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The results of a preliminary experiment suggested that a complexed form of cobalt was more efficacious in promoting growth of a marine phytoplankton than ionic cobalt. The phytoplankton used in this experiment was Coccolithus huxleyi, a vitamin B_{12} producer, and the cobalt complex was cobalt(II)-ethylenediaminetetracetic acid [Co(II)-EDTA]. A review of the biochemistry of vitamin B_{12} indicates that a B_{12} producer might prefer, if not require, Co(III) instead of Co(II). Since some of the Co(II)-EDTA in the preliminary experiment might have become oxidized to Co(III)-EDTA, the observed stimulation of growth could have been due to Co(III)-EDTA.

Two experiments were performed to determine if Co(III)-EDTA is more efficacious in stimulating growth than Co(II)-EDTA. Coccolithus huxleyi, grown in batch cultures with constant illumination, was used in both experiments. One experiment had cobalt concentrations of 10 and 1 μg/l; the other had concentrations of 1 and 0.1 μg/l. In

both experiments there were no observed differences in specific growth rates between treatments of Co(III) as the EDTA complex with 10^{-6} M additional EDTA and Co(II) with 10^{-6} M EDTA. Both of these treatments resulted in a specific growth rate larger than controls without added EDTA or cobalt. It is not possible to measure the amount of Co(II)-EDTA that is oxidized to Co(III)-EDTA at the concentrations used in these experiments. Consequently these results cannot be used as a basis for rejecting the hypothesis that Co(III) is the required form of cobalt. Since Co(III)-EDTA without the additional 10^{-6} M EDTA was apparently able to stimulate growth in relation to the controls, it is assumed that C. huxleyi is capable of utilizing this form of cobalt.

Growth Response of a Marine Phytoplankton *Coccolithus huxleyi*, to Various Chemical Forms of Cobalt

by

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INTRODUCTION

General Statement

Like many other biologically important trace metals, cobalt may be a cofactor for several enzymes. When cobalt is present in a biochemical system as a cofactor, it can be replaced by other divalent cations with one exception. Cobalt has only one known essential function and that is as the metal atom of the corrin ring in vitamin B_{12} and its derivatives (Evans, 1967). Because cobalt is a structural component of vitamin B_{12}, any organism that produces this vitamin must have a requirement for cobalt.

The amount of cobalt available to a vitamin B_{12} producer could influence its growth rate. Kliewer *et al.* (1964) found that a near optimal growth rate of *Rhizobium meliloti*, a B_{12} synthesizer, was obtained with the addition of only 0.04 μg/l cobalt to a cobalt-free medium. A similar concentration of cobalt gave the same results for a blue green alga, *Nostoc muscorum* (Holm-Hansen *et al.*, 1954). Since the concentration of cobalt in sea water is estimated to range from 0.008 to 0.4 μg/l (Brewer and Spencer, 1970), it is unlikely that cobalt limits the growth rates of vitamin B_{12} producers in the oceans.

Even if the amount of cobalt available to a vitamin B_{12} producer

is sufficient for optimal growth, it is still possible for that amount of cobalt to limit the amount of vitamin B_{12} produced. Perlman and O'Brien (1954) reported that the maximum efficiency of conversion of added inorganic cobalt to vitamin B_{12} by Streptomyces griseus was 13% over an unspecified period of time and at a cobalt concentration of 100 μg/l. The maximum yield of B_{12} under the same conditions was at a cobalt concentration of 200 μg/l or higher. If the values for this species are representative for B_{12} producing organisms, the cobalt concentration in sea water is too low to support maximum B_{12} production.

In studying the cobalt requirements of vitamin B_{12} producing organisms, it is too simplistic to take into consideration just the total amount of cobalt in the organisms' environment. For example, all the cobalt in a vitamin B_{12} producer's environment could be in a physical-chemical form that is unavailable to the organism. Consequently, considerations of total cobalt present are trivial if some is not in a physical-chemical form utilizable by the organism. Provasoli (1963) stated "the deficiency of trace metals depends quantitatively far more on the physical status governing their availability to the cells than on total amount." It is therefore imperative for the physical-chemical form of cobalt to be taken into consideration when studying the effect of adding cobalt to cultures of organisms that produce vitamin B_{12}.

The results of one of Perlman and O'Brien's (1954) experiments contrast the differences in response to two different chemical forms of cobalt. They found that an unidentified cobalt complex fixed by yeast was far more effective than ionic cobalt in stimulating vitamin B_{12} production. The results of an experiment performed during my study (see section on Results and Discussion) were analogous to those of Perlman and O'Brien (1954). In this experiment cobalt added as a complex to phytoplankton cultures promoted a faster population growth than cultures receiving no additional cobalt. Cultures receiving 10.0 µg/l of ionic cobalt had a population growth no different than those receiving no additional cobalt. The results of Perlman and O'Brien (1954) and my preliminary experiment noted above suggest that there is a difference in biological response between ionic and complexed cobalt.

There are several alternatives to the hypothesis that complexed cobalt is more effective than ionic cobalt in stimulating B_{12} production or the specific growth rate of B_{12} producing organisms. Free (uncomplexed) cobalt exists primarily as Co(II) in aqueous solutions, but complexed cobalt can be either Co(II) or Co(III) (see section on Cobalt Chemistry). Consequently the differences in biological response between added ionic and complexed cobalt could be due to differences in its oxidation state. Since the oxidation state of cobalt in the free (non-coenzyme) form of B_{12} is trivalent, it is possible for B_{12}

producing organisms to prefer, if not require, Co(III). It is the purpose of this work to determine if Co(III) complexed with ethylenediaminetetracetic acid (EDTA) is more effective than Co(II)-EDTA in stimulating the specific growth rate of a vitamin B_{12} producing marine phytoplankter, Coccolithus huxleyi.

By comparing the growth rates of phytoplankton cultures with added Co(III)-EDTA against those with Co(II)-EDTA one of three results can occur. (1) If the growth rates of cultures with added Co(III)-EDTA are significantly higher than those with Co(II)-EDTA, the hypothesis that Co(III) is the preferred form of cobalt is not rejected. (2) If Co(II)-EDTA promotes a higher growth rate than Co(III)-EDTA, the hypothesis is rejected. (3) If there is no difference between treatments of Co(III)-EDTA and Co(II)-EDTA, the hypothesis can neither be rejected nor accepted.

Ecology and Physiology of Coccolithus huxleyi

The phytoplankton used in these experiments is the marine coccolithophorid, Coccolithus huxleyi (Lohmann) Kamptner. C. huxleyi is the commonest of all the marine coccolithophorids (Braarud, 1961) and, with the exception of a short diatom bloom in the spring, is the predominant phytoplankton species of the Sargasso Sea (Menzel et al., 1963). A culture of clone BT-6 of C. huxleyi was obtained from R. R. L. Guillard in 1969. Guillard originally isolated this

clone from the Sargasso Sea.

Coccolithus huxleyi is spherical in shape and has a mean diameter of about 5 micrometers. Cell volume is considerably reduced in old cultures. The cells have two flagellae, and motility is observed. Nevertheless, they tend to collect at the bottom of culture flasks. Although coccolith (calcareous plate) formation of clone BT-6 in laboratory cultures has been reported (Wilbur and Watabe, 1963), coccoliths were not observed during my experiments. The only known preformed organic requirement of C. huxleyi is thiamine, and the species produces both biotin and vitamin B_{12} (Carlucci and Bowes, 1970a).

Clone BT-6 of C. huxleyi has been in culture under laboratory conditions for at least ten years. Over this period of time, clone BT-6 could have evolved. Consequently, conclusions drawn using this clone as an experimental organism may not be relevant to natural populations of C. huxleyi.

A review of the literature suggests that there are different geographical strains of C. huxleyi. Wilbur and Watabe (1963) compared coccolith formation between a strain isolated from the North Sea (strain 92-A) and clone BT-6. They reported several differences between the two. One of the reported differences was that BT-6 but not 92-A could produce coccoliths in a nitrogen-rich medium. Strain 92-A could produce coccoliths only in a nitrogen-deficient medium. Wilbur and Watabe (1963) also reported that the chemical form of

$CaCO_3$ in the coccoliths of BT-6 varied with temperature. This variation of the chemical form of $CaCO_3$ with temperature was not observed in the coccoliths of 92-A. Another difference between BT-6 and 92-A is that 92-A, but not BT-6, is able to utilize uric acid as a nitrogen source (Guillard, 1963). An additional basis for comparison is growth rate. Using a clone isolated from Oslo Fjord, Paasche (1964) reported that C. huxleyi underwent about one fission per day when in the log phase of growth. Mjaaland (1956) reported a similar division rate for two clones (P_3 and P_8) from the Marine Biological Laboratory, Plymouth and a clone from the Norwegian Sea. During my experiments the division rate of BT-6 in the log phase of growth was about one fission per 15 hours. Using the data of Carlucci and Bowes (1970a), a similar division rate for BT-6 was calculated. If the various strains of C. huxleyi used in all the experiments reported in the literature are representative of their respective natural populations, it would appear that C. huxleyi in the Sargasso Sea is a different geographical strain than those found in more northern waters. It is clear that the results of any experiments on the growth or physiology of C. huxleyi must take into account the strain involved.

Ecological Importance and Chemistry of Vitamin B_{12}

The reported values for the concentration of B_{12} in sea water are from 0-20 ng/l with most of the values occurring in the range of

1-3 ng/l (Cowey, 1956; Daisley and Fisher, 1958; Kashiwada et al., 1956). The concentration varies on a seasonal basis, with high levels in surface waters occurring during the winter months and low levels during the summer (Cowey, 1956). Carlucci (1970) found that the concentration of B_{12} was highest when the phytoplankton standing stock was high.

Vitamin B_{12} or its analogs have been found necessary for the growth of some marine phytoplankton (Provasoli and Pintner, 1953; Droop, 1954, 1955; Carlucci and Bowes, 1970b). Carlucci and Bowes (1970b) in studying the utilization of vitamins found that both Skeletonema costatum and Stephanopyxis turris grew better in enriched media containing B_{12} at 12 ng/l than at 2 ng/l. This would indicate that the average B_{12} range of 1-3 ng/l could affect the growth rate of marine phytoplankton that require B_{12}.

With the exception of C. huxleyi, the only known producers of B_{12} are bacteria, actinomyces and some blue-green algae (Perlman, 1959; Pratt, 1972). Some marine macroalgae have been reported to produce B_{12} but it appears that the production was due to symbiotic bacteria rather than the algae (Goodwin, 1963). Although it is surprising to find a marine coccolithophorid that produces B_{12}, Carlucci and Bowes' (1970a) evidence for B_{12} production by C. huxleyi in an axenic culture is convincing. It would be interesting to determine if other coccolithophorids produce B_{12}.

Vitamin B_{12} (Figure 1) is the largest of the vitamins, with a molecular weight of 1355.4, and is the only one that requires a metal (Pratt, 1972). Cobalt is the metal constituent of the corrin ring in B_{12} and is bound to the corrin ring with four equatorial ligands. One of the axial ligands is with 5, 6-dimethylbenzimidazole (Figure 1). The cyano group in Figure 1 is an artifact of the procedure used in isolating B_{12}. In organisms, the cyano group is not present and usually has in its place a methyl group, deoxyadenosyl (Figure 2), or a hydroxyl group (Pratt, 1972). The free or non-coenzyme form usually has the hydroxyl group and the cobalt is probably in the trivalent oxidation state (Goodwin, 1963). Vitamin B_{12} found in organisms is about 80% in the coenzyme form (Goodwin, 1963) with either the methyl group or deoxyadenosyl as one of the axial ligands. The oxidation state of cobalt with the coenzyme form of B_{12} is evidently univalent or trivalent (Weissbach et al., 1965).

Although a number of steps in the biosynthesis of B_{12} have been elucidated, it is not known whether the cobalt atom is introduced into the corrin structure before or after closure of the ring (Pratt, 1972). The oxidation state of the cobalt atom in the "freshly made" corrinoid is also not known. Since the oxidation state of cobalt in the free or non-coenzyme form of B_{12} is normally trivalent, it is not unreasonable to assume that the "freshly made" corrinoid also has Co(III). If this is true, a B_{12} producer might prefer Co(III) over Co(II).

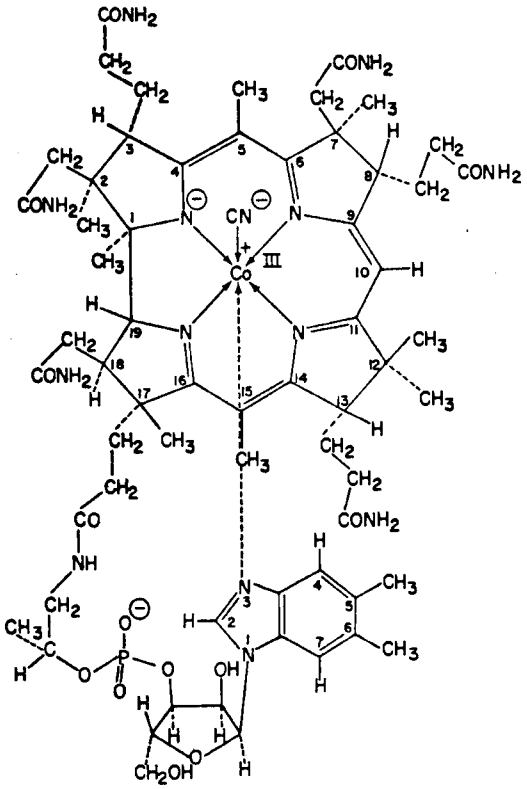

Figure 1. Molecular structure of B_{12} with the axial ligand 5, 6-dimethylbenzimidazole (from Pratt, 1972).

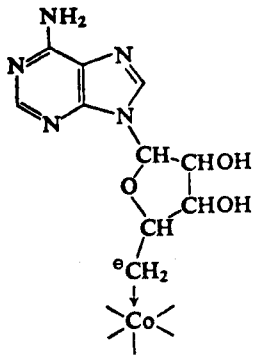

Figure 2. Molecular structure of deoxyadenosyl (from Pratt, 1972).

Cobalt Chemistry

The actual concentration of cobalt in sea water is not known with certainty. Brewer and Spencer (1970) state that the high variability of reported cobalt concentrations in sea water (0.004-1.0 μg/l) may be due to methodology. In their study, several laboratories working independently determined the concentrations of several trace elements in two separate samples of sea water. The results for cobalt are in Table 1. Four of the laboratories analyzed for cobalt by neutron activation of the freeze dried salts. This technique should give the abundance of the total metal. Five of the laboratories used solvent extraction or adsorption concentration methods prior to the analysis. These techniques might miss strongly complexed cobalt. In both samples the difference of the values found between the two techniques was significant as tested by the Wilcoxon Rank Sum Test. It is tempting to reject the values obtained by the extraction or adsorption methods on the basis of possible cobalt contamination and accept the values obtained using the freeze dry method because it is probably less

Table 1. Difference between "total" and "extraction" methods for cobalt (from Brewer and Spencer, 1970).

Sample	Mean Total Methods (μg/kg)	S. D. Total Methods	Mean Extraction Methods (μg/kg)	S. D. Extraction Methods
1	0.052	0.044	0.282	0.153
3	0.030	0.018	0.194	0.129

susceptible to contamination and supposedly measures the total cobalt. These gounds for accepting one method over another are somewhat speculative. Combining both techniques and both samples, the cobalt concentration in sea water is assumed to lie in the range of 0.008 - 0.435 μg/l. Due to the differences between techniques, the grand mean of 0.17 μg/l can be used only as a rough approximation of the cobalt concentration in sea water.

In light of the difficulty in obtaining a reasonably precise estimate of the concentration of cobalt in sea water, it is apparent that a direct determination of its major forms is not currently feasible. However it is possible by using thermodynamic data to predict what the major forms might be at equilibrium (Sillén, 1961). Such predictions, based solely on thermodynamic considerations, are at best first approximations of what may be expected. The predictions will be trivial if important species are not considered. One of the assumptions underlying this approach is that the system under consideration is at equilibrium. This may not be true for sea water. Pressure and temperature effects and adsorption are usually not considered. Another problem in making these predictions is that the value of Eh for sea water is not known. Assuming that the O_2-OH^- couple sets the redox potential, the theoretical Eh value for sea water saturated with O_2, at a temperature of 15°C, and at a pH of 8.3 is 0.750 volts (Cooper, 1938). Measured values of surface sea water are about 0.40

volts (Baas Becking et al., 1960).

Sillén (1961), in developing his equilibrium sea water model, assumed that the two solid phases of cobalt entering the oceans are CoO_2 and CoOOH. His calculations show that of these two only the CoOOH would be stable and that it undergoes the reaction:

$$CoOOH_{(s)} + 3H^+ + e^- \rightleftarrows Co^{+2} + 2H_2O;\ \log K = 29.3$$

Assuming a pH of 8.1 and pE of 12.5, then the $\log[Co(II)] = -6.7$. This calculation implies that there is a difference of about two orders of magnitude between the calculated cobalt concentration and the measured concentration. Sillén (1961) suggests that this difference is possibly due to CoOOH being substituted in hydrous ferric oxides. The formation constants (K_1) for both $[Co(II)Cl]^+$ and $[Co(II)SO_4]$ are much greater than the K_1 for $[Co(II)OH]^+$. Using the appropriate formation constants from Sillén and Martell (1964, 1971) it can be shown that both $CoCl^+$ and $CoSO_4$ could be important forms of cobalt in sea water. These cobalt complexes could account for part of the difference between the measured and predicted cobalt concentrations in sea water. Due to its high oxidizing strength, free Co(III) would not exist in sea water; it could exist only as a complex.

Sillén's (1961) suggestion that some cobalt exists as a solid solution with FeOOH has empirical support. Kurbatov et al. (1951) studied the adsorption of cobalt on hydrous ferric oxide from 0.048 N

NH_4Cl solutions. The results of one of their experiments are shown in Table 2 and these data were used to make Figure 3. The ratio of iron to cobalt in sea water is about 140:1. If iron and cobalt behave in sea water as they do in a 0.048 N NH_4Cl solution, then > 90% of the cobalt in sea water would be associated with hydrous ferric oxides. Krauskopf (1956) determined Co(II) adsorption from sea water on several different adsorbents. His data for iron and cobalt are plotted on Figure 3. He used different levels for both iron and cobalt. The adsorption values obtained at the highest concentration of iron oxide (300 mg/l as Fe) are unexpectedly independent of the cobalt concentration. If these points are excluded from the analysis, then a fairly good fit ($r^2 = 0.85$) is obtained. The slope for data obtained using sea water as a medium is less than that when NH_4Cl was used but as can be seen in Figure 3, the amount of cobalt adsorbed on the iron

Table 2. Effect of Fe concentration on cobalt adsorption.

Total Adsorbant (gram atoms of Fe x 10^6)	Cobalt Adsorbed in 24 Hours (percent)
4	52.6
6	66.0
8	71.1
10	75.3
20	87.8
60	96.9
100	98.6

Constant factors: Co, 6.4×10^{-8} gram-atom; pH, 8.0; NH_4Cl, 0.048 N; temperature, 27°C ± 1°; volume, 34.5 ± 0.1 ml.
Taken from Kurbatov et al. (1951).

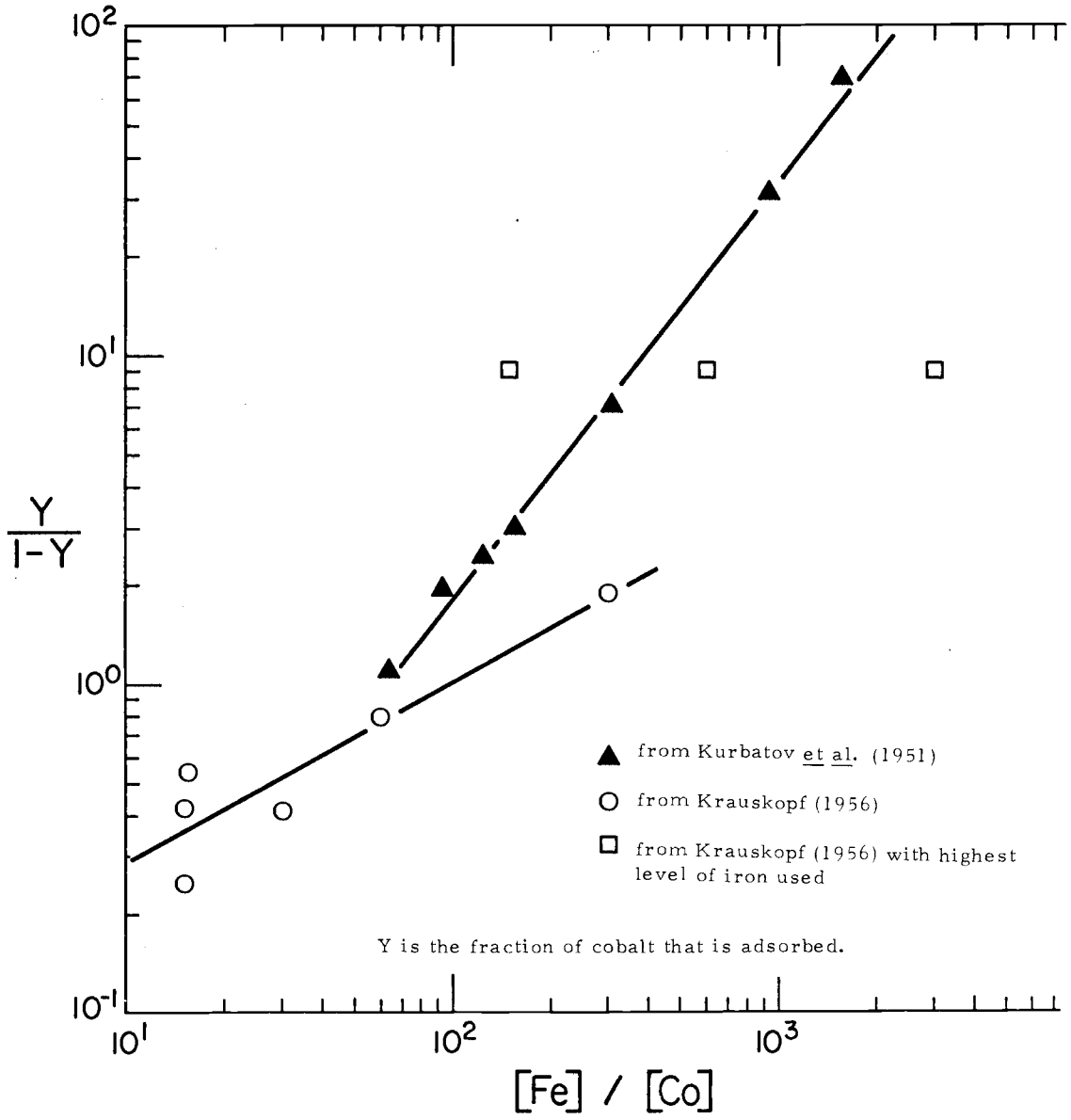

Figure 3. Effect of the ratio of iron to cobalt on cobalt adsorption onto hydrous ferric oxide.

oxide is greater than 50% at a [Fe]:[Co] of 140:1. This decrease in slope is consistent with Kurbatov's et al. (1951) observation that an increase in the chloride concentration decreases the adsorption of cobalt onto iron oxide. The possible importance of cobalt associations with solid phase iron oxide in seawater is further supported by the observation that the enrichment factor for cobalt in iron oxide-rich sediments is from 1 to 13 on the average (Krauskopf, 1955). Enrichment factor is the ratio of the concentration in the sediments to that in average crustal material. Even though the association of cobalt with iron is apparently important in sea water, the type of association is not known. It is probably a combination of both adsorptive and solid solution processes.

The observation that cobalt is highly enriched in MnO_2-rich sediments (Krauskopf, 1955) implies that the Co-MnO_2 association could represent a significant cobalt pool in the oceans. The enrichment factor is from 87 to 870 (Krauskopf, 1955). The high specific adsorption of Co on MnO_2 (Murray et al., 1968) could explain this high enrichment factor. The reason for this high specific adsorption is not known but could be due to the oxidation of Co(II) to Co(III) at the MnO_2-water interface (Murray et al., 1968).

Some of the cobalt in sea water could be associated with both dissolved and particulate organic matter. The amount of cobalt associated with marine phytoplankton and zooplankton is about 3 μg/g

ash (Goldberg et al., 1971). If the concentration of cobalt in sea water is assumed to be 0.3 μg/l, then, in order for 1% of the cobalt to be associated with the particulate organic matter, there would have to be a particulate carbon concentration of 1 mg/l. This conclusion assumes that the cobalt concentration in particulate organic material is equal to that in live organisms. The amount of particulate carbon below the surface waters ranges from 3-10 μg/l and the values in surface waters range up to about 1 mg/l (Menzel and Ryther, 1970). Consequently it is unlikely that an appreciable portion of the cobalt would be associated with the particulate organic matter.

The association of cobalt with dissolved organic compounds is more difficult to evaluate. The amount of dissolved organic carbon in sea water ranges from 0.35 to 2.0 mg/l with values ranging from 0.35 to 0.70 mg/l below a depth of 200 to 300 m (Menzel and Ryther, 1970). The presence of dissolved organic compounds in surface water might be important in that they could chelate a significant portion of the trace metals. If the identity of all the dissolved organics were known along with their stability constants for cations, then it would be possible to predict how much cobalt would be associated with dissolved organic compounds. Some of the dissolved organic compounds in sea water have been identified, but there are probably many more yet to be discovered. It only takes one organic chelator in sea water with the properties of EDTA, and occurring at a concentration of about

10^{-6} M, to effectively complex many of the biologically important trace metals. It is quite possible that a small but significant portion of the cobalt is associated with organic compounds and not in equilibrium with the surrounding medium. This fraction could be taken up preferentially by organisms over ionic or other forms of cobalt. This possibility is especially important for Co(III) organic complexes because they are kinetically inert.

EDTA is not a natural constituent of sea water. However it is commonly used as an additive to laboratory cultures of phytoplankton and it is used in these experiments. Generally it is used to maintain slightly soluble metals such as iron in solution and to detoxify toxic levels of metals such as copper. Conceptually EDTA can be thought of as a trace metal buffer. Duursma (1970) computed that 10^{-6} M EDTA is sufficient to complex all of the Pb^{+2}, Cd^{+2}, Co^{+2}, Cr^{+3}, Ni^{+2}, Cu^{+2}, Zn^{+2}, Fe^{+3}, and Al^{+3} in sea water. The equilibrium constants for some biologically important trace metals and EDTA are given in Table 3.

As can be seen from Table 3 the stability constant for Co(III) EDTA is very high. This complex is also kinetically inert as evidenced by the fact that ionic Co(II) does not exchange with Co(III)-EDTA (Long et al., 1948; Adamson and Vorres, 1956). Consequently it is assumed that Co(III)-EDTA added to seawater does not change its chemical form.

Table 3. Stability constants of some trace metals and EDTA.

Metal	Temperature	Medium	log K_1
Mn^{+2}	20	0.1 (KNO_3)	14.04
Fe^{+2}	25	0.1 (KNO_3)	14.33
Co^{+2}	20	0.1 (KNO_3)	16.31
Zn^{+2}	20	0.1 (KNO_3)	16.50
Cu^{+2}	20	0.1 (KNO_3)	18.80
Fe^{+3}	25	0.1 (KNO_3)	25.1
Co^{+3}	25	0.2 (KNO_3)	40.6

(Taken from Sillén and Martell, 1964)

The Co(II)-EDTA complex is much more labile than Co(III)-EDTA. For instance the reaction rate of isotopic exchange of (Co(II))-(CO(II)-EDTA) is measured in minutes (Long et al., 1948; Krishnan and Jervis, 1967). It is assumed that 10^{-6} M EDTA is sufficient to keep all the Co(II) in sea water as the EDTA complex.

The oxidation of Co(II)-EDTA to CO(III)-EDTA in sea water represents a potential problem in relation to my study. The oxidation couple of Co(II)-Co(III) in the presence of EDTA has an E° value of 0.60 volt (Chaberek and Martell, 1959). If the theoretical value for Eh in sea water (0.75 volt) is assumed to be the actual Eh, the oxidation of Co(II)-EDTA to Co(III)-EDTA would be expected. But if the measured values of Eh (0.40 volt) are correct, only about 0.1% of

the Co(II)-EDTA would be expected to be oxidized to Co(III)-EDTA. Consequently it is uncertain if Co(II)-EDTA becomes oxidized to Co(III)-EDTA in sea water. This uncertainty makes it impossible to make any conclusions about any observation in which there is no difference between treatments of Co(II)-EDTA and Co(III)-EDTA.

METHODS AND MATERIALS

General Laboratory and Culturing Techniques

With the exception of the culture flasks (250 ml Erlenmeyer) all glassware and plasticware were washed with detergent followed by at least three rinses with distilled deionized water. They were then rinsed three times with reagent grade 6N HCl followed by three rinses with distilled deionized water. The culture flasks were subjected to an additional treatment of a one-hour soak in alcoholic sodium hydroxide. After soaking they were rinsed ten times with distilled deionized water.

Sea water to be used for an experiment was treated with activated charcoal (2 g/l) for four hours in a wrist-action shaker. The purpose of the charcoal treatment was to remove dissolved organic compounds which could complex trace metals. After this treatment the sea water was filtered through a 0.22 μm filter. The sea water was then enriched with nutrients (Table 4) and the medium sterile-filtered through a 0.22 μm filter. The culture flasks were covered with aluminum foil and sterilized prior to addition of the sterile media.

All chemicals used were reagent grade, and the solutions were

Table 4. Concentration of added nutrients in enriched media.

Nutrient	Concentration (mg/l) in Media after Addition
KNO_3	100
KH_2PO_4	7.81
Thiamine	0.2

made using distilled deionized water. The concentration of the solutions used for the various treatments was such that 1 ml added to the culture flasks would give the required concentration. The trivalent cobalt used in these experiments was a 1:1 molar ratio of Co(III): EDTA. The Co(III)-EDTA complex was prepared in accordance with Dwyer et al. (1965). The procedure is reproduced in the Appendix.

The inoculations for each experiment were made with cells in the log phase of growth. The cultures were not axenic. Both bacteria and a small (< 1 μm) flagellate were observed in the cultures. Observations of cultures in the log phase of growth indicated that neither the bacteria nor flagellate were present in significant numbers. The cells used for the inoculum were in the same type of medium as the "controls." The term control is used to designate treatments where there are no additions other than the standard nutrients and water blanks. Water blanks of distilled deionized water were used to insure that all treatments had the same salinity. For example, if the treatments for some flasks required two separate additions and for others

only one, those flasks receiving one addition would receive a water blank.

The culture flasks were maintained at a temperature of 18 ± 1°C under seven, continuously operating, General Electric Cool White fluorescent tubes at a height of 60 cm above the culture flasks. The cell concentrations were determined using a Fuchs-Rosenthal counting chamber in the first two experiments. A Coulter Counter, Model B, with a 70 μm aperture was used in experiment number three.

Analysis of Data

Two parameters were used to test for differences between treatments. One was the specific growth rate (k). Specific growth rate is defined as: (increase in number of cells) · (number of cells present^{-1}) · (time)$^{-1}$. The specific growth rate of the population in each culture flask was determined by performing a simple linear regression on the model

$$\ln Y = \ln a + kt$$

Y = cell concentration at the time of sampling

a = Y axis intercept at t = 0

k = specific growth rate

t = time of sampling

The other parameter used to test for differences between treatments was the cell concentration at senescence (k = 0). A one-way analysis

of variance (5%) was used to determine if differences existed between treatments.

Two alternative methods for testing individual comparisons were considered: a sequential variant of the Q method (Snedecor and Cochran, 1967) and the least significant difference (LSD) method. The LSD was not used since this method has the property that as the number of treatments increase, the probability of making an incorrect claim of significance increases. The Q method takes into account the number of treatments so that the probability of no erroneous claim of significance remains ≥ 0.95. The price paid for this protection is that fewer actual differences will be detected. The sequential variant of the Q method provides the same type of protection as the Q method but is more sensitive in detecting real differences.

RESULTS AND DISCUSSION

The hypothesis that trivalent cobalt is the required form of cobalt for a B_{12} producing phytoplankter was suggested by a preliminary experiment to test the toxicity of cobalt to C. huxleyi. The objectives of this experiment were threefold: 1) to determine the concentration at which cobalt was toxic, 2) to determine if EDTA would detoxify toxic levels of cobalt, and 3) to determine how much cobalt became adsorbed onto the walls of the culture flasks.

The sea water used for this experiment was surface water originally collected approximately 500 miles off the Oregon coast, but that had been in a polyethylene carboy for over two years prior to the experiment. After 49 ml of media were placed into the culture flasks, 3.2 nCi (2×10^{-13} M) of isotopically pure (>99%) $^{58}Co(II)$ were added to each flask. The flasks were then inoculated with cells that were in the log phase of growth. At the end of a three-day period, after cell counts showed that the cells were growing, various treatments were added. The treatments (Table 5) were various levels of Co(II) with and without EDTA. The amount of EDTA used was one micromole plus a molar concentration equal to the molar concentration of the added cobalt. The cobalt was premixed with the EDTA.

Table 5. Results of experiment no. 1.

5a. Mean ($\bar{k}$) and standard deviation (s) of specific growth rates.

Treatment	$\bar{k}(hr^{-1})$	s
10 µg/l Co, EDTA	0.0320	±0.0077
1 mg/l Co	0.0238	±0.0052
100 mg/l Co, EDTA	0.0226	±0.0004
1 mg/l Co, EDTA	0.0209	±0.0007
EDTA	0.0148	±0.0046
10 µg/l Co	0.0102	±0.0023
Control	0.0049	±0.0041
100 mg/l Co	(no growth)	------

5b. Analysis of Variance

Source	df	Sum of Squares	Mean Square
Between	6	1004×10^{-6}	167×10^{-6}
Within	7	126×10^{-6}	18×10^{-6}
Total	13	1130×10^{-6}	

F = 9.28

F(5%) = 3.87

Note: The data for 100 ppm was not included in the analysis.

5c. Test Criteria

a[1]	$Q_{0.05} \times S_{\bar{x}}$
7	0.0168
6	0.0161
5	0.0152
4	0.0140
3	0.0125
2	0.0100

[1] a is numerically equal to two plus the number of treatments lying between the means being compared.

Table 5 continued.

5d. Differences[1] of specific growth rates between treatments.

	Control	10 µg/l Co	EDTA	1 mg/l Co, EDTA	100 mg/l Co, EDTA	1 mg/l Co	10 µg/l Co, EDTA
10 µg/l Co, EDTA	0.0271	0.0218	0.0172	0.0111	0.0094	0.0082	0
1 mg/l Co	0.0189	0.0136	0.0090	0.0029	0.0012	0	
100 mg/l Co, EDTA	0.0177	0.0124	0.0078	0.0017	0		
1 mg/l Co, EDTA	0.0160	0.0107	0.0061	0			
EDTA	0.0090	0.0046	0				
10 µg/l Co	0.0053	0					
Control	0						

[1] All differences to the left and above the line are significant ($Q_{0.05}$).

In order to preclude bias in adding the treatments, each flask was randomly assigned a treatment. All treatments were run in duplicate. After the addition of the treatments, the cell concentrations were determined every 48 hours over a six-day period.

The results of the adsorption portion of the experiment are not critical in relation to this thesis; consequently, they are presented in the Appendix. Because the amount of cobalt adsorbed onto the glass was in all cases less than 5%, it was not considered to be a significant problem. The means and standard deviation of the specific growth rate (k) for two flasks for each treatment are given in Table 5a. The means are ordered from highest to lowest. Table 5b gives the results of the analysis of variance used to calculate the F ratio. Table 5c gives the test criteria ($Q_{0.05}$) used in determining what significant differences existed between the specific growth rates of the treatments. Table 5d gives the difference of specific growth rate between treatments with the first column being the difference between the treatment with the lowest specific growth rate and each of the other treatments. All differences lying above or to the left of the line are significant ($Q_{0.05}$).

The data indicate that cobalt at a concentration of 100 mg/l is toxic, but this same level of cobalt with EDTA is not. Based on this one experiment with duplication of treatments, a toxic level of cobalt is detoxified by EDTA. A high level of cobalt (1 mg/l) or cobalt added

with EDTA resulted in a specific growth rate significantly higher than the control, but EDTA by itself or 10 μg/l cobalt did not result in a significant increase in k over the control. Because a cobalt concentration of 10 μg/l is at least an order of magnitude higher than that in normal sea water, it would appear likely that 10 μg/l would be more than adequate to replace any cobalt deficiency. This apparently was not the case in this experiment; only a relatively high concentration of cobalt (1 mg/l) without EDTA was able to stimulate the apparent growth deficiency. Because cobalt with EDTA resulted in a specific growth rate higher than the control in all cases, it appears as though complexed cobalt is more efficacious than the ionic form in promoting growth. The fact that EDTA by itself did not have a significant effect in relation to the control suggests that the amount of cobalt in the medium was insufficient for optimum growth. It is assumed that a micromole of EDTA is sufficient to complex all the cobalt.

There are alternatives to the conclusion that complexed cobalt is more available than ionic cobalt. One alternative is that some trace metal contaminant was added with the cobalt and the observed differences in growth rate were due to a response to the contaminant and not the cobalt. To test this hypothesis the concentrations of various biologically important trace metals in the original cobalt stock solution were determined. The determination was made with a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer. The

results are in Table 6. The contamination of any of the metals tested is at least two orders of magnitude lower than natural concentrations. Even though other metals that were not tested for could be present in significant amounts, it was considered unlikely that the observed response was due to contamination.

There is another alternative to the conclusion the complexed form of cobalt is more available than the ionic form. The cobalt in the cobalt-EDTA stock solution was supposedly Co(II)-EDTA but it is possible that some portion of the cobalt became oxidized to Co(III)-EDTA. If this oxidation occurred, the observed differences in response in the first experiment could be attributed to differences in the oxidation state of the complexed cobalt.

The testing of the hypothesis that Co(III)-EDTA is better in

Table 6. Trace element contamination in the stock cobalt solution.

Trace Metal	Concentration Found Stock Solution (mg/l)	Increase in Concentration in Culture Flask with 1 ppm Cobalt (ng/l)	Amount in Seawater[1] (µg/l)
Ni	12	24	7
Cr	2.5	5	0.5
Mn	0.34	0.68	2.0
Fe	0.15	0.3	3
Cu	0.15	0.3	3
Zn	0.10	0.2	10
Cd	<0.004	<0.008	0.1

[1] Taken from Goldberg (1972).

stimulating growth than Co(II)-EDTA requires media that are deficient in cobalt. Unfortunately, the water that was used in the first experiment was totally used up. A search was made to find sea water that was cobalt-deficient. Water from several sources was tested for apparent cobalt deficiency. Three of the seven waters tested appeared to be deficient in cobalt. The one that seemed to be the most deficient was of uncertain origin and had been stored in a polyethylene carboy for about two years. It is believed to be surface water because of its low salinity ($\sim$ 30‰). It was decided to use this water for the following experiments.

One interesting observation that came out of the search to find sea water deficient in cobalt was that water taken from below the thermocline was less productive for C. huxleyi than surface water. The productivity of this deep water could be improved by the addition of EDTA. This result is consistent with Barber's et al. (1971) observation that the productivity of freshly upwelled water could be increased by the addition of EDTA, and suggests that the trace metal availability and/or toxicity of deep water is different than surface water.

The objective of the second experiment was to determine which oxidation state and level of added cobalt resulted in specific growth rates higher than the control. One hundred milliliters of medium was pipetted into the culture flasks. The treatments were added and the

flasks inoculated with cells in the log phase of growth. Treatments were made in duplicate. The culture flasks were sampled every 48 hours over a six-day period. The results of the experiment are in Table 7.

The treatment with 1 μg/l Co(III)-EDTA resulted in the highest specific growth rate and it is significantly higher than any other treatment. This result indicates that C. huxleyi is able to utilize a cobalt complex with a very high stability constant ($\log k_1 = 40.6$; Sillén and Martell, 1971). The treatment with 10 μg/l Co(II) and all the treatments with cobalt-EDTA resulted in a significantly higher specific growth rate than the control. The treatments with EDTA alone or with 1 μg/l Co(II) did not have a specific growth rate significantly greater than the control. These results are consistent with the first experiment in that complexed cobalt or a relatively high level of ionic cobalt resulted in a specific growth rate greater than the control. There was no difference between treatments of Co(II) and Co(III)-EDTA when EDTA was added. As was discussed in the section on Cobalt Chemistry, it is uncertain if Co(II)-EDTA becomes oxidized to Co(III)-EDTA. Due to this uncertainty, the observation that there was no difference between Co(II) and Co(III)-EDTA when a micromole of EDTA was added is not grounds for rejecting the hypothesis that Co(III)-EDTA is preferred over Co(II)-EDTA. There is no obvious reason for 1 μg/l Co(III)-EDTA to promote a higher specific growth rate than

Table 7. Results of experiment no. 2.

7a. Mean ($\bar{k}$) and standard deviation (s) of specific growth rates.

Treatment	$\bar{k}(hr^{-1})$	s
1 µg/l Co(III)-EDTA	0.0458	±0.0007
10 µg/l Co(II)	0.0427	±0.0009
1 µg/l Co(III)-EDTA,EDTA	0.0425	±0.0004
10 µg/l Co(II), EDTA	0.0424	±0.0007
1 µg/l Co(II), EDTA	0.0419	±0.0006
EDTA	0.0406	±0.0002
1 µg/l Co	0.0400	±0.0008
Control	0.0384	±0.0019

7b. Analysis of Variance

Source	df	Sum of Squares	Mean Square
Between	7	68.40×10^{-6}	9.77×10^{-6}
Within	8	6.72×10^{-6}	0.84×10^{-6}
Total	15	75.12×10^{-6}	

F = 11.63
F(5%) = 3.50

7c. Test Criteria

a[1]	$Q_{0.05} \times S_{\bar{x}}$
8	0.0035
7	0.0034
6	0.0033
5	0.0031
4	0.0029
3	0.0026
2	0.0021

[1] a is numerically equal to two plus the number of treatments lying between the means being compared.

Table 7 continued.

7d. Differences[1] of specific growth rates between treatments.

	Control	1 µg/l Co(II)	EDTA	1 µg/l Co(II), EDTA	10 µg/l Co(II), EDTA	1 µg/l Co(III)-EDTA, EDTA	10 µg/l Co(II)	1 µg/l Co(III)-EDTA
1 µg/l Co(III)-EDTA	0.0075	0.0058	0.0053	0.0039	0.0034	0.0034	0.0032	0
10 µg/l Co(II)	0.0043	0.0027	0.0021	0.0008	0.0003	0.0002	0	
1 µg/l Co(III)-EDTA, EDTA	0.0041	0.0025	0.0019	0.0006	0.0001	0		
10 µg/l Co(II), EDTA	0.0041	0.0024	0.0019	0.0005	0			
1 µg/l Co(II), EDTA	0.0036	0.0019	0.0014	0				
EDTA	0.0022	0.0006	0					
1 µg/l Co(II)	0.0017	0						
Control	0							

[1] All differences to the left and above the line are significant ($Q_{0.05}$).

1 μg/l Co(III)-EDTA, EDTA. It can only be assumed that the observed difference was due to the EDTA. Consequently, any test to distinguish between the efficacy of complexed forms of cobalt versus the oxidation state must include EDTA as a constant treatment, not as a variable one.

Since the results of the second experiment do not provide the basis for a rejection of the hypothesis that Co(III)-EDTA is preferred over Co(II)-EDTA, a third experiment was done. There are four primary reasons why the second experiment was not an adequate test of the hypothesis. The first is that with only duplicates of the same treatment, statistical tests of differences between treatments are not very sensitive and actual differences may not be detected. Consequently, there are five replicates of each treatment in the third experiment. The second pertains to the uncertainty of Co(II)-EDTA becoming oxidized to Co(III)-EDTA. If there is an actual preference for Co(III)-EDTA and if a sufficient amount of Co(II)-EDTA becomes oxidized to Co(III)-EDTA, differences between treatments of Co(II)-EDTA and Co(III)-EDTA will not be observed. One way of reducing the amount of Co(III)-EDTA being oxidized from Co(II)-EDTA is to reduce the concentration of the Co(II)-EDTA. The levels of the added cobalt were changed from 10 and 1 μg/l in the second experiment to 1 and 0.1 μg/l in the third experiment. The third reason pertains to the use of EDTA. The interaction of EDTA complexes with organisms is not known,

consequently the addition of EDTA might cause an unknown confounding affect on the cultures. Since EDTA is added to phytoplankton cultures to make trace metals available it was believed that the addition of a combination of trace metals could possibly replace any beneficial effect of EDTA. The third experiment has a combination of trace metals as one of the treatments. The concentration of each metal used is listed in Table 8. With the exception of copper, all the metals are added at approximately the same level as they are found in natural sea water. Due to the potential toxicity of copper, only 1 μg/l was added. The fourth reason is that the results of the first two experiments were based only on differences between the specific growth rates of the various treatments. These differences may not be due to some intrinsic response of C. huxleyi, but rather to the rate at which cobalt becomes available in the media. Assuming that there

Table 8. Concentration of trace metals added in experiment no. 3.

	Concentration (μg/l) above Ambient Level in Flask after Addition	Concentration (μg/l) in Seawater[1]
Al	1	1
Cr	0.5	0.5
Cu	1	3
Fe	5	3
Mn	2	2
Mo	10	10
Zn	10	10

[1] From Goldberg (1971).

is an unavailable form (UF) and available form (AF) of cobalt, the following model can be used to describe the availability of cobalt to an organism:

$$\boxed{\text{UF}} \rightleftarrows \boxed{\text{AF}} \rightarrow \boxed{\text{Organism}}$$

If the amount of the available form is always in excess of the organism's needs, then the growth rate should be independent of the cobalt concentration. But if the available form is produced at a rate lower than required to sustain the organism's needs, the growth rate of the organism should reflect the rate of formation of the available form. Another test criterion that can be used (beside specific growth rate) is the cell concentration of the senescent population ($k = 0$). This should reflect the total amount of cobalt available in a cobalt-deficient medium. A problem with this test criterion is that exocrines might change the chemical form of the added cobalt. In the third experiment, data to determine both the specific growth rate and the concentration of cells in the senescent population were obtained.

The source of sea water used for the final experiment was the same as that used for the second experiment. In addition to the standard nutrients, 2.36 mM $NaHCO_3$ were added. The purpose for adding $NaHCO_3$ was to increase the buffering capacity of the medium so that the change of pH during the course of the experiment would be minimized. The enriched medium was placed in a plastic carboy and inoculated with cells in the log phase of growth. The enriched

medium and cells were well mixed and 150 ml were placed into each culture flask. The treatments were then added. The treatments are listed in both Tables 9 and 10. All EDTA additions were one micromolar. In order to insure that the Co(II)-EDTA treatments were added as the complex, the Co(II)-EDTA stock solution was adjusted to pH 1-2 with 0.3 N HCl and heated at 80°C for 30 minutes. After this treatment the solution was brought to pH 7 by the addition of 0.3 N NaOH. In order to preclude the HCl-NaOH from becoming a treatment, a blank made of the HCl and NaOH used above was made and added as appropriate. The individual components of the trace metals (TM) to be added were dissolved separately and brought to the correct volume. Aliquots of the metal solutions were taken and added together so that a 1 ml addition of the mixed solution would give the proper trace metal concentration. The 0.1 μg/l Co(III)-EDTA, TM treatment was originally intended to be 1.0 μg/l Co(III)-EDTA, TM but there was an error in dilution that was not detected until after the start of the experiment. Using a Coulter Counter Model B, cell concentration was measured every 12 hours over a period of five days. At the end of this time, the end of the log phase was detected and the cell concentration was then measured on a daily basis until senescence had been reached. Data from the first nine sampling times were used to calculate the specific growth rate of each flask. An average of two separate samples was used to determine the cell concentration at

Table 9. Results of experiment no. 3 for specific growth rate.

9a. Mean ($\bar{k}$) and standard deviation (s) of specific growth rates.

Treatment	$\bar{k}(hr^{-1})$	s
EDTA	0.0403	±0.0003
0.1 µg/l Co(II), EDTA	0.0401	±0.0005
0.1 µg/l Co(III)-EDTA, EDTA	0.0400	±0.0007
1.0 µg/l Co(II), EDTA	0.0400	±0.0003
1.0 µg/l Co(III)-EDTA, EDTA*	0.0397	±0.0005
1.0 µg/l Co(II), TM	0.0382	±0.0002
Control	0.0376	±0.0005
0.1 µg/l Co(III)-EDTA, TM*	0.0351	±0.0004
TM	0.0345	±0.0008

*One flask spilled during course of experiments. Consequently, there are only four replicates.

9b. Analysis of Variance

Source	df	Sum of Squares	Mean Square
Between	8	184.83×10^{-6}	23.10×10^{-6}
Within	34	8.92×10^{-6}	0.26×10^{-6}
Total	42	193.75×10^{-6}	

F = 88.8
F(5%) = 2.23

9c. Test Criteria

a[1]	$Q_{0.05} \times S_{\bar{x}}$
9	0.0011
8	0.0011
7	0.0010
6	0.0010
5	0.0010
4	0.0009
3	0.0008
2	0.0007

[1] a is numerically equal to two plus the number of treatments lying between the means being compared.

Table 9 continued.

9d. Differences[1] of specific growth rates between treatments.

	TM	0.1 µg/l Co(III)-EDTA, TM	Control	1.0 µg/l Co(II), TM	1.0 µg/l Co(III)-EDTA, EDTA	1.0 µg/l Co(II), EDTA	0.1 µg/l Co(III)-EDTA, EDTA	0.1 µg/l Co(II), EDTA	EDTA
EDTA	0.0058	0.0052	0.0027	0.0020	0.0005	0.0003	0.0003	0.0002	0
0.1 µg/l Co(II), EDTA	0.0056	0.0050	0.0025	0.0018	0.0003	0.0001	0.0001	0	
0.1 µg/l Co(III)-EDTA, EDTA	0.0055	0.0049	0.0024	0.0018	0.0003	0.0000	0		
1.0 µg/l Co(II), EDTA	0.0055	0.0049	0.0024	0.0018	0.0003	0			
1.0 µg/l Co(III)-EDTA, EDTA	0.0052	0.0046	0.0021	0.0015	0				
1.0 µg/l Co(II), TM	0.0037	0.0031	0.0006	0					
Control	0.0031	0.0020	0						
0.1 µg/l Co(III)-EDTA, TM	0.0010	0							
TM	0								

[1] All differences lying to the left and above the line are significant ($Q_{0.05}$).

Table 10. Results of experiment no. 3 for cell concentration at senescence.

Treatment	$\bar{x}$ (cells/mm^3)	s
1.0 µg/l Co(II), TM	4580	±253
1.0 µg/l Co(III)-EDTA, EDTA*	4532	±637
1.0 µg/l Co(II), EDTA	4341	±771
0.1 µg/l Co(II), EDTA	4258	±529
0.1 µg/l Co(III)-EDTA, EDTA	3597	±376
EDTA	3268	±108
0.1 µg/l Co(III)-EDTA TM*	3112	±357
Control	3029	±400
TM	2446	±689

*One flask spilled during course of experiments. Consequently, there are only four replicates.

10b. Analysis of Variance

Source	df	Sum of Squares	Mean Square
Between	8	22720×10^3	2840×10^3
Within	34	8507×10^3	250×10^3
Total	42	31227×10^3	
F = 11.35			
F(5%) = 2.23			

10c. Test Criteria

a[1]	$Q_{0.05} \times S_{\bar{x}}$
9	1071
8	1046
7	1014
6	977
5	934
4	874
3	792
2	659

[1] a is numerically equal to two plus the number of treatments lying between the means being compared.

Table 10 continued.

10d. Differences[1] of cell concentration at senescence between treatments.

	TM	Control	0.1 µg/l Co(III)-EDTA, TM	EDTA	0.1 µg/l Co(III)-EDTA, EDTA	0.1 µg/l Co(II), EDTA	1.0 µg/l Co(II), EDTA	1.0 µg/l Co(III)-EDTA, EDTA	1.0 µg/l Co(II), TM
1.0 µg/l Co(II), TM	2134	1552	1469	1313	983	323	239	48	0
1.0 µg/l Co(III)-EDTA, EDTA	2086	1503	1420	1264	935	274	190	0	
1.0 µg/l Co(II), EDTA	1895	1313	1230	1074	744	84	0		
0.1 µg/l Co(II), EDTA	1811	1229	1146	990	660	0			
0.1 µg/l Co(III)-EDTA, EDTA	1151	569	486	330	0				
EDTA	821	239	156	0					
0.1 µg/l Co(III)-EDTA, TM	665	83	0						
Control	582	0							
TM	0								

[1] All differences lying to the left and above the line are significant ($Q_{0.05}$).

senescence. The results of this experiment are presented in Tables 9 and 10.

The results show that each treatment with EDTA resulted in a higher specific growth rate than the control (Table 9). With the possibility of one exception, this observation is consistent with experiment no. 2. The exception is that in the second experiment, EDTA without added cobalt did not result in a specific growth rate significantly higher than the control. The standard deviation of the control treatment in the second experiment is about three times as large as the standard deviations of the other treatments in that experiment. If the standard deviation of the control in the second experiment were to be arbitrarily reduced to the same level as the rest of the treatments, the results of the EDTA treatment in the second experiment would be significantly greater than the control. With this reduction, the differences in results between experiments 2 and 3 in regard to the EDTA treatment would not exist.

As in the second experiment, there were no differences in the specific growth rates between treatments of Co(II) and Co(III)-EDTA when EDTA was added. If it could be demonstrated that Co(II)-EDTA in these cultures did not oxidize to Co(III)-EDTA, this observation and the corresponding one in experiment no. 2 could be a basis for rejecting the hypothesis that Co(III)-EDTA is preferred over Co(II)-EDTA. Unfortunately it is not possible to measure the amount of Co(III)-EDTA

oxidized from Co(II)-EDTA at the concentrations used in these experiments. Consequently these observations of no differences between treatments cannot be used to reach any conclusion regarding the hypothesis.

As has been noted, the purpose of the trace metals was to replace any beneficial effect of EDTA. This obviously did not happen. Both the TM and 0.1 μg/l Co(III)-EDTA, TM had a specific growth rate significantly lower than the control. There was no difference in the specific growth rate between the control and 1.0 μg/l Co(II), TM. The treatment 0.1 μg/l Co(III)-EDTA, TM had a higher specific growth rate than the TM. Since this particular treatment was done in only one experiment any interpretation of the observations must be done with caution. One interpretation is that the TM decreased the rate at which cobalt became available. This decrease in cobalt availability could have been due to cobalt sorbtion onto solid phases of Fe, Al, and/or Mn. The observation that 1 μg/l Co(II) was able to remove any limiting effects due to the TM treatment could indicate that C. huxleyi does not require Co(III) as a cobalt source.

When the cell concentration at senescence is used as a test criterion (Table 10), there is some evidence to reject the hypothesis that Co(III)-EDTA is preferred over Co(II)-EDTA. With the exception of 0.1 μg/l Co(III)-EDTA, EDTA, all additions of EDTA with cobalt, and the treatment with 1 μg Co(II), TM, resulted in a higher final cell

concentration than the control. If Co(III)-EDTA is the preferred form, then the treatment with 0.1 μg/l Co(III)-EDTA, EDTA should have resulted in a cell concentration at least as high as 0.1 μg/l Co(II), EDTA. The treatment with EDTA alone did not result in a value significantly higher than the control. This could indicate, in conjunction with the results of the specific growth rate, that EDTA in batch culture experiments affects only the rate at which cobalt becomes available and not the total amount available. The observation that the treatment with TM resulted in a final population no different than the control implies that while the TM apparently affects the rate at which C. huxleyi grows, it does not affect the final total population in a batch culture. It is suspected that the level of dissolved organic compounds is significant in media in which the cells have reached senescence. Since these dissolved organic compounds could affect the physical-chemical forms of the trace metals present, the conclusions above are drawn with some uncertainty.

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APPENDIX

APPENDIX

Sorbtion of Cobalt onto Culture Flasks

The purpose of this experiment was to determine how much cobalt became sorbed onto the surfaces of the culture flasks. Cobalt sorbed onto the surface of the culture flasks might not be available to the organisms. If sorbed cobalt is not a linear function of the cobalt concentration, it would be a problem in interpreting the response of C. huxleyi to various levels of added cobalt.

The level of radioactivity of ^{58}Co to be added to each flask in experiment no. 1 was measured prior to addition. All measurements of radioactivity were made using gamma-ray spectrometry. The amount of radioactivity removed during the course of the experiment was measured. At the end of the experiment the radioactivity in the remaining media was measured. The culture flasks were rinsed with filtered sea water and the radioactivity removed was assumed not to represent ^{58}Co sorbed to the flasks. The flasks were then rinsed with 3 N HCl. The radioactivity removed with the HCl was assumed to be sorbed ^{58}Co. The percent ratio of the radioactivity recovered to that added was calculated. This is labeled as % Recovery in Table 11. The percent sorbed was calculated using the amount of radioactivity

Table 11. Percent ^{58}Co sorbed to the culture flasks.

Treatment	Flask	Percent Sorbed	Percent Recovered
Control	A	2.3	100.7
	B	1.7	101.4
EDTA	A	0.1	99.8
	B	0.0	100.5
10 µg/l Co(II)	A	3.6	101.6
	B	0.2	100.2
1 mg/l Co(II)	A	0.0	101.4
	B	3.1	102.0
100 mg/l Co(II)	A	0	102.5
	B	0	101.7
10 µg/l Co(II), EDTA	A	0	99.9
	B	0.5	100.3
1 mg/l Co(II), EDTA	A	0	101.7
	B	0	101.3
100 mg/l Co(II), EDTA	A	0	99.3
	B	0	99.6

removed by the HCl rinse and the amount of radioactivity recovered.

Preparation of Potassium Ethylenediaminetetra-acetocobaltate (III) Dihydrate

A solution of cobalt (II) chloride hexahydrate (8 g), potassium acetate (20 g) and ethylenediametetraacetic acid (10 g) in distilled deionized water (60 ml) was heated to nearly boiling. Thirty milliliters of 3% hydrogen peroxide was gradually added to the solution.

The solution was cooled to room temperature and approximately 400 ml of 95% ethyl alcohol (ETOH) was added. The flask containing the solution was placed in an ice water bath overnight. The liquid was then decanted from the precipitate. The precipitate was brought into solution by the addition of a minimal amount of distilled deionized water. It was then filtered through a 0.22 μm filter. The solution was then reprecipitated by the addition of 95% ETOH and cooling in an ice water bath. The process of decanting, bringing the salt into solution and precipitation was done one more time. After the final precipitation, the liquid was decanted and the remaining salt dried for 24 hours at a temperature of 95°F (70.6°C).