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Zinc–cobalt colimitation of *Phaeocystis antarctica*

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Abstract

We present evidence demonstrating the capability of *Phaeocystis antarctica* colonies to substitute cobalt (Co) and zinc (Zn) as micronutrients, in which Co limitation is alleviated by additions of Zn and vice versa. Maximal growth rates and biomass were determined by fluorescence and the values obtained under replete Zn and no added Co conditions were significantly higher than under replete Co and no added Zn conditions, suggesting a preference for Zn over Co. The observation of Zn-Co substitution in this high-latitude member of the Prymnesiophyceae class, coupled with similar previous observations in the coccolithophore *Emiliana huxleyi* and several centric diatoms, suggests that Zn-Co substitution could be a widespread global phenomenon in eukaryotic phytoplankton. The Zn-Co biochemical substitution seen in *Phaeocystis* might be the result of evolutionary pressure for maintaining growth rates in high export environments in which rapid depletion of Zn, Co, and carbon occur simultaneously in the upper water column.

The influence of trace element nutrition on phytoplankton has been studied in a variety of representative species, but relatively little work has been done on phytoplankton from high-latitude environments. *Phaeocystis* sp. is a cosmopolitan marine phytoplankter found in low-temperature marine environments throughout the oceans. Species of *Phaeocystis* are known to be key components of the phytoplankton community in many environments (Schoemann et al. 2005). For example, the strain *Phaeocystis antarctica* is a major component of the phytoplankton community structure in the Ross Sea of Antarctica, exerting its biogeochemical influence through both carbon export and dimethyl sulfide production (DiTullio et al. 2000 and references therein). During the annual bloom, *P. antarctica* often forms large spherical colonies with cells distributed outside of a mucilaginous center (Scott and Marchant 2005). The phytoplankton productivity of the Ross Sea is known to be subject to seasonal iron limitation (Sedwick et al. 2000), whereas the influence of zinc (Zn) and manganese (Mn) has not been observed to have any noticeable effect on chlorophyll *a* (Chl *a*) concentrations in bottle incubations (Sedwick et al. 2000; Cochlan et al. 2002). The influences of other micronutrients such as cobalt (Co), cadmium (Cd), and vitamin B_{12} have not been studied in this environment until recently (Bertrand et al. 2007). Yet, analyses of field concentrations of metals have found that elements such as iron, Co, Cd, and Zn can all be drawn down to low concentrations in the Ross Sea surface waters (Fitzwater et al. 2000; Bertrand et al. 2007).

The nutritional importance of elements such as Co, Cd, and Zn to marine phytoplankton has been the subject of numerous studies in recent years (Morel et al. 1994; Saito et al. 2003, and references therein). In several species of centric marine diatoms (*Thalassiosira weissflogii, Thalassiosira oceanica,* and *Thalassiosira pseudonana*), a physiological Zn requirement can be replaced by either Co or Cd (Price and Morel 1990; Sunda and Huntsman 1995). Moreover, this requirement is connected to the carbon acquisition system on the basis of physiological evidence demonstrating colimitation by carbon when under Zn limitation (Morel et al. 1994), as well as molecular evidence demonstrating the presence of Zn and Cd in the active site of two carbonic anhydrase enzymes purified and sequenced from these phytoplankton (Roberts et al. 1997; Lane et al. 2005). In contrast to these studies, the diatom *Chaetoceros calcitrans* appears to lack the Zn-Co substitution capability (Timmermans et al. 2001), although the molecular basis for this is unknown. Among the coccolithophores, *Emiliania huxleyi* demonstrates a Co-Zn substitution capability (Sunda and Huntsman 1995), although a Cd substitution capability for Zn has not yet been reported for this organism (Sunda and Huntsman 2000). In stark contrast to these centric diatoms and coccolithophores, the marine cyanobacteria (e.g., *Prochlorococcus* and *Synechococcus*) have an absolute Co requirement and a small to nondetectable zinc requirement, and appear incapable of Zn-Co substitution (Sunda and Huntsman 1995; Saito et al. 2002). In addition to these biochemical differences, the metal acquisition capabilities of different groups of phytoplankton can also vary significantly. This is largely because of the presence of organic metal–ligand complexes in seawater that can interfere with the metal ion transport machinery of the phytoplankton cells (Bruland 1992; Saito and Moffett 2001; Ellwood 2004 and references therein). At this time, little is known about how different eukaryotic phytoplankton groups interact with metal–ligand complexes, although there is some evidence that the cyanobac-

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teria may be both synthesizing and utilizing Co–ligand complexes (Saito et al. 2002, 2005) and that many eukaryotic algae have iron (Fe) reductases to acquire Fe from Fe–ligand complexes (Maldonado and Price 1999).

The extent to which other trace elements can substitute for the Zn requirement in phytoplankton is only known for the handful of laboratory cultures described above, and currently, very little information exists for high-latitude, low-temperature phytoplankton strains. Moreover, the trace metal nutritional requirements of *Phaeocystis* have not been examined, with the exception of a recent study of its Fe requirements (Sedwick et al. 2007). In this manuscript, we describe the trace metal physiology of *P. antarctica* with respect to the micronutrients Co and Zn under colimiting conditions. This notion of bioinorganic colimitation is distinct from other types of colimitation often described in oceanography and limnology, as described in the accompanying manuscript (Saito et al. 2008).

Materials and methods

Phaeocystis antarctica (strain CCMP 1871) was obtained from the Bigelow Provasoli–Guillard National Center and maintained by means of sterile technique until required for experiment inoculation. Sample, media, and reagent bottles were rigorously cleaned to remove trace metal contamination with a minimum washing procedure of a 48-h detergent soak, five rinses in milli-Q water, a 7-d soak in 10% HCl (Baker Instra-Analyzed), and several final rinses in very dilute acid (HCl, pH 2). Experiments were performed in cleaned and sterilized 28-mL polycarbonate tubes, and all solutions were pipetted after three tip rinses with 10% trace metal–grade HCl and sterile dilute HCl (pH 2). Culture and laboratory work were conducted in a clean room environment that exceeded Class-100 specifications.

The trace metal culture medium was designed to be comparable to that used by Sunda and Huntsman (1995) for trace metal experimentation. The medium was made from a 0.2-μm–filtered Sargasso seawater base and was microwave-sterilized before amendments. Macronutrients were added to a final concentration of 88.2 μmol L^{-1} $NaNO_3$, 41.7 μmol L^{-1} NaH_2PO_4, and 106 μmol L^{-1} Na_2SiO_3 and were chelexed before use (Price et al. 1988/1989). Vitamin amendments consisted of 0.4 nmol L^{-1} biotin, 60 nmol L^{-1} thiamine, and 0.074 nmol L^{-1} vitamin B_{12} and were also chelexed before use. Trace elements were added to final concentrations of 10 μmol L^{-1} $FeCl_3$, 48 nmol L^{-1} $MnCl_2$, 40 nmol L^{-1} $CuCl_2$, 100 nmol L^{-1} $NiCl_2$, and 10 nmol L^{-1} Na_2O_3Se within a 100–μmol L^{-1} ethylenediamine tetraacetic acid (EDTA) metal ion buffer system to regulate the free metal concentrations. Zn and Co were added separately from sterile, weakly acidified stocks (pH 2) at a range of concentrations as reported in Tables 1 and 2. The macronutrient concentrations used here are similar to that of f/2 media, whereas the trace metal mix is similar to the Sunda and Huntsman (1995) recipe. We do not believe these high-nutrient concentrations had an effect on metal speciation or solubility given the high media concentrations of EDTA, use of microwave sterilization before nutrient amendments instead of the standard autoclaving of media (the latter of which often causes precipitation), and the reproducibility of our experiments. All amendments were sterile-filtered, and media were equilibrated for at least 24 h before inoculation.

After first establishing cultures in our trace metal clean media for several months, *Phaeocystis* cells were then acclimated in a low-metal media with 10 nmol L^{-1} total Zn and Co on the basis of preliminary experiments that identified the range of onset of limitation. Free ion concentrations are calculated using thermodynamic databases with $M^{2+}:M_{Total}$ ratios (where M = metal) of $10^{-3.99}$ and $10^{-3.63}$ for Zn and Co (Sunda and Huntsman 1995), respectively, and specific to this metal ion buffer concentration. The trace quantities of Co and Zn in the Sargasso seawater base were measured and included in calculations as shown in Tables 1 and 2.

This acclimated *Phaeocystis* culture (10 nmol L^{-1} Zn and Co) was used to initially inoculate all our experiments with volumes of 3.6%. Two types of experiments were conducted. First, simple limitation experiments were performed with Zn omitted and a range of Co concentrations, as well as vice versa (Table 1). Second, matrix experiments were conducted wherein Zn and Co were both varied (Table 2), allowing a three-dimensional (3D) image of growth rates, as we have used previously (Saito et al. 2002). The T1 (transfer 1) cultures in the simple experiment were inoculated with the acclimated *Phaeocystis* culture (10 nmol L^{-1} Zn and Co) described above. The T2 (transfer 2) cultures in the simple experiment were inoculated with the T1 culture of equivalent or the closest equivalent concentrations. In contrast, the first and second transfers of the matrix experiments were inoculated in each case from a culture with 10 nmol L^{-1} total Co and Zn. This difference in inoculation source was used for practical reasons and appears to have slightly influenced the degree of limitation observed for each metal, likely because of some small residual carryover. As a result, the 3D matrix experiment is most useful for visualization of biochemical substitution, whereas the simple limitation experiments are better suited for assessments of K_m values.

Cultures were grown in incubators set at 2°C with a 16 : 8 light : dark cycle at 60 μmol m^{-2} s^{-1}, comparable to previous *Phaeocystis* culture studies (Schoemann et al. 2005), with cultures randomly repositioned each day to avoid subtle variations in light intensity. Growth rate was measured by monitoring fluorescence (Turner Instruments TD-700) as a proxy for Chl *a* and is reported as relative fluorescence units. Growth rates were calculated from the exponential portion of the growth curve. It should also be pointed out that the measurement of growth rates in *Phaeocystis* is confounded by its formation of colonies. The measurement of in vivo relative fluorescence should be proportional to Chl *a* in the cultures and hence should be able to measure the initial solitary forms as well as the colonial forms that develop, albeit with some experimental variability associated with self-shading when the colonies are particularly dense. Half-saturation constants for growth and maximal growth rates in Table 3 were generated with a nonlinear fitting function.

Table 1. Effects of Zn and Co on growth rates and relative biomass yields for simple experimental design (only one element varied). Added Zn and Co are adjusted for the initial seawater metal blank then converted to free metal ion concentrations (M^{2+}) on the basis of equilibrium with the metal ion buffer EDTA. Two transfers (T1 and T2) and their range of metal treatments are listed. Each treatment was conducted in duplicate (A and B).

Transfer	Added [Co] (nmol L^{-1})	Added [Zn] (nmol L^{-1})	Total [Co]* (nmol L^{-1})	Total [Zn]† (nmol L^{-1})	log [Co^{2+}] (mol L^{-1})	log [Zn^{2+}] (mol L^{-1})	Growth rate replicate A	Growth rate replicate B	Growth rate average	Yield,‡ replicate A, RFU_{max}	Yield,‡ replicate B, RFU_{max}	Yield average
T1-1	0	0	0.25	0.40	−13.23	−13.39	0.08	0.10	0.09	144.50	168.20	156.35
T1-2	0.3	0	0.55	0.40	−12.89	−13.39	0.10	0.10	0.10	182.00	171.70	176.85
T1-3	1	0	1.25	0.40	−12.53	−13.39	0.11	0.10	0.11	228.40	225.20	226.80
T1-4	3	0	3.25	0.40	−12.12	−13.39	0.14	0.15	0.14	374.10	365.90	370.00
T1-5	10	0	10.25	0.40	−11.62	−13.39	0.16	0.14	0.15	424.20	497.50	460.85
T1-6	30	0	30.25	0.40	−11.15	−13.39	0.15	0.13	0.14	522.30	625.50	573.90
T1-7	0	0.3	0.25	0.70	−13.23	−13.14	0.11	0.10	0.10	173.40	188.80	181.10
T1-8	0	1	0.25	1.40	−13.23	−12.84	0.12	0.11	0.11	199.10	228.70	213.90
T1-9	0	3	0.25	3.40	−13.23	−12.46	0.14	0.15	0.15	375.40	378.40	376.90
T1-10	0	10	0.25	10.40	−13.23	−11.97	0.21	0.19	0.20	706.80	767.30	737.05
T1-11	0	30	0.25	30.40	−13.23	−11.51	0.19	0.20	0.19	937.20	952.60	944.90
T2-1	0.00	0	0.25	0.40	−13.23	−13.39	0.04	0.04	0.04	7.77	6.22	6.99
T2-2	0.3	0	0.55	0.40	−12.89	−13.39	0.05	0.04	0.05	9.69	5.81	7.75
T2-3	1	0	1.25	0.40	−12.53	−13.39	0.07	0.07	0.07	13.23	10.41	11.82
T2-4	3	0	3.25	0.40	−12.12	−13.39	0.09	0.08	0.09	19.39	12.42	15.91
T2-5	10	0	10.25	0.40	−11.62	−13.39	0.09	0.11	0.10	16.25	28.25	22.25
T2-6	30	0	30.25	0.40	−11.15	−13.39	0.09	0.10	0.10	17.76	33.90	25.83
T2-7	100	0	100.25	0.40	−10.63	−13.39	NA	0.12	0.12	NA	71.79	71.79
T2-8	300	0	300.25	0.40	−10.15	−13.39	0.14	0.14	0.14	71.05	70.54	70.80
T2-9	0	0.3	0.25	0.70	−13.23	−13.14	0.04	0.04	0.04	9.10	6.99	8.05
T2-10	0	1	0.25	1.40	−13.23	−12.84	0.04	0.04	0.04	14.62	9.01	11.81
T2-11	0	3	0.25	3.40	−13.23	−12.46	0.06	0.06	0.06	126.50	56.90	91.70
T2-12	0	10	0.25	10.40	−13.23	−11.97	0.13	0.13	0.13	148.70	159.80	154.25
T2-13	0	30	0.25	30.40	−13.23	−11.51	0.18	0.22	0.20	276.90	201.90	239.40
T2-14	0	100	0.25	100.40	−13.23	−10.99	0.20	0.20	0.20	161.90	141.30	151.60
T2-15	0	300	0.25	300.40	−13.23	−10.51	0.25	0.26	0.26	116.80	119.20	118.00

* Includes 0.252 nmol L^{-1} Co in media base.
† Includes 0.4 nmol L^{-1} Zn in media base.
‡ RFU = relative fluorescence units.

Total Co analysis of the seawater media blank was performed by adsorptive cathodic stripping voltammetry with an electrochemical hanging mercury drop system as described previously (Saito and Moffett 2002). The media blank for total dissolved Zn was analyzed by isotope dilution magnesium (Mg) precipitation preconcentration inductively coupled plasma mass spectrometry (ICP-MS) according to a slightly modified technique for Fe, Cd, and Mn from previous studies (Saito and Schneider 2006). In this instance, 10 mL of media was acidified with trace metal–clean concentrated HCl (Seastar Inc.) to a pH of ~2, spiked with a ^{67}Zn isotope, and allowed to equilibrate overnight. The sample was then precipitated with ammonia (Seastar Inc.), centrifuged at 3,000 rpm (1,460 × g) in a Brinkmann 5810 swinging bucket centrifuge, decanted, recentrifuged, and decanted again. The precipitate was resuspsended in 0.5 mL of 5% nitric acid (Seastar Inc.) and analyzed at medium resolution on an Element 2 ICP-MS equipped with an Aridus and autosampler (Saito and Schneider 2006). Isotope dilution calculations were performed with the use of ^{66}Zn and ^{67}Zn isotopes.

Results and discussion

Zn-Co substitution in Phaeocystis: Influence on growth rates and biomass—In this study, we present physiological evidence demonstrating the capability of *P. antarctica* to substitute Zn and Co as micronutrients. The influence of Zn and Co on phytoplankton was assessed both in terms of growth rates as well as biomass yields. In the simple substitution experiments in which one micronutrient was varied with none of the second nutrient added, Co substitution for Zn was evident in the calculated growth rates (Fig. 1A,B; Table 1). The converse scenario, Zn substitution for Co, led to a more pronounced increase in growth rates, suggesting a "preference" for Zn (Fig. 1A,B; Table 1). This Zn preference was also evident in the biomass yield (Fig. 1C,D; Table 1), defined here as the maximal biomass on reaching stationary phase for the culture. The experiments in which Co was withheld and Zn modulated had significantly higher biomass yields than vice versa. In addition, biomass yields decreased significantly with decreases in Co or Zn, demonstrating limitation of

Table 2. Effects of Zn and Co on growth rates and relative biomass yields for matrix experimental design (Zn and Co covaried). Added Zn and Co are adjusted for the initial seawater metal blank prior, then converted to free metal ion concentrations (M^{2+}) on the basis of equilibrium with the metal ion buffer EDTA. Two transfers (T1 and T2) and their range of metal treatments are listed.

Transfer	Added [Co] (nmol L^{-1})	Added [Zn] (nmol L^{-1})	Total [Co]* (nmol L^{-1})	Total [Zn]† (nmol L^{-1})	log[Co^{2+}] (mol L^{-1})	log[Zn^{2+}] (mol L^{-1})	Growth rate (d^{-1})	Yield‡ (RFU_{max})
T1-1	0	0	0.18	0.40	−13.38	−13.39	0.14	112.4
T1-2	0.3	0	0.48	0.40	−12.95	−13.39	0.15	111.6
T1-3	3	0	3.18	0.40	−12.13	−13.39	0.17	140.8
T1-4	30	0	30.18	0.40	−11.15	−13.39	0.21	232.7
T1-5	300	0	300.18	0.40	−10.15	−13.39	0.23	276.9
T1-6	0	0.3	0.18	0.70	−13.38	−13.14	0.09	66.1
T1-7	1	0.3	1.18	0.70	−12.56	−13.14	0.13	135.9
T1-8	10	0.3	10.18	0.70	−11.62	−13.14	0.17	287.1
T1-9	100	0.3	100.18	0.70	−10.63	−13.14	0.20	251.1
T1-10	0.3	1	0.48	1.40	−12.95	−12.84	0.13	129.8
T1-11	3	1	3.18	1.40	−12.13	−12.84	0.22	298.3
T1-12	30	1	30.18	1.40	−11.15	−12.84	0.25	347.4
T1-26	300	1	300.18	1.40	−10.15	−12.84	0.31	433.1
T1-13	0	3	0.18	3.40	−13.38	−12.46	0.17	191.5
T1-14	1	3	1.18	3.40	−12.56	−12.46	0.17	274.3
T1-15	0.3	10	0.48	10.40	−12.95	−11.97	0.25	426.9
T1-16	10	10	10.18	10.40	−11.62	−11.97	0.27	187.5
T1-17	300	10	300.18	10.40	−10.15	−11.97	0.35	650.2
T1-18	0	30	0.18	30.40	−13.38	−11.51	0.28	602.8
T1-19	1	30	1.18	30.40	−12.56	−11.51	0.27	601.1
T1-20	30	30	30.18	30.40	−11.15	−11.51	0.38	781
T1-21	0.3	100	0.48	100.40	−12.95	−10.99	0.44	747.5
T1-22	0	300	0.18	300.40	−13.38	−10.51	0.34	602.5
T1-23	1	300	1.18	300.40	−12.56	−10.51	0.39	831.9
T1-24	10	300	10.18	300.40	−11.62	−10.51	0.43	833.3
T1-25	300	300	300.18	300.40	−10.15	−10.51	0.47	832
T2-1	0	0	0.18	0.40	−13.38	−13.39	0.16	30.78
T2-2	0.3	0	0.48	0.40	−12.95	−13.39	0.23	44.77
T2-3	3	0	3.18	0.40	−12.13	−13.39	0.24	54.71
T2-4	30	0	30.18	0.40	−11.15	−13.39	0.30	79.65
T2-5	300	0	300.18	0.40	−10.15	−13.39	0.27	82.80
T2-6	0	0.3	0.18	0.70	−13.38	−13.14	0.13	35.73
T2-7	1	0.3	1.18	0.70	−12.56	−13.14	0.17	48.22
T2-8	10	0.3	10.18	0.70	−11.62	−13.14	0.22	67.17
T2-9	100	0.3	100.18	0.70	−10.63	−13.14	0.26	91.10
T2-10	0.3	1	0.48	1.40	−12.95	−12.84	0.22	64.49
T2-11	3	1	3.18	1.40	−12.13	−12.84	0.21	75.83
T2-12	30	1	30.18	1.40	−11.15	−12.84	0.30	117.50
T2-26	300	1	300.18	1.40	−10.15	−12.84	0.29	133.40
T2-13	0	3	0.18	3.40	−13.38	−12.46	0.18	65.05
T2-14	1	3	1.18	3.40	−12.56	−12.46	0.20	85.74
T2-15	0.3	10	0.48	10.40	−12.95	−11.97	0.22	233.10
T2-16 A	10	10	10.18	10.40	−11.62	−11.97	0.26	220.70
T2-16 B	10	10	10.18	10.40	−11.62	−11.97	0.26	241.00
T2-17	300	10	300.18	10.40	−10.15	−11.97	0.40	254.40
T2-18	0	30	0.18	30.40	−13.38	−11.51	0.31	228.10
T2-19	1	30	1.18	30.40	−12.56	−11.51	0.27	240.10
T2-20	30	30	30.18	30.40	−11.15	−11.51	0.36	258.00
T2-21	0.3	100	0.48	100.40	−12.95	−10.99	0.38	311.30
T2-22	0	300	0.18	300.40	−13.38	−10.51	0.44	408.40
T2-23	1	300	1.18	300.40	−12.56	−10.51	0.39	458.90
T2-24	10	300	10.18	300.40	−11.62	−10.51	0.49	471.40
T2-25	300	300	300.18	300.40	−10.15	−10.51	0.47	532.30

* Includes 0.177 nmol L^{-1} Co in media base.
† Includes 0.4 nmol L^{-1} Zn in media base.
‡ RFU = relative fluorescence units.

Table 3. Maximum growth rate and half-saturation constants for Zn and Co limitation.

Species	Zn		Co	
	μ_{max}	K_m	μ_{max}	K_m
*Phaeocystis antarctica**	0.25	9.5×10^{-13} (r^2=0.97)	0.12	1.9×10^{-13} (r^2=0.89)
Thalassiosira pseudonana†	1.56	1.2×10^{-12} (r^2=0.97)	0.84	3.6×10^{-12} (r^2=0.95)
Emiliana huxleyi†	1.03	6.5×10^{-13} (r^2=0.96)	1.23	1.0×10^{-12} (r^2=0.98)

* Data from this study, Table 1, Transfer 2.
† Data from Sunda and Huntsman (1995).

overall biomass by these elements. Moreover, although the substitution effect was consistent between the two transfers, overall relative biomass yields decreased in the subsequent transfer, perhaps because of increasing Zn limitation in the successive transfers (Fig. 1E). This decrease in relative biomass yields does not seem to be the result of lack of acclimation or changes in chlorophyll per cell given the significant acclimation time described above, as well as the consistent light environment throughout the experiment.

Zn-Co substitution was also clearly evident in the matrix experiments, in which the Zn and Co concentrations were covaried (Fig. 2A; Table 2). Optimal growth rates occurred when both Co and Zn were added to the growth medium (far corner), as well as the replacement of Co for Zn, and Zn for Co (front edges). Relative biomass yields in the matrix experiments (Fig. 2B; Table 2) showed maximal yield when Zn concentrations were high (back edge). Modulations in Co and no Zn slightly influenced the biomass yield (front edge), whereas changes in Zn without Co resulted in large influence on biomass yield (right edge).

Comparison of Phaeocystis Zn and Co physiology with other phytoplankters—We compared the growth rates generated in this study to the Co and Zn physiological studies of Sunda and Huntsman (1995) for diatoms and coccolithophores. Half-saturation constants for growth and maximal growth rates were calculated from the second transfer of the simple Co limitation and Zn limitation datasets presented here (Fig. 1B; Tables 1 and 3). This dataset had a slightly wider dynamic range of growth rates compared with the first transfer and hence seemed more applicable to calculations of half-saturation constants. Half-saturation constants for growth and maximal growth rates were also calculated from Sunda and Huntsman (1995) for the coastal diatom *T. pseudonana* and coccolithophore *Emiliana huxleyi* (Fig. 3; Table 3). Although the Zn half-saturation constant for growth of *P. antarctica* is between that of the eukaryotic phytoplankton strains studied (*T. pseudonana* and *E. huxleyi*), the Co half-saturation constant of *P. antarctica* was 0.19 pmol L^{-1}, more than five times lower than that of *T. pseudonana* and *E. huxleyi* (Table 3). This might partly be the effect of fitting data with a low μ_{max} for *P. antarctica* when grown under conditions of replete Co with no added Zn, as well as a genuine difference in Co half-saturation constants. Interestingly, *T. oceanica* also appears to have lower Zn and Co requirements, likely because of adaptation to more oligotrophic and Zn-poor waters. K_m values could not be calculated for *T. oceanica* because of the incomplete Zn-Co limitation in that study. It should be pointed out that this cobalt limitation effect in *Phaeocystis antarctica* is likely distinct from a vitamin B12 limitation effect (B12 contains an atom cobalt), as we have previously reported (Bertrand et al. 2007) since this culture medium contains added B12.

Modeling of the diffusive boundary layers of *Phaeocystis* colonies has suggested that the colonial form should have higher K_m values than the unicellular form for nutrients (Ploug et al. 1999). Our results likely reflect growth rates for the colonial form given their prevalence at late exponential growth in our experiments. Interestingly, Fe and Mn have been observed to accumulate in the mucilage found in the center of *Phaeocystis* colonies, suggesting that this material might be able to serve as a trace metal storage facility for the colony (Schoemann et al. 2001). As a result, the acquisition of trace metals by *Phaeocystis* colonies might not be consistent with that predicted increase (and may result in the reverse of that predicted) for macronutrients because of this potential for complexation and storage of trace elements within the mucilaginous center of the colony. More study is needed on the trace metal complexation characteristics of the *Phaeocystis* colony mucilage.

The notion of a physiological preference for Zn over Co described above is a somewhat vague term that must reflect a biochemical complexity within each cell. This notion is explored more extensively in the accompanying manuscript on colimitation in phytoplankton (Saito et al. 2008), including the use of data from this study. We hypothesize that the particular case of Zn-Co colimitation described here is the result of metal substitution within the active site of a carbonic anhydrase enzyme, similar to that found in the marine centric diatoms. The enzyme DCA1 (diatom carbonic anhydrase, formally described as TWCA1) is known to be the metalloenzyme that confers the Co-Zn substitution capability in the centric diatoms, with an active site that is believed to utilize either Zn or Co in vivo (Roberts et al. 1997). It is possible that *P. antarctica* has a homologous enzyme that maintains the Zn-Co flexibility in its active site. As discussed above, Co limitation is observed in *P. antarctica* at lower concentrations than in the diatom *T. pseudonana* (Fig. 3; Table 3), whereas the Zn limitation level appears to be similar for both at $\sim10^{-13}$ mol L^{-1} Zn^{2+}. Several interpretations are possible for this result. First, the membrane transport capability for Co could differ between the diatoms studied and *P. antarctica*. Second, assuming that the Co-Zn physiological substitution observed is conferred by an enzyme homologous to DCA1, the differences in the enzyme amino acid sequence could result in greater activity with Co in *P. antarctica* than

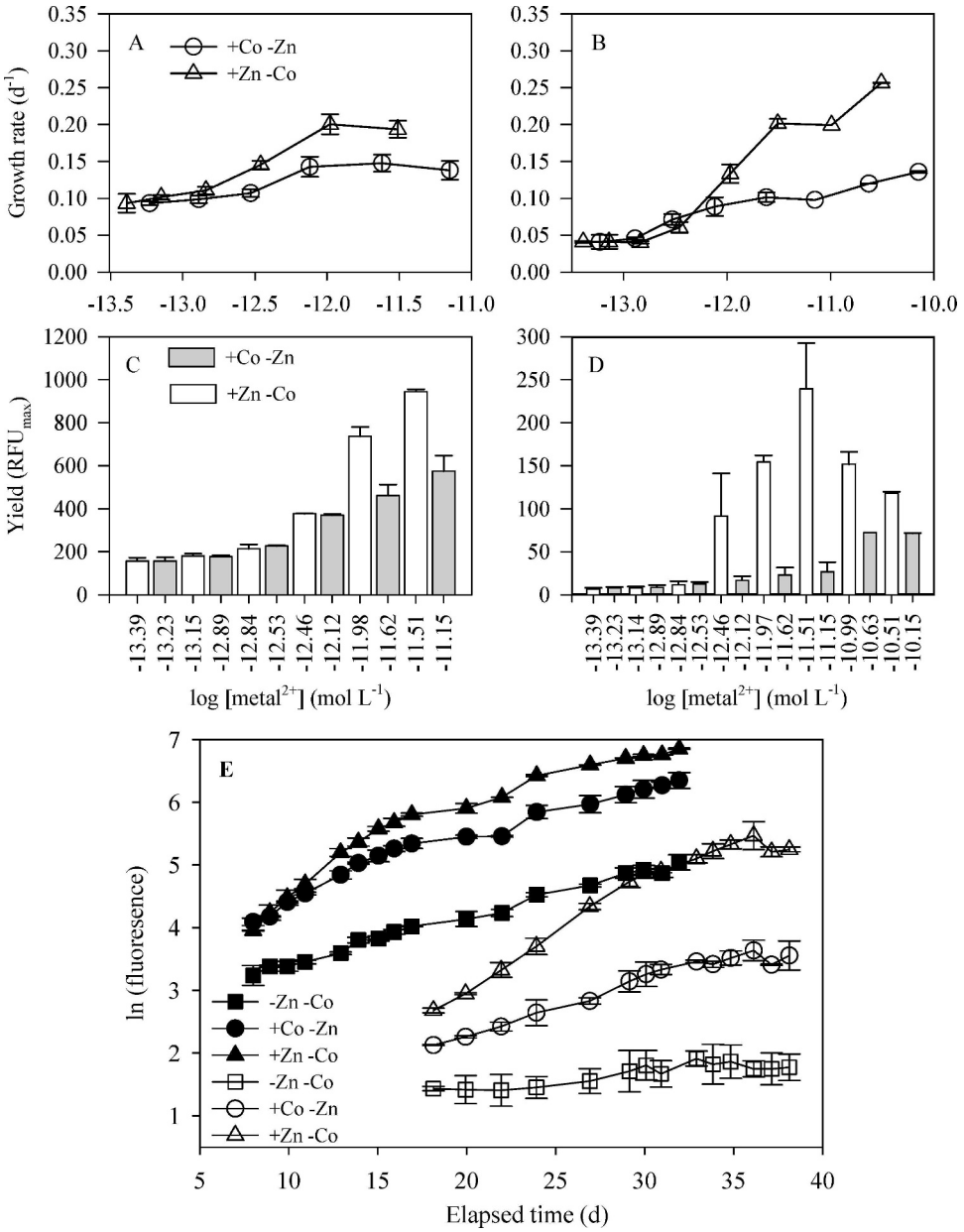

Fig. 1. Growth rates of *Phaeocystis antarctica* for (A) transfer 1 and (B) transfer 2 (Table 1), varying zinc concentrations with no added cobalt (+Zn –Co) and varying cobalt with no added zinc (+Co –Zn), demonstrated substitution of these two micronutrients under colimiting conditions. Relative biomass yields in each of these experiments increased with added Zn or Co and were highest when Zn was replete for (C) transfer 1 and (D) transfer 2 (RFU = relative fluorescence units). These growth rate and relative biomass results both show improved growth with Zn relative to Co in *P. antarctica,* implying a preference for Zn. (E) A subset of growth curves for both transfers with no metal added, or either $\log[Co^{2+}] = -11.49$ mol L^{-1} or $\log[Zn^{2+}] = -11.13$ mol L^{-1} added, showed decreased biomass yields in the second transfer. Filled symbols indicate transfer 1 (T1), and open symbols indicate transfer 2 (T2).

in *T. weissfloggii*. This is a distinct possibility: In vitro studies of carbonic anhydrase enzymes have demonstrated that different metals in the active sites can result in large changes in enzyme activity and that single amino acid modifications can result in loss of activity (Tripp et al. 2002). Alternatively, the Co-Zn substitution capability could be conferred by another metalloenzyme such as alkaline phosphatase, which is known to contain zinc yet is hypothesized to substitute other metals such as Co in marine phytoplankton (Wisniewski, 2006).

The presence of an extracellular carbonic anhydrase, evident in cultures of *Phaeocystis* from isotope disequilibria experiments, is believed to allow them to maintain a high carbon uptake capability even in bloom conditions when

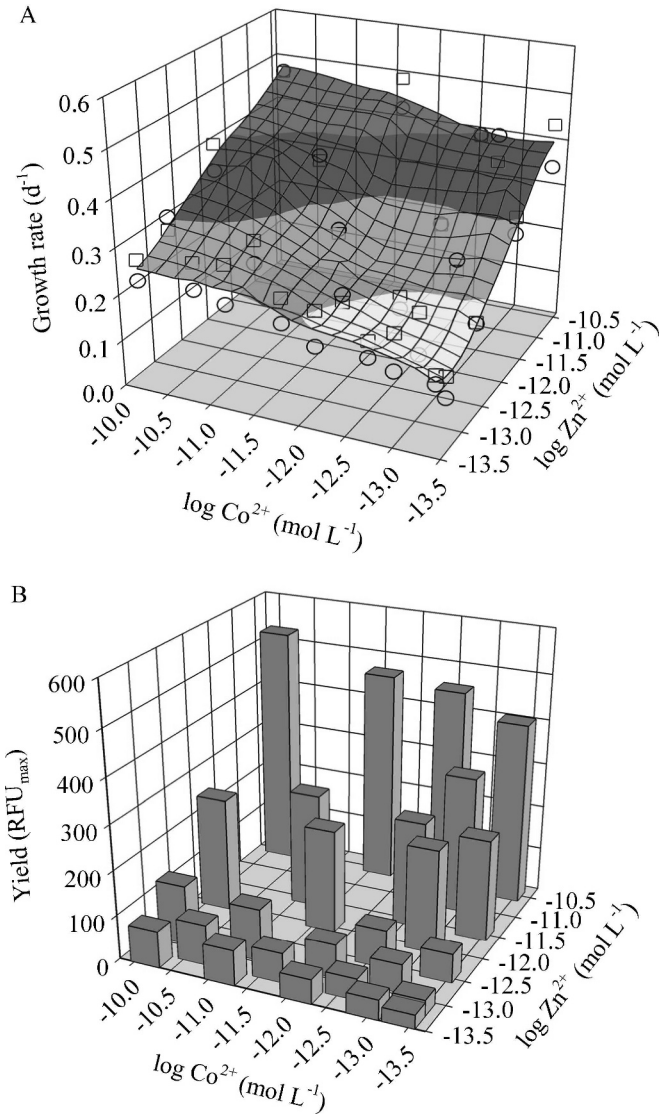

Fig. 2. (A) Growth rates of *Phaeocystis antarctica* under covarying cobalt and zinc concentrations, demonstrating substitution of these two micronutrients under colimiting conditions. Data from both transfers (Table 2) were averaged to generate the surface, and the discrete growth rate data are overlaid on the transparent surface (circles for transfer 1 and squares for transfer 2, faint points are below the surface). (B) Relative biomass yields in the matrix experiment, only transfer 2 shown, increased with added Zn or Co, were higher when Zn was replete (RFU = relative fluorescence units), and were at a maximum at the highest Zn and Co concentrations.

the environmental pH can become significantly elevated (8.7) (Elzenga et al. 2000; Schoemann et al. 2005). Moreover, carbon dynamics might be important in influencing the phytoplankton species composition: In bottle experiments *Phaeocystis* sp. was observed to increase significantly in cellular abundance when pCO_2 concentrations were lowered to 150 ppm (Tortell et al. 2002).

Although much can be learned on a physiological and molecular level about the utilization of carbon by *Phaeocystis*, it is already clear from previous laboratory studies that carbonic anhydrases are abundant enzymes and future characterization and sequencing of these enzymes will provide the necessary information as to the exact nature of their metal-containing active sites. This study provides a starting point in demonstrating the physiological capability for Co-Zn substitution in *Phaeocystis* and the potential implications for their carbonic anhydrase metalloenzymes.

Comparison with field Co-Cd-Zn seawater concentrations—It is intriguing that *Phaeocystis* should be observed to have a Co-Zn substitution capability, given that it is found in nearshore environments such as the large coastal shelf of the Ross Sea, Antarctica (*P. antarctica*), and Cape Cod Bay, Massachusetts (*P. pouchetii*); however, we hypothesize that the intense drawdown of trace elements during bloom conditions such as those found in the Ross Sea would create the selection pressure for biochemical flexibility with regard to Zn and Co utilization. At first consideration, the Zn-Co substitution capability might seem most advantageous in open ocean environments with extremely low levels of these metals, rather than in coastal environments that have a variety of sources in close proximity (e.g., riverine, upwelling, aeolian, and sedimentary). However, the cyanobacteria that dominate the oligotrophic gyres are the organisms that actually lack a Co-Zn substitution capability, as well as having an absolute requirement for Co but not for Zn (Sunda and Huntsman 1995; Saito et al. 2002), perhaps because of their evolution in an ancient ocean that was scarce in Zn and abundant in Co (Saito et al. 2003). Moreover, the rapid recycling of nutrients via grazing of cyanobacteria as part of the microbial loop avoids the intense depletion and export associated with diatom and *Phaeocystis* blooms. Hence, the presence of Co-Zn substitution in not only *P. antarctica*, but also coastal diatoms (*T. pseudonana* and *T. weissflogii*), could reflect the blooming behavior of these phytoplankton and their potential capability to completely remove and export these micronutrients from the dissolved milieu surrounding themselves, a phenomenon we recently observed in Fe-enriched bottle incubations in the Ross Sea (complete labile Co depletion in Fe-stimulated bottle incubation experiments; Bertrand et al. 2007). Hence, the Co-Zn biochemical substitution seen in these eukaryotic algae may be the result of evolutionary pressure for maintaining growth rates in these high-biomass and high-export environments in which rapid depletion of Zn and carbon occur simultaneously.

The Ross Sea, with its massive annual *P. antarctica* blooms and seasonal Fe limitation (Sedwick et al. 2000), has relatively high Co and Zn at shallow depths (e.g., >150 m) and significant depletion of these elements in the upper water column (Fitzwater et al. 2000). Although no effect of Zn or Co has been observed on the total productivity of phytoplankton assemblages in the Ross Sea (Cochlan et al. 2002; Coale et al. 2003; Bertrand et al. 2007), it is possible that these metals influence the phytoplankton species composition (Saito et al. 2004) or that an incubation experiment setup in the center of a large bloom might yield significant Zn or Co stimulation effects.

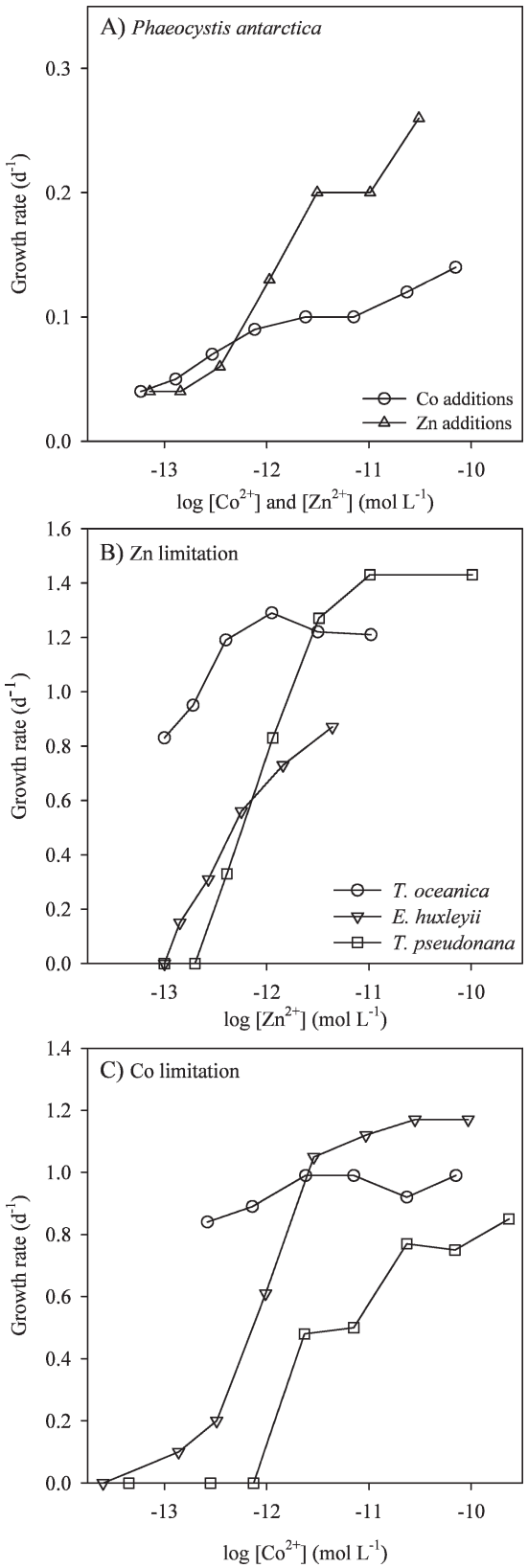

Fig. 3. Comparison of (A) *Phaeocystis antarctica* growth rates with those of *Thalassiosira pseudonana*, *Thalassiosira oceanica*, and *Emiliania huxleyi* under conditions of (B) Zn and (C) Co limitation. *P. antarctica* data is from this study, and other phytoplankton strains are from Sunda and Huntsman (1995). Although half-saturation growth constants for Zn for *P. antarctica* are intermediary between *E. huxleyi* and *T. pseudonana,* cobalt K_m values are significantly lower than *T. pseudonana* and somewhat lower than *E. huxleyi* (Table 3), suggesting a possible no-zinc low-cobalt niche for *P. antarctica.* Maximal growth rates for *P. antarctica* are significantly lower than the other phytoplankton strains because of the low-temperature culturing conditions.

We have recently observed colimitation by both Fe and vitamin B_{12} (a Co-containing biomolecule) in the Ross Sea, suggesting this subcomponent of the Co biogeochemical cycle is important in controlling the ecology of the Ross Sea, and that *Phaeocystis antarctica* may have a distinct ecological advantage through the production of B12 by heterotrophic bacteria living within the colony mucilage (Bertrand et al. 2007). Moreover, given the intense drawdown of carbon dioxide in the Ross Sea environment, it is conceivable that Zn, Co, and Cd availability could have direct effects on carbon acquisition in the phytoplankton assemblages, as has been observed with CO_2 concentrations on Cd uptake in the coastal California environment (Cullen et al. 1999). It should also be noted that there is a possibility of Cd-Zn substitution in *Phaeocystis* that should be investigated in future studies.

In this manuscript, we present experiments demonstrating Co-Zn micronutrient substitution in *P. antarctica*. These results show that the Co-Zn substitution strategy extends geographically to the high latitudes and taxonomically to another important eukaryotic phytoplankton strain. Moreover, the concentrations of Co in which we observed a limitation response in *P. antarctica* were significantly lower than those of previous studies on coastal marine diatoms. It is proposed that the Co-Zn biochemical flexibility might have been selected for in eukaryotic phytoplankton because of their rapid bloom growth patterns in natural waters, resulting in rapid trace metal depletion and export as well as significant carbon dioxide depletion.

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