

Accumulation of NO₂-cobalamin in nutrient-stressed ammonia-oxidizing archaea and in the oxygen deficient zone of the eastern tropical North Pacific

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Summary

Cobalamin (vitamin B₁₂) is a precious resource in natural systems that is produced by select prokaryotes and required by a broad range of organisms. In this way, the production of cobalamin reinforces numerous microbial interdependencies. Here we report the accumulation of an unusual form of cobalamin, nitrocobalamin (NO₂-cobalamin), in a marine oxygen deficient zone (ODZ), isolates of ammonia-oxidizing archaea (AOA), and an anaerobic ammonium-oxidizing (anammox) bacteria enriched bioreactor. Low oxygen waters were enriched in NO₂-cobalamin, and AOA isolates experiencing ammonia or copper stress produced more NO₂-cobalamin, though there is wide strain-to-strain and batch-to-batch variability. NO₂-cobalamin has no known biochemical role. We hypothesize that AOA and anammox bacteria are a source of marine NO₂-cobalamin in the environment via a reactive nitrogen intermediate. These findings suggest connections between cobalamin forms and nitrogen transformations, physiological stress and ocean deoxygenation.

Introduction

Cobalamin (vitamin B₁₂) supply and demand play an important role in regulating metabolic processes in

microbial communities because this structurally complex, cobalt-containing cofactor is produced only by select members of the cobalamin-dependent community (Croft *et al.*, 2005; Bertrand *et al.*, 2015). Cobalamin and cobalamin-like molecules are chemically diverse and different chemical forms can have differing bioavailabilities in both prokaryotes and eukaryotes (Droop, 1957; Ayers, 1960; Helliwell *et al.*, 2016; Heal *et al.*, 2017), indicating that the chemical diversity of cobalamin has wide ranging ecological implications. Despite the importance of its chemical diversity, few studies measure multiple forms of cobalamin in natural systems or environmentally relevant organisms (Heal *et al.*, 2014; 2017).

Reactive nitrogen species (RNS) such as nitric oxide (NO) can rapidly react with cobalamin replacing the alpha ligand of more bioavailable forms of the cofactor (Fig. 1). In humans, the product of this reaction acts to inhibit cobalamin-dependent enzymatic processes, essentially depleting functional cobalamin (Nicolaou *et al.*, 1996; Kambo *et al.*, 2005). However, this chemical reactivity has not been investigated in the context of marine microbiology, despite the fact that RNS are known intermediates in some cobalamin-producing microbes. One study hypothesized that RNS play some role in stabilizing dissolved cobalt in the water column (including dissolved cobalamin) based on a strong correlation between nitrous oxide (N₂O) and the dissolved cobalt pool in the North Atlantic (Noble *et al.*, 2012), though there have been no direct measurements of compounds that could be supporting this phenomenon.

An unusual form of cobalamin, nitrocobalamin (NO₂-cobalamin), has been shown to be an important product of reactions between cobalamin and RNS. Unlike other forms of cobalamin like adenosyl-, methyl- and hydroxocobalamin, NO₂-cobalamin has no known role in biochemistry (forms shown in Fig. 1). Here we show that NO₂-cobalamin can be abundant in the ocean and marine microbial isolates, and we identify likely microbial origins for this unfamiliar form of cobalamin.

Results and discussion

Our group recently quantified several chemical species of particulate cobalamin in oxygenated waters in the North

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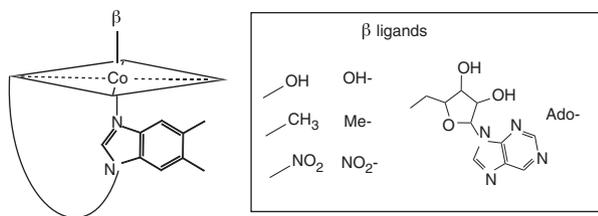


Fig. 1. Forms of cobalamin detected and discussed in this study.

Pacific Ocean (Heal *et al.*, 2017). We re-analysed a subset of these samples to monitor NO₂-cobalamin in natural communities and quantify the compound when detected. In these samples, NO₂-cobalamin comprised low but detectable proportions (~ 2%–4%) of the particulate cobalamin pool, ranging from below detection (~ 0.001 pM) to 0.004 pM (Supporting Information Table S1). We also analysed the particulate cobalamin pools at two stations in the eastern tropical North Pacific (ETNP) including samples in the oxygen deficient zone (ODZ). For both stations, we have one sample in the oxycline and at least two samples in the ODZ. The ETNP stations displayed markedly different particulate cobalamin composition than other marine community samples we analysed in this study, with NO₂-cobalamin contributing up to 66% of the total particulate cobalamin pool within the ODZ, reaching concentrations up to 0.03 pM (Fig. 2, Supporting Information Table S1).

To investigate plausible sources of NO₂-cobalamin, we analysed five strains of cultured ammonia-oxidizing archaea (AOA) isolated from marine waters (four strains) and soil (one strain). AOA have been implicated as major suppliers of cobalamin in the ocean (Doxey *et al.*, 2014; Heal *et al.*, 2017), produce RNS as intermediates during ammonia oxidation (Martens-Habbenha *et al.*, 2015; Kim *et al.*, 2016), and have been well-described and cultured axenically (Qin *et al.*, 2014; 2015; 2017). Some ammonia-oxidizing bacteria also produce RNS as intermediates (Schmidt *et al.*, 2004; Caranto and Lancaster, 2017), but they are present in much lower abundance in marine environments compared with AOA (Mincer *et al.*, 2007; Horak *et al.*, 2018) and do not appear to have the genetic capacity for cobalamin biosynthesis (Chain *et al.*, 2003; Klotz *et al.*, 2006; Norton *et al.*, 2008). In the AOA cultures we tested, there was wide strain-to-strain and batch-to-batch variability in the proportion of cobalamin present as NO₂-cobalamin, but we consistently found that a greater proportion of the detected cobalamin pool was present as NO₂-cobalamin in AOA cells under ammonia depletion, copper limitation, or copper toxicity (Table 1; Fig. 3; Supporting Information Fig. S1, Table S2).

AOA are capable of ammonia oxidation at remarkably low O₂ concentrations (Bristow *et al.*, 2016; Qin *et al.*, 2017) and are transcriptionally active in the low oxygen boundary region of the ODZ (Ulloa *et al.*, 2012). Thus,

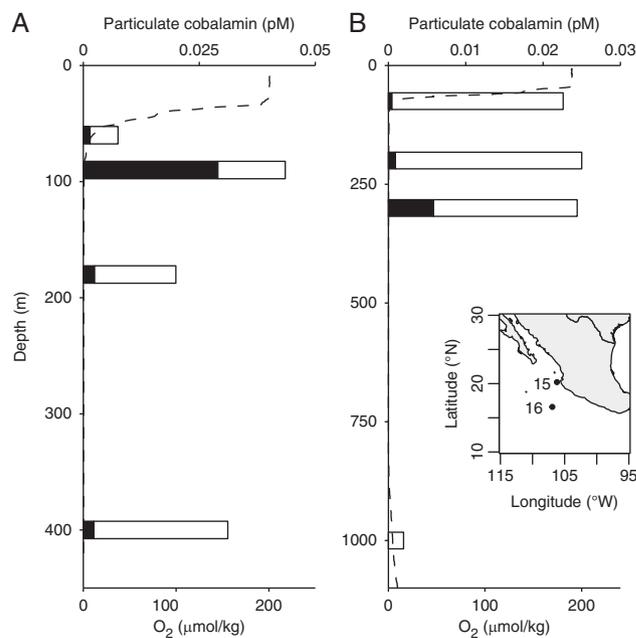


Fig. 2. NO₂-cobalamin detected in the eastern tropical North Pacific. Particulate cobalamin (bars) and O₂ concentrations (dashed lines) at Stations 15 (A), and 16 (B) with NO₂-cobalamin (black) portion of the total particulate cobalamins. Inset shows station locations.

considering both their environmental distribution and activity in culture, AOA are a plausible source of the observed NO₂-cobalamin in the oxycline and the upper ODZ. We hypothesized that anaerobic ammonium oxidizing (anammox) bacteria (Schmid *et al.*, 2000) are another likely source of NO₂-cobalamin within the ODZ. Anammox bacteria are one of the most active populations within the ODZ (Ulloa *et al.*, 2012), possess the genetic capacity for cobalamin biosynthesis (Heal *et al.*, 2017; Lawson *et al.*, 2017), and process RNS such as NO (Strous *et al.*, 2006; Kartal *et al.*, 2011). Together,

Table 1. Percent of total detected cobalamins (NO₂⁻, Me-, OH- and Ado-) present as NO₂-cobalamin in pure cultures of AOA under ammonia-replete and ammonia-depleted conditions.

Strain	NH ₄ ⁺ -replete		NH ₄ ⁺ -depleted	
	<i>n</i>	% NO ₂ -cobalamin	<i>n</i>	% NO ₂ -cobalamin
<i>Nitrosopumilus cobalaminigenes</i> HCA1	3	nd	1	1.2%
<i>Nitrosopumilus oxycloinae</i> HCE1	3	nd	2	0.8%–1.3%
<i>Nitrosopumilus ureiphilus</i> PS0	3	nd	3	0.3%–0.4%
<i>Nitrosopumilus maritimus</i> SCM1	4	nd–1.4%	2	50%–75%
<i>Nitrososphaera viennensis</i> EN76	1	0.4%	1	28%

nd = not detected. Full results are given in Supporting Information Table S2.

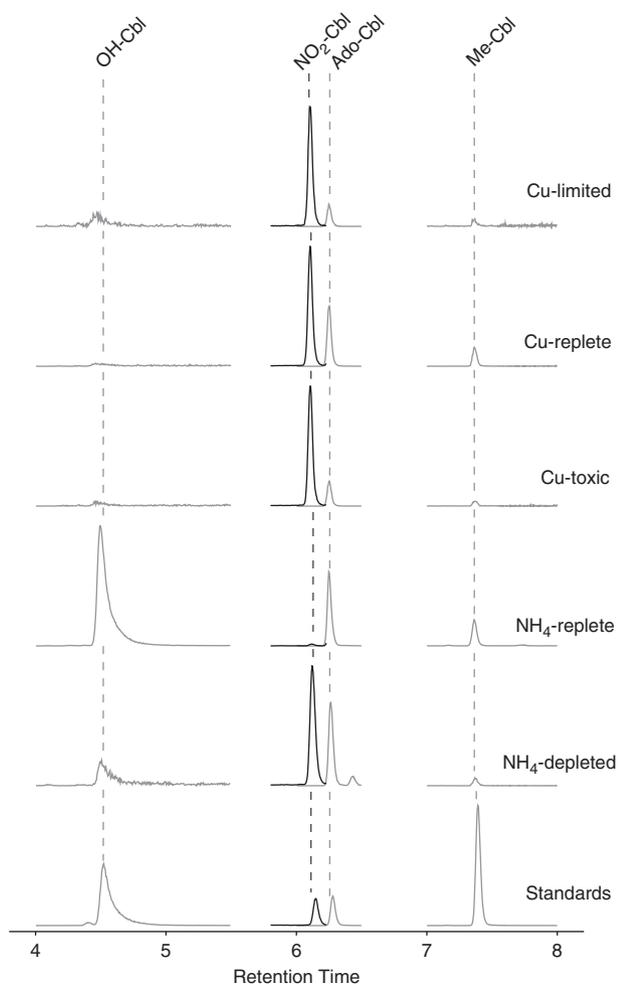


Fig. 3. NO₂-cobalamin in copper and ammonia-stressed *N. maritimus*. Chromatograms of *N. maritimus* strain SCM1 cells harvested under three copper regimes (described in Qin *et al.*, 2017c), ammonia-replete and ammonia-depleted conditions. Full results are reported in Supporting Information Table S2 for all conditions. Standards of each of the compounds are shown at the bottom. Y axes represent intensity.

these factors strongly support the possibility for NO₂-cobalamin production by anammox bacteria. Corroborating this conjecture, we analysed bioreactors enriched with anammox bacteria similar to those previously reported (Winkler *et al.*, 2011; 2012). In those bioreactors, we found that NO₂-cobalamin comprised 5%–14% of the total detected cobalamin pool (Supporting Information Table S2).

NO₂-cobalamin can be formed via a slow reaction between reduced cobalamin and nitrite (Wolak *et al.*, 2000), and nitrite is the major product of ammonia oxidation in AOA. However, nitrite production is thought to occur in AOA's pseudo-periplasmic space (Lehtovirta-Morley *et al.*, 2016), limiting interactions between nitrite and cobalamin in the cytoplasm. We did not observe any correlation between variable nitrite concentrations (0.2–2 mM) and intracellular

NO₂-cobalamin in three AOA strains (Supporting Information Table S2, Supporting Information Fig. S2), nor did we detect any extracellular NO₂-cobalamin during nitrite accumulation (0.003–0.9 mM) in *Nitrosopumilus maritimus* strain SCM1. Together, these findings suggest that the reaction between reduced cobalamin and nitrite has a minor, if any, contribution to the NO₂-cobalamin we detected in AOA.

A more plausible source of NO₂-cobalamin in AOA is the rapid reaction between reduced cobalamin and intracellular NO (Sharma *et al.*, 2003; Dereven'kov *et al.*, 2016). Gaseous NO is an intermediate of ammonia oxidation in both AOA and anammox bacteria (Kartal *et al.*, 2011; Martens-Habbena *et al.*, 2015; Kozłowski *et al.*, 2016) and may diffuse into the cell to react with the reduced cobalamin that forms during cellular processing, forming nitrosylcobalamin (NO-cobalamin). This rapidly formed NO-cobalamin is readily oxidized to NO₂-cobalamin after exposure to O₂ (within cells or during sample processing) (Subedi and Brasch, 2013).

Although the production and consumption of NO are tightly controlled during normal growth of AOA, previous work has shown that excess NO can accumulate during unbalanced growth of AOA stemming from suboptimal growth conditions. For example, NO accumulates in AOA cultures after O₂ depletion (Kozłowski *et al.*, 2016) and transiently when ammonia is nearly depleted (Martens-Habbena *et al.*, 2015). Since many Cu-dependent enzymes mediate ammonia oxidation (Amin *et al.*, 2013; Hosseinzadeh *et al.*, 2016), disruption of Cu homeostasis may also alter the flux of NO in AOA cells. Together, these observations suggest that under ammonia depletion or Cu stress, NO accumulation within AOA results in the formation of NO-cobalamin (ultimately detected as NO₂-cobalamin). Our work supports the results of recent transcriptional analyses of *N. maritimus*, showing increased expression of the cobalamin biosynthetic pathway under ammonia-depletion and Cu-stress (Qin *et al.*, 2017). Furthermore, we saw that *N. maritimus* cells recovering from ammonia starvation harboured residual NO₂-cobalamin, unlike ammonia replete cells (data not shown). With this building evidence, we hypothesize that NO depletes functional cobalamin causing AOA to over-express cobalamin synthesis genes to compensate for loss of bioactive cobalamin when they experience nitrosative stress.

Cobalamin plays a fundamental role in structuring microbial community diversity and activity, therefore recognizing factors controlling its production, transformation and exchange is essential to developing an improved understanding of marine microbial ecology. Our analyses indicate that we now must explicitly consider the biogeochemical consequences of RNS reactions with cobalamin in natural marine communities.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Appendix S1: Supporting information.

Table S1: Full results from reanalyzed particulate cobalamins in the North Pacific Ocean (from Heal *et al.*, 2017; cruise ID KM1314) and in the ENTP (this study, cruise ID SKQ16–17). Percent of detected cobalamins (NO₂⁻, Me⁻, OH⁻, and Ado⁻) present as NO₂-cobalamin. Note that values for Me⁻, OH⁻, and Ado⁻-cobalamin were taken from our previous work on the same samples (Heal *et al.*, 2017), *dl* notes when values were below the detection limit.

Table S2: Full results from individual cultures of AOA summarized in Table 1, Cu experiment described in Qin, *et al.* (2017), and anammox bioreactor. Percent of detected cobalamins (NO₂⁻, Me⁻, OH⁻, and Ado⁻) present as NO₂-cobalamin in pure cultures of AOA, initial ammonia concentrations, and final nitrite concentrations at time of harvest (for AOA grown under different ammonia concentrations). When possible, we provided the summed total of cobalamins detected for each of the samples.