Constraining the contribution of aerobic nitrite oxidation to oxygen consumption in oceanic oxygen minimum zones

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Abstract

Oceanic oxygen minimum zones (OMZs) are globally significant sites of biogeochemical cycling where microorganisms deplete dissolved oxygen (DO) to concentrations <20 μM. Amid intense competition for DO in these metabolically challenging environments, aerobic nitrite oxidation may consume significant amounts of DO and help maintain low DO concentrations, but this remains unquantified. Using parallel measurements of oxygen consumption rates and ^{15}N-nitrite oxidation rates, applied to both water column profiles and oxygen manipulation experiments, we show that the contribution of nitrite oxidation to overall DO consumption systematically increases as DO declines below 2 μM. Nitrite oxidation was dominant only under DO concentrations <393 nM. These patterns were consistent across sampling stations and experiments, reflecting coupling between nitrate reduction and nitrite-oxidizing *Nitrospina* with high oxygen affinity (based on isotopic and omic data). Collectively our results provide new insight into the contribution of nitrite oxidation to the maintenance and biogeochemical dynamics of OMZs.

Introduction

Aerobic nitrite oxidation is pervasive throughout much of the oceanic water column, playing a central role in deep ocean chemoautotrophy and carbon cycling[1,2], as well as in the oceanic nitrogen (N) cycle[3,4]. Nitrite oxidation rates are typically undetectable using $^{15}NO_2^-$ isotopic tracer in the sunlit euphotic zone, peak at the base of the euphotic zone, and subsequently decline with depth[5]. However, nitrite oxidation rate profiles deviate from this pattern in oceanic oxygen minimum zones (OMZs) that are depleted in dissolved oxygen (DO): rapid rates have been reported despite low DO concentrations[6-9], and nitrite oxidation itself may consume DO to low levels. OMZs are ultimately generated by high sinking fluxes of organic matter combined with reduced ventilation at depth, and are typically defined by DO concentrations <20 μM (ref. 10). Regions of the tropical oceans containing no measurable DO—termed anoxic marine zones (AMZs; ref. 11)—are further distinguished by the accumulation of nitrite to comparatively high (>1 μM) concentrations[12]. These secondary nitrite maxima (SNM) result from anaerobic nitrate reduction to nitrite under low DO[11-13]. Accumulated nitrite may be subsequently reduced via denitrification and anaerobic ammonium oxidation—resulting in gaseous N loss[14]—or, alternatively, oxidized by NOB. Nitrite is therefore rapidly produced and consumed via multiple processes in OMZs, linking the oceans' N, carbon (C), and oxygen cycles.

Despite its centrality in OMZ biogeochemical cycles, nitrite oxidation is still poorly understood in these dynamic regions of the ocean. In particular, DO can be photosynthetically produced[15] or physically introduced to OMZs[12,16,17], requiring subsequent DO consumption in order to maintain the existence of the OMZ itself[4]. High rates of nitrite oxidation within OMZs suggest that marine nitrite-oxidizing bacteria potentially contribute to DO depletion, and therefore to the ultimate generation and maintenance of OMZs[4,6-9,15,17] . However, the contribution of nitrite oxidation to DO drawdown has not been directly quantified. While nitrite oxidation has been measured in several OMZs[6-9], oxygen consumption rate (OCR) measurements in OMZs are rare[16,18,19]. Yet simultaneous measurements are necessary to directly quantify the contribution of nitrite oxidation to oxygen consumption and to the maintenance of OMZs.

The central question addressed by our research is whether or not nitrite oxidation is a significant DO sink in OMZs, and, if so, over what range of DO concentrations does this occur? This has direct implications for our understanding of how OMZs form and expand[4,11]—as well as associated consequences for C and N cycling and loss[3,14]. For example, an additional diagnostic

feature of AMZs is the presence of a secondary chlorophyll maximum (SCM) where distinct ecotypes of the globally important cyanobacterium *Prochlorococcus* produce DO within low DO waters[15,20,21]. Photosynthetic DO production is non-trivial, as production rates of 211-2,520 nM-O_2 d^{-1} occur in the SCM—requiring corresponding DO consumption so that DO does not accumulate[15]. SCM-based DO production can overlap with nitrite supply via nitrate reduction, but the degree to which nitrite oxidation (versus other processes) consumes DO in the SCM remains poorly constrained[15]. Greater DO consumption via nitrite oxidation implies that less DO is used to respire organic C, while the oxidation of nitrite precludes its further reduction to gaseous forms of N[3,14].

Our understanding of how nitrite-oxidizing bacteria (NOB) oxidize nitrite in OMZs and other low-DO habitats is also evolving. NOB gain energy by oxidizing nitrite to nitrate while using DO as a terminal electron acceptor. NOB appear to have high affinity for DO (K_m values of 778-900 nM DO or lower), allowing them to aerobically oxidize nitrite even at nanomolar concentrations of DO[22,23]. Omic data further indicate that some NOB are metabolically flexible[24], and perhaps able to survive anaerobically through the use of alternative electron donors and acceptors[13,25]. Adaptations to low DO are evident in different NOB, and both *Nitrospina* and *Nitrococcus* may contribute to oceanic nitrite oxidation varying degrees[25,26]. But although *in situ* nitrite oxidation rates are typically highest under low DO, experimental oxygen manipulations show the opposite pattern: declining rates with declining DO[22]. This may be explained by variations in genomic content among different NOB that allow them to occupy distinct, highly-resolved DO niches throughout the water column[4,7,22,23,25]—including those that occur from the edge (20 μM) to the core (<5 nM DO) of OMZs. These details are essential to understand in the context of ocean deoxygenation, as seemingly small variations in DO may have significant biogeochemical implications if different ecotypes within different functional groups (such as nitrite oxidizers, nitrate reducers, and organisms respiring organic matter aerobically) are adapted to specific DO concentration ranges, and so display varying sensitivities to DO.

Here we address these open questions through parallel measurements of nitrite oxidation and overall OCR in the oceans' largest OMZ, the eastern tropical North Pacific Ocean (ETNP). Nitrite oxidation rate measurements (using $^{15}NO_2^-$), OCR measurements (using optical sensor spots), and ammonia oxidation rate measurements (using $^{15}NH_4^+$) were made along depth

106 profiles at six stations in the ETNP, including three OMZ stations and three AMZ stations
107 (Figure 1). At each station, we sampled in the upper 100 m to capture the primary nitrite
108 maximum and an expected peak in nitrite oxidation rates at the base of the euphotic zone (EZ).
109 We then sampled a range of DO values (from 200 μM to the anoxic SNM at AMZ stations) to
110 quantify rate variations in response to vertical gradients in DO. How nitrite oxidation varies
111 from the OMZ edge to its anoxic core is critical to our understanding of ocean biogeochemical
112 cycles; we therefore conducted oxygen manipulation experiments and examined the response of
113 nitrite oxidation and OCR. Combined with natural abundance stable isotope data, 16S rRNA
114 sequencing, and metagenomes, we show that nitrite oxidation is a substantial—and occasionally
115 even dominant—sink for oxygen in the ETNP.
116
117 **Results**
118 *Nitrite oxidation rates in the ETNP*
119 We sampled six stations in the ETNP OMZ with DO concentrations <20 μM at depth (Figs 1 and
120 2). Three stations extending out from the coast of Mexico (Stations 1-3) were AMZ stations
121 with distinct SCMs and accumulations of nitrite in SNMs indicative of anaerobic N cycling
122 (Figure 2). We expected that these would be hotspots of nitrite oxidation given oxygen supply
123 via photosynthesis in the SCM overlapping with nitrite supply via nitrate reduction. Across all
124 stations and depths, we observed the highest nitrite oxidation rates at the AMZ stations (Fig. 2C,
125 G, and K). Station 1 is located nearest the coast and had a shallow OMZ at the time of our
126 sampling, with 20 μM DO at 28 m depth, and nitrite > 1 μM at 100 m (Fig. 2A). Chlorophyll
127 concentrations were also high in the upper water column (up to 5 mg m^{-3} at 20 m), with an SCM
128 spanning 70-125 m (Fig. 2B). Nitrite oxidation displayed a local maximum at the base of the EZ
129 at Station 1 (20-30 m), and then increased to higher levels (>100 nmol L^{-1} d^{-1}; Fig. 2C). This
130 increase at 100 and 125 m corresponded with the overlap between the bottom of the SCM and
131 the top of the SNM. Nitrite oxidation rates then reached higher values at 150 m within the SNM
132 at Station 1. Stations 2 and 3 displayed similar nitrite oxidation rate profiles to each other,
133 including elevated rates in the SCM (Fig. 2G and K). Nitrite oxidation rates were similar in
134 magnitude, and peak values at the base of the EZ and in the OMZ were also similar (69-96 nmol
135 L^{-1} d^{-1}). Depth patterns tracked oceanographic differences across the three AMZ stations, as the
136 depth of all features increased moving offshore from Station 1 to 2 to 3. For example, the SCM

137 extended from 105-155 m at Station 2, while nitrite concentrations began to increase below 100
138 m; nitrite oxidation rates were elevated at 140 m and declined slightly with increasing depth (Fig.
139 2E-G). At Station 3, the SCM (120-180 m) and SNM (>140 m) depths were deeper, and nitrite
140 oxidation rates increased from 100 to 200 m (Fig. 2I-K).
141 In contrast to these three AMZ stations (Stations 1-3), rate profiles at Stations 4-6 showed
142 peaks at the base of the EZ followed by decreases with depth, and lacked a pronounced rate
143 increase within the OMZ (Supplementary Figure 1). Parallel measurements of ammonia
144 oxidation rates also showed this type of pattern at all stations (Supplementary Figure 1).
145 Subsurface maxima in ammonia oxidation tracked variations in the EZ across all six stations, but
146 rates were not elevated in OMZ/AMZ waters—again contrasting with nitrite oxidation rate
147 profiles at the AMZ stations. These data accord with earlier work in OMZs showing contrasting
148 ammonia and nitrite oxidation rate profiles, and particularly high rates of nitrite oxidation in
149 OMZ waters[6-8,27-29].
150 Initial DO concentrations for these measurements closely matched *in situ* values above
151 the SCM (where DO concentrations are higher), and starting DO ranged from 260-1500 nM for
152 measurements in and below the SCM. These DO concentrations are generally lower than those
153 used for previous nitrite oxidation rate measurements in OMZs[6,9], but similar to work examining
154 the oxygen affinity of nitrite oxidation[22] and overall oxygen consumption[16,19]. Elevated nitrite
155 oxidation in the limited number of samples (n=5) collected below the SCM (> 125 m at Station
156 1, >155 m at Station 2, and >180 m at Station 3)—where little to no DO is available—could have
157 a number of possible explanations discussed below. Within the SCM, our data support the idea
158 that nitrite oxidation contributes to 'cryptic' oxygen cycling[15]—i.e., that DO produced via
159 oxygenic photosynthesis is rapidly consumed.
160
161 *Oxygen consumption via nitrite oxidation*
162 We determined the contribution of nitrite oxidation to overall oxygen consumption via parallel
163 measurements of oxygen consumption rates (OCR) using *in situ* optical sensor spots—which are
164 non-invasive, provide nearly identical results as other low-level measurement approaches[30], are
165 the only effective means of achieving of substantial replication, and for which sensitivity
166 increases as DO decreases[30,31]. Decreases in DO were measured in both nitrite and ammonia
167 oxidation rate sample bottles, as well as in three additional replicates, to leverage statistical

168 power for increased sensitivity to low-level DO consumption (see Materials and Methods).
169 Water column OCR profiles at all stations showed exponential declines with depth and
170 decreasing DO concentrations (Fig. 2D, H, and L and Supplementary Figure 1). Rates were
171 highest in the upper water column, and declined sharply within the upper portion of the OMZ
172 above the SCM. The majority of measurements within the SCM—where DO may be produced
173 via photosynthesis—were 100s of nmol L^{-1} d^{-1}, with an overall range of 160-1380 nmol L^{-1} d^{-1}.
174 Below the SCM, DO would be available more rarely (e.g., ref. 16), and OCR measurements
175 represent potential rates should oxygen be supplied; OCR ranged from 120-390 nmol L^{-1} d^{-1}.
176 OCR also tracked variations in DO across stations, with progressively steeper declines in OCR
177 with depth from Station 6 through Station 1.
178 These OCR results are similar to the limited previous measurements that have been
179 conducted in OMZ regions, with some key differences. In particular, they are consistent with
180 previous measurements of rapid DO production and consumption in the SCM, with rates ranging
181 from 482-1,520 nmol-O_2 L^{-1} d^{-1} in the ETSP, and from 55-418 nmol-O_2 L^{-1} d^{-1} in the ETNP[15].
182 Earlier OCR measurements conducted in the ETNP near Stations 1 and 3 (across a wide range of
183 DO values) likewise ranged from 420-828 nmol L^{-1} d^{-1} in the SCM near Station 1, and from 101-
184 269 nmol L^{-1} d^{-1} in the SCM near Station 3 (ref. 16). Above the SCM, previous OCR
185 measurements in the ETNP spanned 2,260 to 662 nmol L^{-1} d^{-1} from the EZ to the edge of the
186 OMZ; these values are lower than our measurements at 44 and 67 m depth at Station 2, but in
187 line with our remaining measurements above the SCM. OCR reached 1610 nmol L^{-1} d^{-1} in the
188 SCM off Namibia, and 200-400 nmol L^{-1} d^{-1} in the SCM off Peru[18]. Kalvelage et al.[18]
189 furthermore observed sharp decreases with depth in the ETSP, with rates declining from >1000
190 nmol L^{-1} d^{-1} above the SCM.
191 This pattern of declining OCR with increasing depth and decreasing DO was also evident
192 in our dataset, and contrasted with that of nitrite oxidation rates, which were notably elevated in
193 the SCM at the AMZ stations (Fig. 2). We directly compared nitrite oxidation rates with OCR,
194 assuming that each mole of nitrite is oxidized using ½ mole of O_2 (ref. 5). We found that nitrite
195 oxidation systematically increased as a proportion of overall OCR at lower DO levels (Figure 3A
196 and B). Nitrite oxidation was responsible for up to 69% of OCR at Station 1, although most
197 values were closer to 10-40% at Stations 2 and 3 (Fig. 3A and B). In contrast, ammonia
198 oxidation contributed < 5% of oxygen consumption in the OMZ (Supplementary Figure 1).

199 Overall, these data demonstrate consistent patterns in the contribution of nitrite oxidation to
200 OCR, as the proportion of DO consumed by nitrite oxidation increased at progressively lower
201 DO concentrations across multiple stations.
202
203 *Effects of oxygen manipulation experiments on nitrite oxidation and OCR*
204 We corroborated these observations by experimentally manipulating DO concentrations to
205 measure the response of OCR, as well as $^{15}NO_2^-$ nitrite oxidation rates, to changing DO (Figure
206 4). To examine responses of different assemblages in different depth regions, we conducted
207 experiments on the edge of the OMZ at Stations 2-4, in the SCM at Stations 2 and 3, and in the
208 SNM at Station 3 and an additional sampling Station 3.5 (Table 1). (We could not conduct
209 additional experiments at Stations 1 and 2 owing to a hurricane in the region at the time of
210 sampling in 2018, and added Station 3.5 as a result.) Oxygen manipulations were designed to
211 quantify OCR and nitrite oxidation across the full spectrum of DO concentrations that occur
212 from the OMZ edge to its core—rather than solely probing their lower limits—in order to
213 constrain rate responses to changing DO within OMZs.
214 Oxygen manipulation experiments were consistent with water column profiles and
215 provide additional evidence that nitrite oxidation can be a substantial oxygen sink. In all
216 experiments, we found that OCR declined with experimentally decreased DO (Fig. 4). These
217 declines were particularly steep below 1-2 μM DO across different experiments. For the SCM
218 and SNM experiments, DO concentrations >1 μM are obviously higher than expected to occur *in*
219 *situ*, but these concentrations were included for comparison across experiments and with earlier
220 studies[9,15]; OCR measurements for SCM and SNM samples conducted at DO concentrations >1
221 μM therefore represent potential rates. For OMZ edge samples, OCR values in the μM range
222 were higher than observed in profiles—most likely due to the effects of bubbling[19], which could
223 physically break down organic matter present in higher concentrations at these depths (Table 1).
224 Throughout all experiments, rate magnitudes in the 100s of nM DO concentration range (11-820
225 nmol L^{-1} d^{-1}) were similar to profile measurements (Fig. 2), as well as to previous measurements
226 in OMZs[15,16,18,19] (see above).
227 DO concentrations were also continuously monitored in a subset of experimental bottles,
228 and DO consumption was consistently linear (see Methods). The few exceptions occurred in
229 several experiments conducted at DO concentrations <235 nM; in these experiments, OCR

230 declined as DO was consumed over time. These observations are consistent with Tiano et al.[16]
231 and allowed us to calculate the low-level DO affinity of the community (following ref. 16), in
232 addition to overall values based on variations across all experimental incubations (following ref.
233 19; Supplementary Note 1). However, we note that both reflect mixed assemblages of
234 microorganisms that use DO to oxidize a variety of substrates (Supplementary Note 1). These
235 substrates can include nitrite, as well as different forms of organic matter, reduced sulfur
236 compounds, and possibly methane[32]. Low-level results were consistent with previous
237 observations indicating high affinity for DO (K_m ranged from 53-127 nM DO), and, importantly,
238 all data demonstrate that OCR decreases as DO decreases (Supplementary Note 1, Table 1).
239 In line with earlier work[22,23], nitrite oxidation rates also declined in response to
240 decreasing DO in most of our experiments (Fig. 4). However, nitrite oxidation rates were less
241 sensitive to declining DO than OCR. Nitrite oxidation was therefore responsible for
242 progressively higher proportions of overall OCR as DO was experimentally decreased (Fig. 3C).
243 In fact, we found that 97% of OCR in the SCM at Station 2 could be explained by measured
244 nitrite oxidation rates—indicating that nitrite oxidation alone can sustain all DO consumption at
245 DO concentrations just below 393 nM. However, most values were closer to 10-20%. Overall,
246 patterns in experiments were notably similar to results from rate profiles: both showed increases
247 in the contribution of nitrite oxidation to DO consumption below 2 μM DO, and both displayed
248 similar values (Fig. 3B and C).
249 Based on these data, we used several approaches to calculate the DO level at which nitrite
250 oxidation may be expected to consume all DO. First, we fit power law curves to the
251 relationships in Figure 3, which suggest that nitrite oxidation would be responsible for all OCR
252 at DO concentrations of 15 nM (based on experiments; r^2=0.40, P<0.0005) to 44 nM (based on
253 profiles; r^2=0.51, P<0.0001). However, as the data above indicate, this contribution is obviously
254 variable across experiments: our experiment in the SCM at Station 2 showed that nitrite
255 oxidation can consume all available DO at ca. 393 nM DO (Fig. 4C), while the experiment with
256 20 μM [DO] water at Station 2 suggests that, even at low DO levels, nitrite oxidation would not
257 consume all DO (Fig. 4B). In general, nitrite oxidation is unlikely to be dominant on the edge of
258 the OMZ due to higher DO and organic matter concentrations, along with lower nitrite
259 concentrations (Figs. 2 and 3, Table 1). We therefore applied multiple approaches to the SCM
260 and SNM experiments, including using regression and calculating kinetic parameters based on

experimental treatments (Supplementary Note 2). These calculations collectively indicate that nitrite oxidation can account for all DO consumption at up 267 nM in the DCM station 2 (based on regression); for the other experiments, nitrite oxidation is estimated to consume all DO below concentrations of 9-46 nM (Supplementary Note 2).

These data tie together multiple aspects of OMZ biogeochemistry into a coherent picture. Although nitrite oxidation rates decline as DO is experimentally decreased at individual depths—and so with particular assemblages of NOB—different depths/assemblages display different properties (Fig. 4 and Supplementary Note 2). More importantly, this decline is always less severe for nitrite oxidation than for overall OCR (Figs. 2-4). NOB are therefore effective at scavenging DO (Figs. 3 and 4; ref. 15), and the concentration range over which this occurs in our experiments matches model predictions (0.2-1 μM DO) involving a nitrogen-oxygen feedback loop in OMZs driven by oxygen depletion via nitrite oxidation[17].

Isotopic, 16S rRNA, and metagenomic constraints on nitrite oxidation

To further verify our findings, we applied natural abundance stable isotope measurements, 16S rRNA sequencing, and metagenome sequencing to water samples and nucleic acid samples collected in parallel with nitrite oxidation rate measurements (Figure 5). The dual N and O isotopic composition of dissolved nitrate ($\delta^{15}N$ and $\delta^{18}O$, respectively; Supplementary Figure 2) is thought to provide an effective constraint on nitrite oxidation under low DO conditions due to isotopic 'overprinting' by nitrite oxidation[33,34]. While the respiratory reduction of nitrate (denitrification) leads to equal 1:1 increases in $\delta^{15}N$ and $\delta^{18}O$ (refs. 35, 36), deviations in 1:1 N:O isotope behavior arise from a decoupling of the N and O systems. These deviations are widely interpreted as reflecting cryptic re-oxidation of nitrite under low DO, where the reduction of nitrate to nitrite removes an O atom, and the subsequent re-oxidation of nitrite to nitrate appends a new O atom derived from ambient water[37,38]. Deviations from the 1:1 relationship are represented via $\Delta(15,18)$ values[39], where more negative values represent larger departures from the 1:1 relationship.

At Stations 1-3, dual nitrate isotope values exhibited non-linear trends (Figure 5B and Supplementary Figure 3) that are consistent with isotopic overprinting by nitrite oxidation[33,34]. $\Delta(15,18)$ values were lowest (i.e., deviations were largest) at 50-150 m at Station 1, 100-140 m at Station 2, and 110-160 m at Station 3. The lower portions of these depth ranges overlapped with

292 the upper portion of the SCM (75-125 m, 105-155 m, and 120-180 m, respectively) and tracked
293 depth variations between stations. Peak deviations at Stations 1 and 2 were just above the SCM
294 (75 and 100 m), while the peak at Station 3 corresponded closely with the SCM (140 m).
295 Throughout these ETNP OMZ sites, our nitrate isotope data are consistent with previous
296 observations and interpretations of rapid recycling between nitrate and nitrite[33,39,40], ultimately
297 evidenced by isotopic overprinting of the nitrate reduction signal by nitrite oxidation.
298 16S rRNA gene and transcript sequencing revealed a similar pattern to rate profiles,
299 experiments, and isotopic data, while also enabling identification of NOB that are abundant and
300 active in the ETNP OMZ. Based on DNA, *Nitrospina* 16S rRNA amplicon sequence variants
301 (ASVs; ref. 41) comprised up to 3.4-5.4% of all ASVs at Stations 1-3 (Fig. 5C), and relative
302 abundances were consistent with the isotopic anomalies—abundances and anomalies were well-
303 correlated (r^2=0.70-0.84, $P<0.05$) at Stations 1 and 3, for instance. These patterns were
304 accentuated for RNA samples, but with discrete peaks in 16S rRNA transcripts generally
305 occurring at deeper depths (Fig. 5D). *Nitrospina* 16S rRNA peaked at 125 m at Sta 1 (1.8% of
306 all 16S rRNA transcripts; with an additional upper water column peak at 50 m), 140 m at Station
307 2 (3.2%), and particularly 140-180 m at Station 3 (1.6-7.1%). All of these depths lie within the
308 SCM at each station. Although 20 *Nitrospina* ASVs were identified, three were dominant (each
309 >1% of all ASVs, together constituting 72-100% of all *Nitrospina* 16S rRNA gene sequences
310 and transcripts). One of these ASVs was found only in OMZ samples at Stations 1-3, and was
311 not detected above the OMZ, nor at Stations 4 and 5. This was also the lone ASV observed in
312 the OMZ at Station 3, where *Nitrospina* were most active based on their comparatively high
313 percentages of all 16S rRNA transcripts. In contrast, no *Nitrococcus* ASVs and only one low-
314 abundance *Nitrospira* ASV were identified out of >11,000 ASVs and >3.5 million 16S
315 sequences from 73 DNA and 73 RNA samples. In line with earlier work in the ETNP[7,25], other
316 OMZs[6,42], and the variations in DO affinity observed across oxygen manipulation experiments
317 (Fig. 4), these results indicate that particular *Nitrospina* ecotypes—and one ASV in particular—
318 are significant for low-oxygen nitrite oxidation, while other ASVs may be more important at
319 different depths and DO concentrations.
320 To validate the genetic potential for nitrite oxidation by *Nitrospina* under low DO
321 concentrations, we sequenced metagenomes collected on the OMZ edge, at the SCM, and within
322 the OMZ core at Stations 1, 2, and 3. Nitrite oxidoreductase genes from *Nitrospina* were

323 prevalent at all stations and depths, establishing the genomic potential for nitrite oxidation
324 throughout the ETNP OMZ (Table 2). In addition, multiple high-affinity cytochrome C oxidase
325 genes from *Nitrospina* were present in all samples—indicating that *Nitrospina* are capable of
326 consuming DO at the low concentrations found within the OMZ. These genes were more
327 common in SCM and SNM metagenomes than at the OMZ edge, consistent with *Nitrospina*
328 ecotype distributions, and with the lower DO concentrations found in the SCM and SNM (Table
329 2). Cytochrome *bd*-type oxidase genes from *Nitrospina* were also detected in all metagenomes
330 except the OMZ edge sample at Station 1, although these lack quinol binding sites and may not
331 function as canonical oxidases[25,43]. Sun et al.[25] also suggested that chlorite dismutase genes may
332 be relevant for anaerobic metabolism in *Nitrospina*, and these were present in all metagenomes
333 (Supplementary Table 1). We also recovered formate dehydrogenase and nitrate reductase genes
334 indicative of anaerobic metabolism in *Nitrospina*[24] (Supplementary Table 1). Both of these
335 genes were absent from OMZ edge metagenomes at Stations 1 and 2, showing higher relative
336 abundances in the SCM, and especially the SNM. *Nitrospina*-derived nitrate reductase genes
337 were present at similar levels as nitrite oxidoreductase in the SNM, while less common in the
338 SCM (Supplementary Table 1). Finally, *Prochlorococcus* genes were prevalent from the OMZ
339 edge to core, and especially within the SCM, supporting the idea that oxygenic photosynthesis
340 and cryptic oxygen cycling occur throughout the ETNP OMZ (Table 2). Overall, metagenomic
341 data are consistent with experimental data and 16S data, and indicate *Nitrospina* are tightly tuned
342 to DO concentrations in the ETNP.
343
344 **Discussion**
345 Assembled together, our results provide a comprehensive view of nitrite oxidation and its
346 contribution to oxygen consumption in the ocean's largest OMZ. Rate measurements, oxygen
347 manipulation experiments, stable isotopic data, and molecular data all converge on the fact that
348 nitrite oxidation is active from the base of the euphotic zone into the OMZ. Nitrite oxidation was
349 particularly significant within the SCM at Stations 1-3: nitrite oxidation rates were elevated (Fig.
350 2), isotopic overprinting of nitrate by nitrite oxidation was evident (Fig. 5B and Supplementary
351 Figure 3), and *Nitrospina* were abundant and active (Fig. 5C and D). All of these data tracked
352 systematic progression in the depth of the SCM across stations (Fig. 2), and indicate that nitrite
353 oxidation is important for DO utilization in the SCM. Based on rate profiles, nitrite oxidation

354 ranged from 10-47% of OCR in the SCM (Fig. 3B), while experiments indicate maximum
355 percentages of 13-97% (Fig. 3C). Stations 4-6—which lack a SCM and SNM—provide a
356 notable contrast, as do ammonia oxidation rate profiles (Supplementary Figure 1).
357 Peaks in the magnitude of departure from a 1:1 nitrate reduction signal (e.g., isotopic
358 overprinting by nitrite oxidation), and in *Nitrospina* ASV abundances, further indicate that nitrite
359 oxidation is active above and below the SCM (Fig. 5). At Stations 2 and 3, nitrite oxidation rates
360 above the SCM were similar to rates deeper in the water column, while Station 1 showed a rate
361 increase within the AMZ (Fig. 2). In OMZs/AMZs, DO may be photosynthetically produced in
362 the SCM[15] or introduced through physical mixing[12,16], but both of these DO sources would be
363 less significant below the SCM. Instead, the introduction of DO during sample manipulation
364 effectively measures the potential for oxygen consumption in samples collected below the SCM.
365 Short of conducting *in situ* incubations, such DO contamination is essentially unavoidable, and is
366 the reason that we also performed oxygen manipulation experiments (Fig. 4)—which also have
367 limitations[19]. In line with earlier work in OMZs facing these same challenges[16,18], oxygen
368 consumption potential was significant even below the SCM. Most importantly, inclusion of DO
369 sensor spots in all bottles allowed us to directly measure DO in every incubation, and therefore
370 to quantify nitrite oxidation and OCR as a function of DO across all profiles and experiments.
371 Patterns in OCR and nitrite oxidation were notably consistent between profiles and experiments
372 (Fig. 3B and 3C). This similarity indicates that—independent of methodology—nitrite oxidation
373 increases in relative importance below 2 μM DO.
374 To make our findings widely relevant to current and emerging OMZ regions, we
375 examined the full range of DO concentrations found in OMZs, rather than in the AMZ
376 exclusively. However, consistent with isotopic data, we found that *Nitrospina* 16S rRNA
377 transcripts increased in relative abundance within the AMZ (Fig. 5). RNA samples were
378 collected from the CTD rosette and rapidly filtered, so if these data capture a response to oxygen
379 exposure, this response is exceptionally rapid and consistent. Alternatively, these data indicate
380 that *Nitrospina* are active within the AMZ. One possibility is that *Nitrospina* survive
381 anaerobically for periods of time until provided DO[24]. Oxygen-evolving mechanisms from
382 nitrite have also been proposed for anaerobic methane-oxidizing bacteria[44] that are active in
383 OMZs[45]. The presence of *Prochlorococcus* below the SCM may allow low-level DO production
384 via oxygenic photosynthesis (Table 2). Finally, Tiano et al.[16] previously detected DO at 500 m

385 depth within the core of the ETNP AMZ. This is substantially deeper than our experiments and
386 well below the SCM (Fig. 2)—suggesting that DO can be periodically introduced even deep
387 within the AMZ. Regardless of the mechanism, isotopic data and 16S rRNA data suggest that
388 transient nitrite oxidation coupled to nitrate reduction occurs below the SCM[17].
389 However, our primary focus was on oxygen consumption via nitrite oxidation and its
390 implications for the formation, maintenance, and expansion of OMZs. Two clear and
391 remarkably consistent patterns emerged from both water column profiles and oxygen
392 manipulation experiments: (i) overall OCR declined with decreasing DO in profiles and
393 experiments (Figs. 2C and 4), while (ii) nitrite oxidation increased as a proportion of OCR as DO
394 declined in both profiles and experiments (Fig. 3B and C). Nitrite oxidation was significant
395 throughout the OMZ and experiments, but increased in relative importance below 2 μM DO—
396 typically ranging from 10-40% of OCR—and was only dominant at low DO concentrations
397 (Table 1). For example, nitrite oxidation consumed nearly all DO produced in the SCM
398 experiment at Station 2 (Fig. 3C). Variation in the proportion of overall OCR attributable to
399 nitrite oxidation could reflect fluctuations through time[17], as well as differences in substrate
400 availabilities and affinities relative to DO for different processes consuming DO. Other than
401 nitrite oxidation, aerobic respiration of organic matter is most significant for OCR given relative
402 substrate concentrations (organic C > CH_4 or reduced S compounds; refs. 45–47), as well as high
403 affinities for DO among heterotrophic bacteria[48-50]. Our data indicate that aerobic respiration is
404 likely the dominant DO-consuming process from the edge of the OMZ (20 μM DO) until OCR
405 declines precipitously (Figure 4 and Table 1).
406 Many aerobic heterotrophs are also facultatively anaerobic, switching to nitrate reduction
407 under low DO[13,51]. Along with a substrate-driven decline in aerobic respiration rates with
408 decreasing DO, this shift to nitrate reduction likely explains the steep declines in OCR observed
409 in our experimental data (Fig. 4). Production of nitrite from nitrate reduction also provides a
410 needed substrate for nitrite oxidation, and is consistent with isotopic data (Fig. 5B and
411 Supplementary Figure 3). We suggest that such cryptic nitrite/nitrate cycling explains the
412 adaptations of particular *Nitrospina* bacteria to AMZs: although these are low-DO environments,
413 they are also high-nitrite environments, representing a trade-off in the availability of electron
414 donor and acceptor. The fact that nitrite oxidation rates increase with increasing nitrite into the
415 micromolar range[9], and that nitrate is reduced at similar rates[52], provides additional support for

416 this idea. Our results further indicate that nitrite oxidation is carried out by just a few *Nitrospina*
417 ASVs under these conditions—in contrast with the diversity of other microbial groups that may
418 be involved in overall oxygen consumption[4,53]. These *Nitrospina* are disproportionately
419 biogeochemically important given their roles in DO consumption and regeneration of nitrate,
420 which precludes its further reduction. For these organisms, the costs of life at low DO are offset
421 by the advantages of persistently elevated nitrite concentrations.
422 At the same time, efficient scavenging of DO by *Nitrospina* can maintain DO at low
423 levels favorable for nitrate reduction[17,54]. This represents a form of biogeochemical symbiosis,
424 in that microorganisms performing nitrite oxidation and nitrate reduction mutually benefit from
425 their biogeochemical interaction. Rate profiles, oxygen manipulation experiments, natural
426 abundance measurements, 16S rRNA sequencing, and metagenomic data all favor this idea: the
427 proportion of DO consumed by nitrite oxidation increased at progressively lower DO
428 concentrations in both profiles and experiments (Fig. 3B and C); isotopic anomalies are
429 indicative of coupled nitrite oxidation and nitrate reduction (Figs. 5B and Supplementary Figure
430 3); and the low-oxygen adaptations of particular *Nitrospina* support high abundances and activity
431 (Table 2, Fig. 5C and D). These data also provide multiple lines of direct evidence that verify
432 model results showing co-existence of nitrite oxidation and nitrate reduction emerging below ~1
433 μM DO (refs. 17, 54). By placing entirely new bounds on the contribution of nitrite oxidation to
434 DO consumption, we resolve the niche of nitrite-oxidizing *Nitrospina* within OMZs, quantify
435 their contributions to coupled oxygen and nitrogen cycling, and convincingly establish that
436 nitrite oxidation is pivotal in the maintenance and biogeochemical dynamics of these important
437 regions of the ocean.
438
439 **Materials and Methods**
440 **Sample Collection.** Samples were collected in April 2017 and June 2018 aboard the *R/V*
441 *Oceanus*. At each station, conductivity/salinity, temperature, depth, pressure, chlorophyll
442 fluorescence, and photosynthetically active radiation (PAR) were measured by a SeaBird SBE-
443 9plus CTD, SBE-3F temperature sensor, SBE-43 DO sensor, WetLabs ECO-FLR Fluorometer,
444 and Biospherical QCP2200 PAR sensor. Initial casts were used to measure DO and nutrient
445 profiles to guide subsequent sampling. Nutrient samples were analyzed for NH_4^+ and NO_2^-
446 aboard the ship, with additional shore-based analyses of combined NO_3^-+NO_2^- and PO_4^{3-} at the

447 University of California Santa Barbara Marine Science Institute Analytical Lab (Supplementary
448 Materials and Methods).
449 At all stations, water samples were collected in the upper 100 m to capture variation in
450 the upper water column, and we then sampled across a range of DO levels and nitrite levels to
451 capture variation in the OMZ. Samples were collected at 200, 100, 50, 20, 10, 5, and 1 μM [DO]
452 at all stations. Because the OMZ is shallower and more intense moving south and nearshore in
453 the ETNP (Fig. 1), these DO levels can occur within the upper 100 m, such that upper 100 m and
454 DO-based sampling overlapped. This overlap was greater at Station 1, followed by 2 and 3. At
455 these AMZ stations, samples were also collected at three depths between the 1 μM [DO] level
456 and the SNM. Experiments were conducted for 24 hours in the dark in a cold van adjusted to
457 ambient temperature, with separate incubations (and therefore temperatures) conducted for
458 samples from the upper 100 m, for oxygen/nitrite-based sampling, and for oxygen manipulation
459 experiments at each station.
460
461 **OCR Measurements and Oxygen Manipulations**. Two types of sensor spots were used to
462 measure OCR and manipulate DO, one with a wider range and detection limit of 100 nM (Fibox,
463 Loligo Systems, Viborg, Denmark), as well as trace-level spots with a detection limit of 10 nM
464 (FireSting, Pyroscience, Aachen, Germany)[31]. Loligo sensor spots were used for water column
465 profiles given the wider DO range, while FireSting trace-level spots were used to establish
466 oxygen manipulation experiments and measure OCR within them. Optical sensors were
467 calibrated using DO-saturated water and sodium sulfite-saturated and He-purged water. On the
468 upper end, Fibox sensor spot DO measurements were highly correlated with CTD DO values
469 (r^2=0.996, slope=0.997, P<0.00001) for [DO] > 1 μM. On the lower end, we regularly checked
470 the '0 nM' concentration with repeated measurements of sodium sulfite-saturated water and He-
471 purged water, and our lowest values measured in experiments were consistently within 10 nM of
472 0. In both profiles and experiments, OCR was measured by the decrease in DO over the course
473 of experiments[30,55]. Oxygen-based measurements such as these are often converted to C-based
474 respiration rates assuming oxidation of Redfield-type organic material, but our results
475 demonstrate that this assumption does not hold in OMZs.
476 Water column profiles of OCR were measured using 5 replicates at each depth, including
477 one each with tracer level (5-10% *in situ* concentration measured at sea) addition of $^{15}NH_4^+$ or

478 $^{15}NO_2^-$ to measure ammonia/nitrite oxidation (see below). Loligo sensor spots were attached to
479 the inside of 300 mL Wheaton BOD glass bottles using silicone glue. At sea, bottles were filled
480 to at least three times overflowing via slow laminar flow, were rapidly capped and transferred to
481 the cold van, and initial DO measurements were made. DO was measured subsequently at 10-14
482 hours, and at the end of the 20-24 hour incubations. The majority of OCR rates calculated from
483 both measurement time points were highly correlated (r^2= 0.968-0.995; all P<0.0001) with slopes
484 ranging from 1.06-1.19 (across different stations), indicating that OCR did not accelerate or
485 decrease substantially over the course of the incubations. The only exceptions were three
486 sampling depths from station 3 (77, 87, 97 m) showing nonlinearity, and so we report data from
487 measurements conducted at similar depths (75, 88, 100 m) the previous day. After incubation,
488 endpoint DO measurements were made and then 50 mL samples from relevant bottles were
489 frozen for nitrite and ammonia oxidation rate measurements.
490 Oxygen manipulation experiments were conducted in 500 mL serum bottles with
491 attached FireSting sensor spots. We conducted a total of seven experiments, with experiments
492 on the OMZ edge and in the SCM at Station 2; on the OMZ edge, in the SCM, and in the SNM at
493 Station 3; in the SNM at Station 3.5; and on the OMZ edge at Station 4. For each experiment, a
494 total of 24 bottles were filled with water collected at a specific depth, sealed with deoxygenated
495 stoppers, and then bubbled with ultrapure He gas (15-30 minutes) while DO was monitored. We
496 introduced an He headspace of 20 mL, and air was injected into bottles to raise DO
497 concentrations when needed. For each experiment, we established initial DO values ranging
498 from 10s to 1000s of nM (Figure 4), and 8 bottles had tracer-level $^{15}NO_2^-$ additions, 8 bottles had
499 tracer-level $^{15}NH_4^+$ additions, and 8 were unlabeled. For all experiments and water column
500 profiles, bottles were cleaned with 0.1 M HCl and triple rinsed with water between incubations,
501 and dedicated bottles were used for the different ^{15}N labels.
502 OCR was measured in all experimental bottles based on starting and ending DO values,
503 and samples for nitrite oxidation were collected from $^{15}NO_2^-$ labeled bottles at the end of the
504 experiments. Four to eight bottles in each experiment were also continuously monitored for DO
505 concentration changes throughout the experiment. We tested DO consumption curves for
506 linearity over time using the Maximal Information Coefficient (MIC; ref. 56.) and linear
507 regression. MIC effectively captures both linear and nonlinear relationships, such that
508 differences between higher MIC scores and lower regression coefficients are indicative of

509 nonlinear relationships, whereas high MIC scores combined with high r^2 values are indicative of
510 strong linear relationships[56]. We found the vast majority of DO time courses were linear (r^2 >0.6,
511 MIC-r^2 <0.2). The few exceptions—one incubation bottle each in the SCM at Stations 2 and 3,
512 one in the SNM at Station 3, two in the SNM at Station 3.5, and one on the OMZ edge at Station
513 4—all showed a decline in OCR over time. All of these incubation bottles had average DO <235
514 nM, and were used to calculate affinity for DO at these low concentrations (Supplementary Note
515 1).
516
517 **Nitrate isotopic measurements.** Both natural abundance and ^{15}N-tracer-based nitrate isotopes
518 were quantified by use of the denitrifier method[57,58]. Samples were collected from the rosette or
519 at the end of incubations, filtered through a 0.2 µm filter, and then frozen until analysis. In the
520 lab, 20-30 nmoles of NO_3^- were quantitatively converted to N_2O by a culture of the denitrifying
521 bacterium, *Pseudomonas aureofaciens*. Product N_2O was cryogenically purified and trapped
522 under flow of ultra-high purity He, before being introduced to an IsoPrime 100 isotope ratio
523 mass spectrometer. Isotope ratios were normalized to international reference materials (USGS
524 32, USGS 34, USGS 35) and reported using standard delta notation. Any nitrite present in the
525 sample was removed by addition of sulfamic acid[35]. In samples from the upper 100 m, where
526 NO_3^- concentrations were low, 'carrier' NO_3^- of known isotopic composition was added and
527 sample compositions were calculated by mass balance. Precision of natural abundance isotope
528 ratio measurements was ±0.3‰ and ±0.4‰ for $\delta^{15}N$ and $\delta^{18}O$, respectively. $\delta^{15}N$ and $\delta^{18}O$
529 values were used to calculate the Δ(15,18) deviation from the 1:1 line following Sigman et al.[39].
530
531 **Nitrite Oxidation Rates.** Nitrite oxidation rates were measured by adding 98 atom percent
532 (atom%) $^{15}NO_2^-$ to a final concentration of 12-184 nmol L^{-1}—representing 5-10% of *in situ* NO_2^-
533 concentrations (with the exception of some samples in the upper 100m with no measurable
534 nitrite)—and measuring the accumulation of ^{15}N label in the NO_3^- pool following Beman et al.[7].
535 After incubation for ~24 hours, samples were frozen at sea. Upon thawing in the laboratory,
536 excess $^{15}NO_2^-$ was removed following Granger and Sigman[35]. In brief, sulfamic acid (~8-10 µL
537 mL^{-1}) was added, the samples were shaken and allowed to sit for >5 minutes, and then samples
538 were neutralized by adding NaOH (4 M, ~11 µL mL^{-1}) prior to analysis.

539 Rates of $^{15}NO_2^-$ oxidation ($^{15}R_{ox}$) were calculated using equation 1 from Beman et al.[7]
540 adapted from Ward et al. (ref. 59):

541 $$^{15}R_{ox} = \frac{\left(n_t - n_{oNO_3^-}\right) \times \left[NO_3^-\right]}{\left(n_{NO_2^-}\right) \times t} \quad (1)$$

542 where n_t is the atom% ^{15}N in NO_3^- measured at time t corrected for $^{15}NO_3^-$ contamination of the
543 $^{15}NO_2^-$ stock; $n_{oNO_3^-}$ is the measured atom% ^{15}N of unlabeled NO_3^-; $[NO_3^-]$ is the concentration of
544 the NO_3^- pool; and $n_{NO_2^-}$ is the exponential average atom% of NO_2^- over time t. $n_{NO_2^-}$ was
545 calculated by isotope mass balance based on initial NO_2^- concentrations, measured isotopic
546 composition, added ^{15}N-labeled NO_2^-, and measured $^{15}NH_4^+$ oxidation rates (which produce
547 unlabeled NO_2^-; ammonia oxidation rate measurements followed Beman et al.[28]).
548
549 **RNA/DNA Extractions, 16S rRNA Sequencing and Analysis, Metagenome Sequencing and**
550 **Analysis, and Organic Matter Analysis** are reported in the Supplementary Materials and
551 Methods.
552
553 **Sequence data** are available in the Sequence Read Archive under BioProjects PRJNA192803
554 (16S data), and PRJNA634212 (metagenomes).
555
556 **Statistical analyses** were carried out in the R statistical environment (RStudio Version 1.0.136).
557 Linear regressions were performed using the 'lm' function in R; Michaelis-Menten curve fits
558 were performed using the 'drm' function in the R package drc (ref. 60).
559
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566
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Figures (for initial submission)

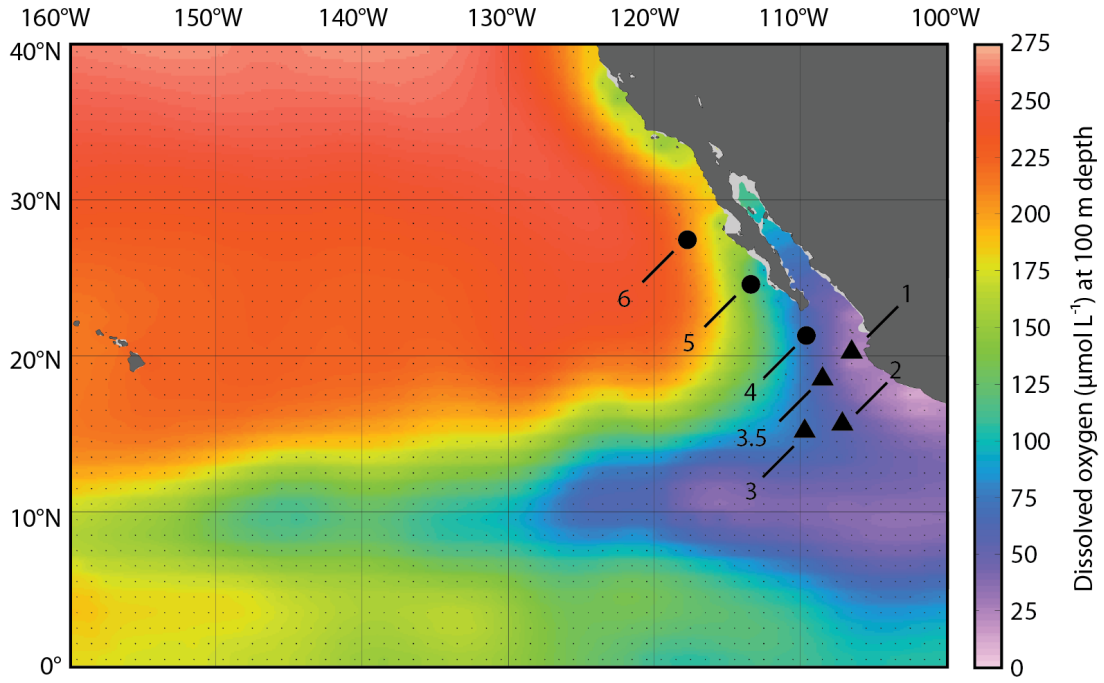

Figure 1: Station locations (numbered symbols) in the ETNP plotted on dissolved oxygen concentrations (in µM) at 100 m depth from the World Ocean Atlas. Triangles denote AMZ Stations 1-3.5 and circles denote OMZ stations 4-6.

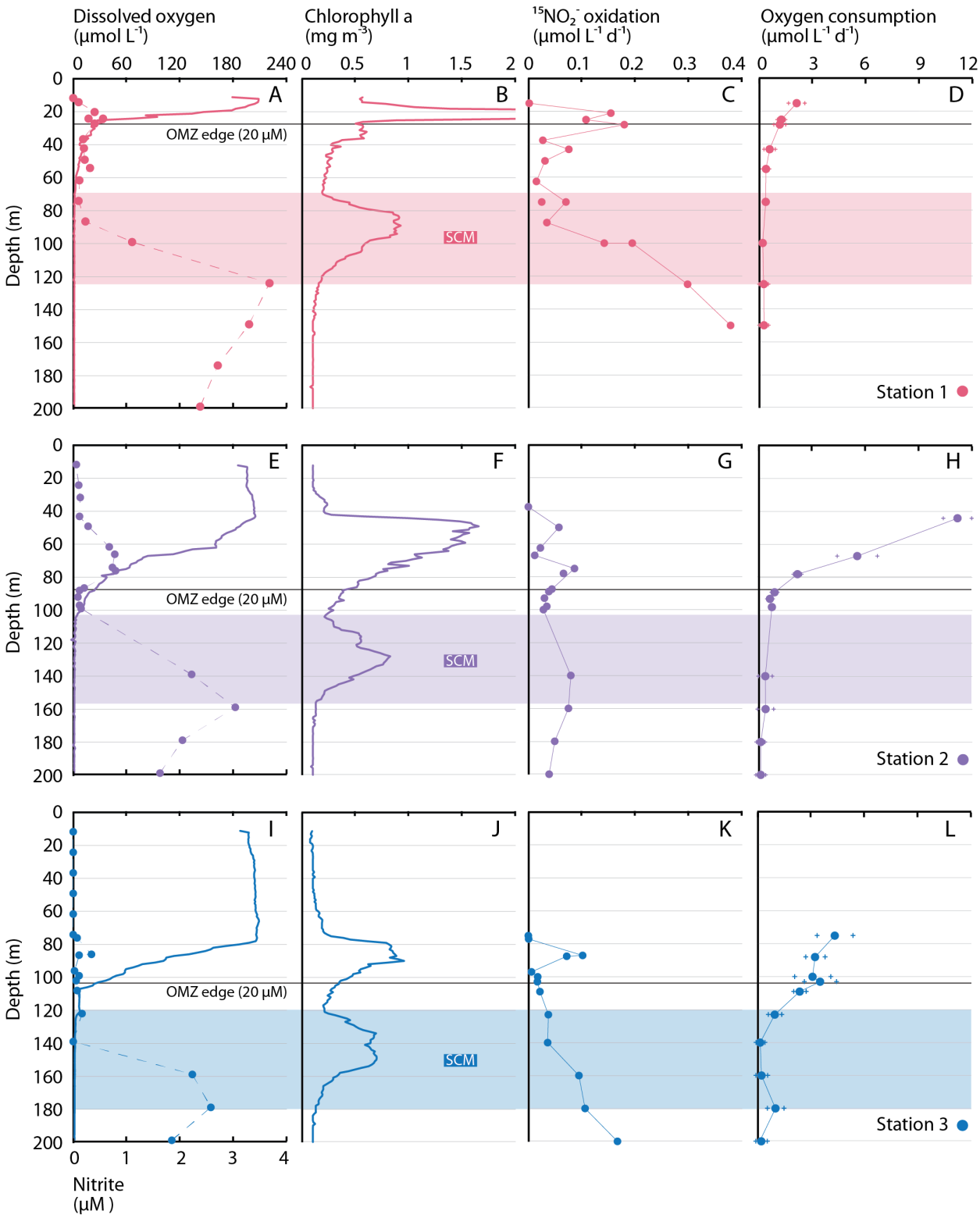

Figure 2: Depth profiles of (A,E,I) DO (solid lines) and nitrite (data points connected by dashed lines), (B,F,J) chlorophyll a, (C,G,K) nitrite oxidation rates, and (D,H,L) OCR (error bars denote

standard deviation of five replicates) show consistent variation across (A-D) Station 1, (E-H) Station 2, and (I-L) Station 3 (denoted by different colors). Black horizontal lines denote the depth of the OMZ, and shaded areas show the SCM at each station. Maximum chlorophyll values at Station 1 plot off-axis.

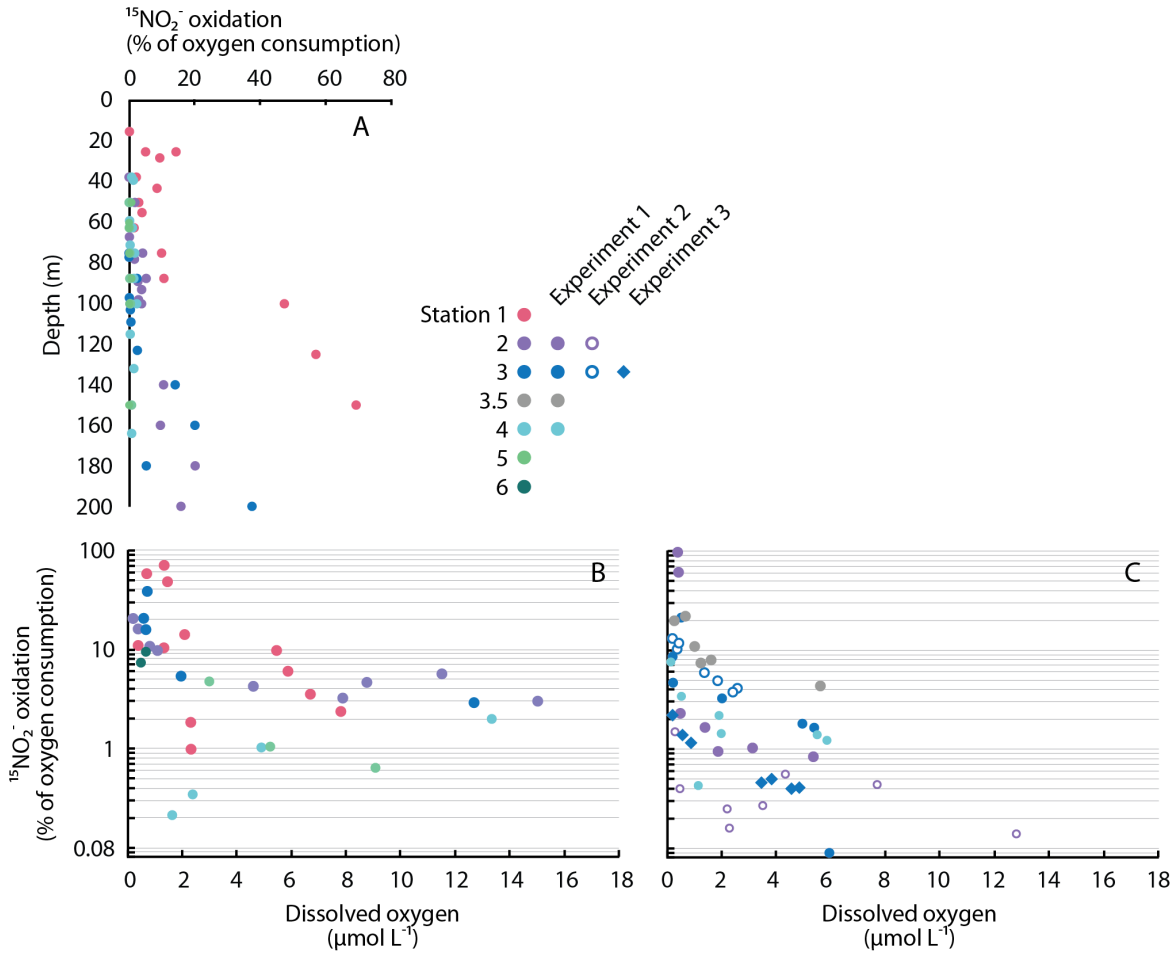

Figure 3: DO consumption via nitrite oxidation as a percentage of OCR with (A) depth, and as a function of average DO in (B) depth profiles and (C) oxygen manipulation experiments. Colors denote different stations. Only measurements for DO <18 µM are included in panel B, and all experimental bottles with dual nitrite oxidation and OCR are shown in C. In panel C, different symbols denote different experiments, with two experiments conducted at Station 2 (SCM and OMZ edge), three at Station 3 (SCM, SNM, and OMZ edge), and one each at Stations 3.5 (SNM) and 4 (OMZ edge).

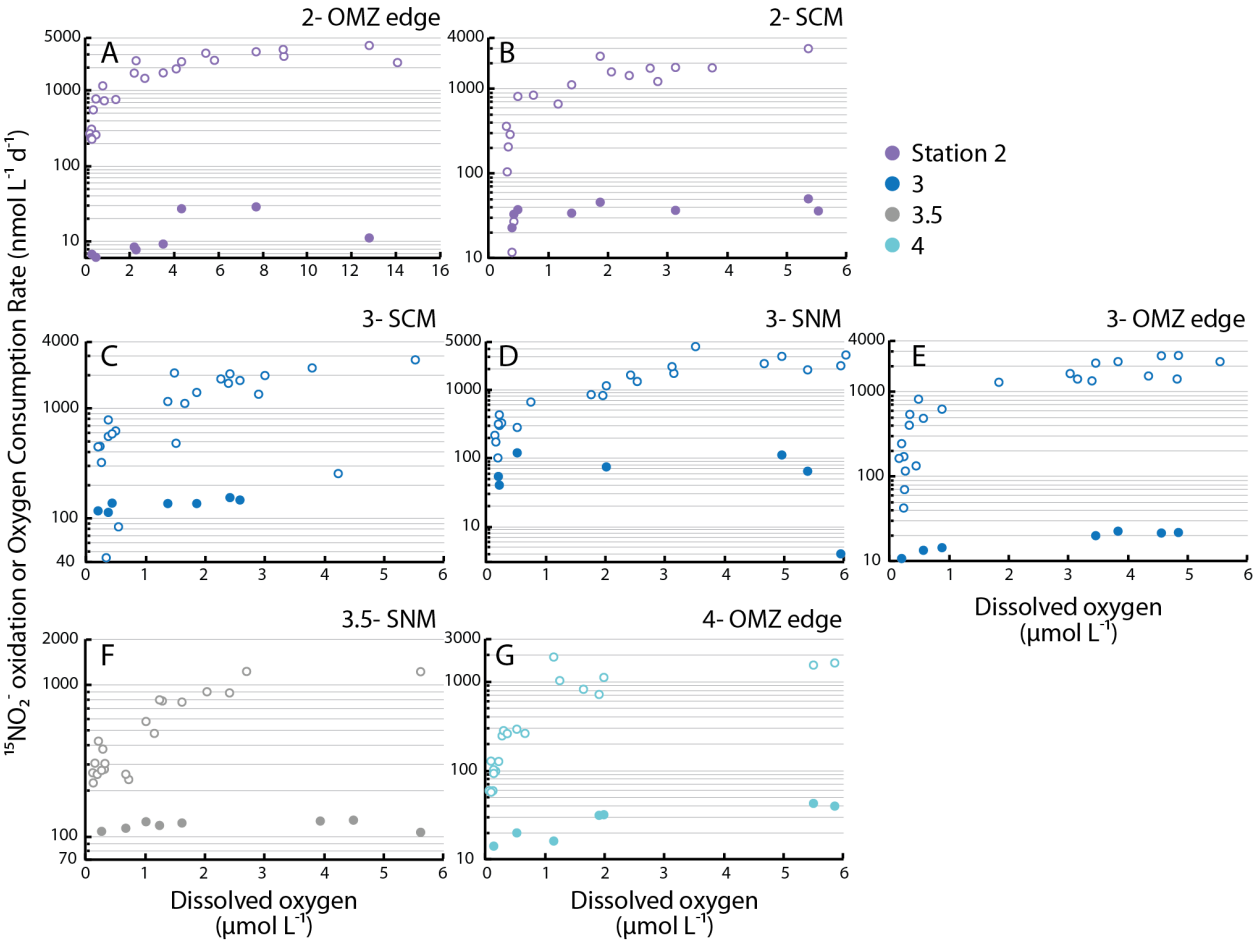

Figure 4: Nitrite oxidation rates (filled symbols) and OCR (open symbols) as a function of average DO in oxygen manipulation experiments. Colors denote different stations, with panels displaying data from different experiments: A) Station 2 OMZ edge (20 µM DO), B) Station 2 SCM, C) Station 3 SCM, D) Station 3 SNM, E) Station 3 OMZ edge, F) Station 3.5 SNM, and G) Station 4 OMZ edge. Note differences in vertical axes between experiments, and differences in horizontal axis in panel A compared with the remaining panels.

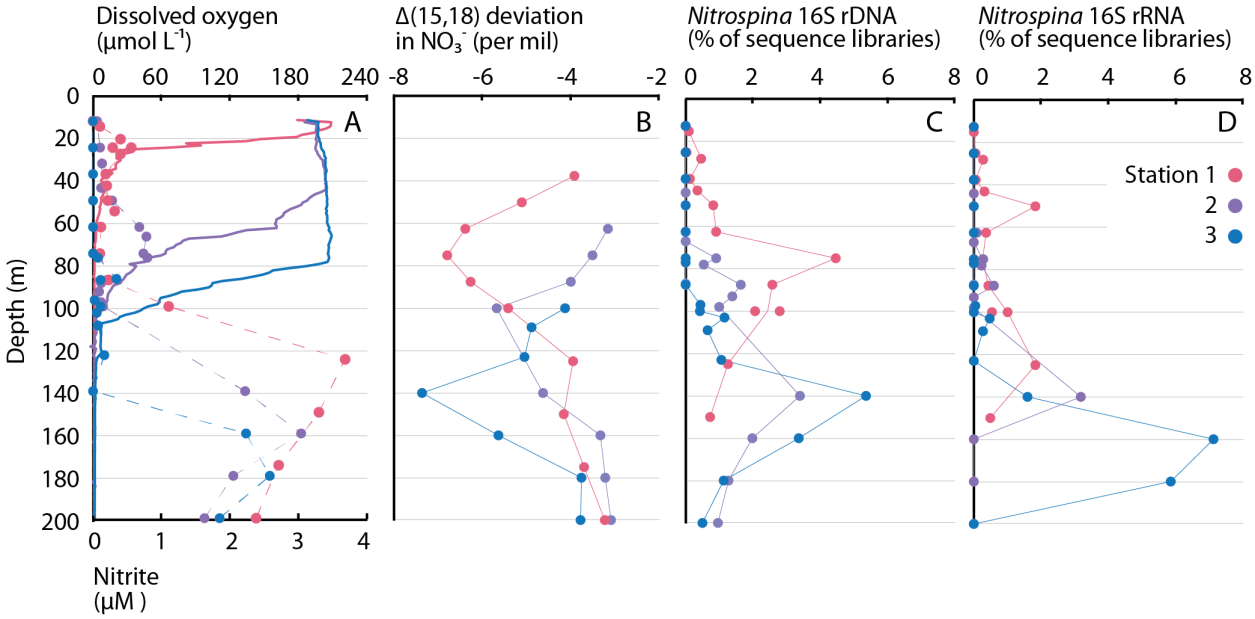

Figure 5: Depth profiles of (A) DO (solid lines) and nitrite (data points connected by dashed lines), (B) Δ(15,18) dual-isotope deviations in nitrate, and *Nitrospina* ASVs as a percentage of (C)16S rDNA sequence libraries and (D) 16S rRNA sequence libraries. Colors denote different sampling stations.

Table 1: Details of oxygen manipulation experiments, including: station, depth, region, and experiment length; starting DO, total organic carbon (TOC), and nitrite concentrations; and calculated parameters for OCR (including both low-level and overall K_m values) and nitrite oxidation rates (K_m values are for dissolved oxygen in all cases). NA=not applicable, and NS=parameter estimate not significant.

Experiment	**2-1**	**2-2**	**3-1**	**3-2**	**3-3**	**3.5-1**	**4-1**
Station	2	2	3	3	3	3.5	4
Depth (m)	90	140	130	170	110	80	100
Depth region	OMZ edge	SCM	SCM	SNM	OMZ edge	SNM	OMZ edge
Length (hr)	22	19	16	16	22	17	24
DO (nM)	19940	480	293	260	12800	274	3480
TOC (nM)	57965	45318	49118	46929	51211	44339	47397*
Nitrite (nM)	203	205	100	2410	73	2650	68
OCR							
Low-level K_m (nM)	NA	124	127	81	NA	53-104	82
Overall K_m (nM)	3262	1787	1804	6382^{NS}	4247^{NS}	1621	1738
v_{max} (nmol L^{-1} d^{-1})	4164	2757	2765	5973	4025	1612	2075
r^2	.878	.811	.569	.757	.868	.846	.776
Nitrite oxidation							
Overall K_m (nM)	1876^{NS}	185	63.8	38.4^{NS}	346	34.1	1015
v_{max} (nmol L^{-1} d^{-1})	23.3^{NS}	43.9	149	71.2	23.0	122	47.5
r^2	.347	.445	.665	.019	.910	.269	.825

*Station 4 TOC sample was collected at 90 m

761 **Table 2:** Relative abundance of *Nitrospina* reads, functional genes from *Nitrospina*, and
762 *Prochlorococcus* reads within metagenomes (expressed per million reads).
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Sta.	Depth	Depth region	DO (μM)	*Nitrospina*	Nitrite oxido-reductase	Cyto-chrome c	Cyto-chrome bd	*Prochloro-coccus*
1	25	OMZ edge	19.9	1084	2	0	0	865
1	87.5	SCM	0.46	31920	65	20	3	47992
1	100	SNM	0.30	21196	37	22	2	10765
2	89	OMZ edge	15.3	22442	38	12	10	57194
2	130	SCM	0.78	33290	57	20	4	33522
2	160	SNM	0.60	21725	37	28	2	4157
3	123	OMZ edge	13.0	18647	34	10	2	54709
3	140	SCM	0.74	34227	55	18	4	37202
3	180	SNM	0.45	17617	30	26	2	4881

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