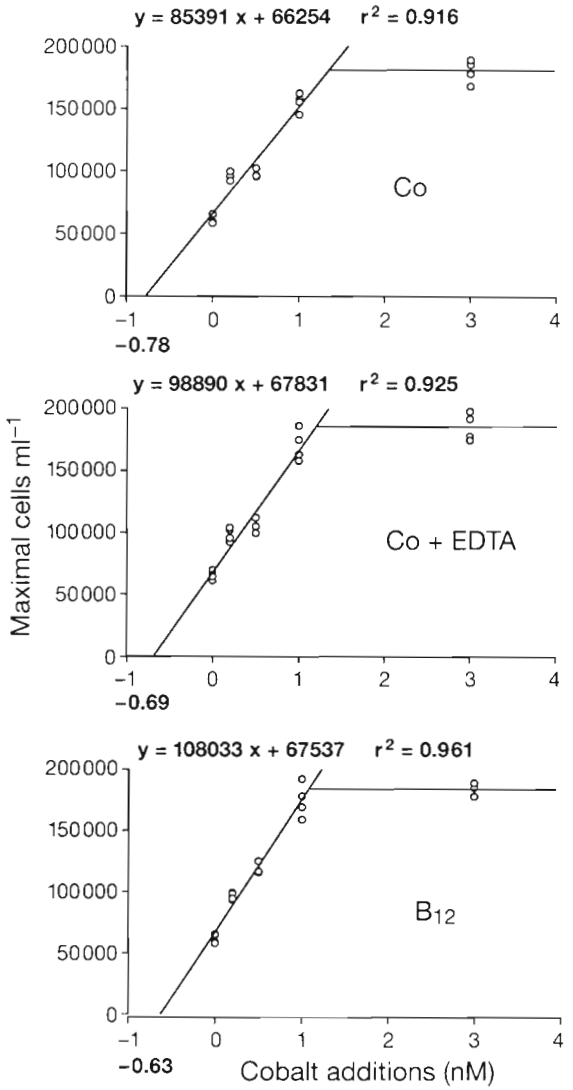

Fig. 4. *Chrysochromulina polylepis*. Maximal numbers of cells produced in relation to increasing Co, Co + EDTA or B_{12} concentrations. Other details as in Fig. 1

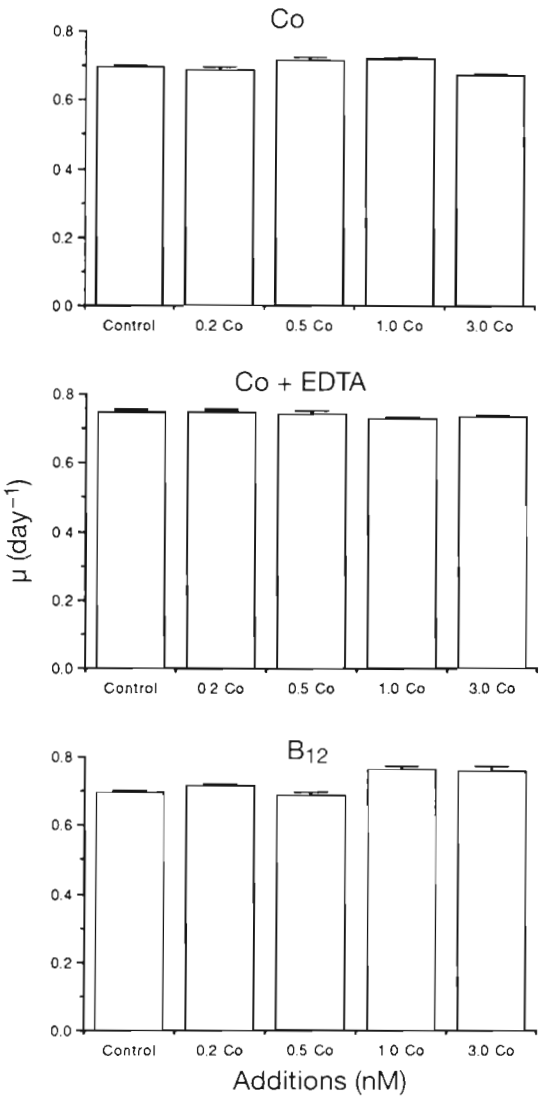

Fig. 5. *Chrysochromulina polylepis*. Growth rates (Days 1 to 6) in relation to Co, Co + EDTA or B_{12} concentrations. Other details as in Fig. 1

since there seemed to be some Co contamination, it cannot be excluded that low levels of Co (below ~1 nM) may affect the growth rate of *C. polylepis* relative to the growth rate in completely Co-free medium. Rhodes et al. (1994) found that Co added to a concentration of 0.23 μM inhibited growth of *C. quadrikonta*, while growth was good at 0.11 μM Co; immediate cell death occurred at 0.57 μM Co. However, these Co levels are at least 3 orders of magnitude higher than our additions, and irrelevant for natural sea water conditions. Thus the conclusion of Rhodes et al. (1994) that Co, contrary to the hypothesis of Sangfors (1988; see also Granéli & Haraldsson 1993, Maestrini & Granéli 1991) is not involved in the growth stimulation of *Chrysochromulina* species finds no support in Rhodes et al.'s own study.

Cell quotas of Co from analyses of filter residues from the cultures with Co additions were 0.55 to 0.70 fg cell^{-1}. It is also possible to make an independent calculation of *Chrysochromulina polylepis* cell quotas for Co by using data on maximal cell production in relation to Co addition (Fig. 4). For additions of 0 to 1.0 nM Co there was very little Co left in the cultures at the end of the experiment, indicating efficient uptake by *C. polylepis*. Only for the highest addition was there a substantial fraction of the added Co left in the medium, indicating that some other chemical element limited cell accumulation. If a straight line is fitted to maximal cell accumulation in relation to the Co additions of 0, 0.2, 0.5 and 1.0 nM Co, the slope of that line gives the cell quota for Co (assuming no residual Co in the medium, which was the case

for these concentrations of added Co). Calculated in this way the cell quota will be 0.69, 0.60 and 0.55 fg Co for cultures with additions of Co, Co + EDTA and vitamin B_{12}, respectively. The value for the Co cultures, 0.69 fg Co $cell^{-1}$, is in close agreement with the Co content in the cells of the 3.0 nM Co addition culture, 0.70 fg Co $cell^{-1}$. This latter value should represent Co-sufficient cells, unless N- or P-limitation causes a depression of Co-uptake, while values based on cultures with 0.2 and 0.5 nM Co should represent Co-limited cells (0.55 and 0.61 fg Co $cell^{-1}$). The rather limited variation in the Co content of *C. polylepis* in this study is somewhat surprising since other studies have shown a large plasticity in trace element cell quotas of phytoplankton. *Heterosigma akashiwo* cells growing under nutrient sufficient conditions had cell quotas for Co of 0.28 to 2.14 fg (Watanabe et al. 1989), which agrees well with our values for *C. polylepis*, considering that *H. akashiwo* is somewhat larger than *C. polylepis*.

Above 1 to 1.4 nM Co (or ~2.0 nM Co if the probable level of initial Co contamination is included) another factor became limiting. This factor is most likely P or N. According to Maestrini & Granéli (1991) cell quotas for *Chrysochromulina polylepis* of N and P in nutrient sufficient medium are 0.26 and 0.02 pmol $cell^{-1}$, respectively. Initial N and P in the medium can be estimated at 29.2 and 2.63 µM, based on the nutrient content in the Atlantic water (0.39 and 0.01 µM), added as nutrient solution (10 and 1.0 µM) and added with the inoculum (18.8 and 1.62 µM). This would be enough for the production of 112×10^6 cells l^{-1} and 131.5×10^6 cells l^{-1}, based on the initial pools of inorganic N and P, respectively. These values are lower than the maximal numbers in the cultures with 3 nM Co additions, 180×10^6 cells l^{-1} (Fig. 2). However, cells were chlorotic after growth had reached the stationary phase, and cell quotas for N and P may therefore have been lower than values for nutrient (N and P) sufficient cells during the last days of the experiment. That cells in the 3.0 nM Co treatments were N or P deficient at the end of the experiment can also be seen from chlorophyll *a* cell quotas (Fig. 3). Initially chlorophyll quotas were identical for all cultures, but at the end of the experiment values were significantly lower than initial quotas for 0.5, 1.0 and 3.0 nM Co additions ($p < 0.05$, Mann-Whitney *U*-test). Latasa & Berdalet (1994) likewise found that the dinoflagellate *Heterocapsa* sp. dramatically decreased the chlorophyll content per cell under N and P limitation. On the other hand, judging from our experiments, chlorophyll cell quotas were not strongly influenced by Co limitation.

Martin & Gordon (1988) and Martin et al. (1989) reported Co concentrations for the North Pacific of between 4 and 50 pM, which Bruland et al. (1991) suggested to be at the limiting level for phytoplankton growth. The amount of Co found in the Atlantic sea water used by us for the Co enrichment bioassays was 20 pM, thus in the same range as for the North Pacific. Co concentrations in the Kattegat, with salinities between 15 and 25‰, are substantially higher than values for oceanic water. According to Granéli & Haraldsson (1993) Co concentration in the Kattegat is between 0.2 and 0.4 nM. With a cell quota of 0.6 fg Co, 0.2 to 0.4 nM of Co would be sufficient for 20 to 40×10^6 cells l^{-1}. Considering that during the *Chrysochromulina polylepis* bloom in the Kattegat and Skagerrak in May/June 1988 cell densities reached 200×10^6 l^{-1} (B. Sundström unpubl.) and values around 100×10^6 cells l^{-1} were common offshore (Kaas et al. 1988, 1991), Co limitation of biomass accumulation cannot be excluded. In the Seto Inland Sea in Japan, blooms of the raphidophytes *Chatonella antiqua* and *Heterosigma akashiwo* have been a common phenomenon for the last 2 decades (Ono & Takano 1980, Honjo 1993). These blooms have been associated not only with nitrogen and phosphorus enrichment, but also with high concentrations of vitamin B_{12} (Nakamura et al. 1988, 1989, Honjo 1993).

The flagellate-dinoflagellate blooms in the Kattegat and Skagerrak, which were noticed for the first time in 1979, have increased during the last 15 yr both in intensity and in the variety of species involved (Granéli et al. 1989). During the same period (and starting at least a decade earlier) there has been an increase in trace metal mobilisation from soils due to acidification (Anonymous 1986, Borg 1988). Granéli & Haraldsson (1993) have shown that there is an inverse correlation between pH and Co concentrations in different river waters draining from the Swedish west coast. The humic/fulvic acid concentrations in riverine water of forest origin have also increased (Forsberg & Petersson 1990, Forsberg 1991). McLaren et al. (1986) showed that Co is easily sorbed by humic and fulvic acids and that Co is also relatively easily desorbed from the humic acids. 70% of the Co adsorbed by the humic fractions remained isotopically exchangeable even after 50 d. The combination of natural chelators and essential trace metals might have played a role in the flagellate/dinoflagellate blooms seen during the last 15 yr in Scandinavian coastal waters.

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