

1 **Dissolved organic matter and heterotrophic**  
2 **prokaryotes diel patterns reveal enhanced**  
3 **growth at the mesopelagic fish layer during**  
4 **daytime**

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21

22 **ABSTRACT**

23 Contrary to epipelagic waters, where biogeochemical processes closely follow the light and dark  
24 periods, little is known about diel cycles in the mesopelagic realm. Here, we monitored the  
25 dynamics of dissolved organic matter (DOM) and planktonic heterotrophic prokaryotes every 2  
26 h for one day at the surface and 550 m (a depth occupied by mesopelagic fish during light  
27 hours) in oligotrophic waters of the central Red Sea. We additionally performed predator-free  
28 seawater incubations of samples collected from the same site at midnight and noon.  
29 Comparable variability in microbial biomass and dissolved organic carbon in situ suggests a diel  
30 supply of fresh DOM in both layers. The presence of mesopelagic fishes during daytime  
31 promoted a sustained, longer growth of larger prokaryotic cells, with specific growth rates  
32 consistently higher in both noon experiments (surface: 0.34 vs. 0.18 d<sup>-1</sup>, deep: 0.16 vs. 0.09 d<sup>-1</sup>).  
33 Heterotrophic prokaryotes in the mesopelagic fish layer were also more efficient at converting  
34 DOM into new biomass. These results suggest that the ocean's twilight zone receives a  
35 continuous diurnal supply of labile DOM from diel vertical migrating fishes, enabling an  
36 unexpectedly active community of heterotrophic prokaryotes.

37

38 Keywords: diel cycles, heterotrophic prokaryotes, mesopelagic fishes, vertical migration, carbon  
39 fluxes.

## 40 INTRODUCTION

41 Planktonic heterotrophic prokaryotes (HP) pertaining to the domains Bacteria and  
42 Archaea rely on labile dissolved organic matter (DOM) for metabolism and growth (Carlson et  
43 al. 1994, Goldman and Dennett 2000, Pomeroy et al. 2007). In surface waters, diel cycles in HP  
44 biomass and activity have been related to the photosynthetic activity of phytoplankton (Gasol  
45 et al. 1998), which obviously follows sunlight. Heterotrophic prokaryotes dependence of DOM  
46 derived from planktonic algae (Baines and Pace 1991) was reported to increase offshore, as we  
47 moved away from coastal inputs, in temperate and polar ecosystems (Morán et al. 2001).  
48 Although this relationship also known as bacterioplankton-phytoplankton coupling was  
49 questioned (Fouilland and Mostajir 2010), but see (Morán and Alonso-Sáez 2011), in regions  
50 with low DOM advection (e.g. at permanently stratified sites without anthropogenic or riverine  
51 inputs nearby, such as oligotrophic tropical waters), we might expect strong diel signals in the  
52 response of heterotrophic prokaryotes coupled with the activity of primary producers (Ruiz-  
53 González et al. 2012). In this regard, the Red Sea offers a unique opportunity to study  
54 biogeochemical processes in oligotrophic ecosystems. With no permanent rivers, the only  
55 allochthonous inputs of DOM come from urban centers such as Suez, Ghardaqa, Jeddah or Port  
56 Sudan, coastal macrophytes (Alongi and Mukhopadhyay 2015, Duarte and Cebrián 1996) or  
57 dust events (Bao et al. 2018, Lekunberri et al. 2010).

58 While epipelagic processes driven by primary production are well known (Henson et al.  
59 2012, Herndl and Reinthaler 2013), large gaps in our understanding of the ecology and  
60 biogeochemistry of the mesopelagic zone (i.e. waters between 200 m and 1000 m) remain  
61 (Robinson et al. 2010). In the mesopelagic realm, trophic interactions between microbes and  
62 metazoa have been long neglected. The available studies have focused mostly on  
63 mesozooplankton (e.g., (Al-Mutairi and Landry 2001, Bianchi et al. 2013, Isla et al. 2015)).  
64 However, recent reports on the large biomass contributed to the ocean's biota by mesopelagic  
65 fishes performing diel vertical migration (DVM, (Irigoien et al. 2014, Klevjer et al. 2016) suggest  
66 they may also play an important role as rapid vectors of labile organic matter. DVM can affect  
67 only a fraction or the entire community (Klevjer et al. 2016). In the Red Sea, the entire  
68 populations of mesopelagic fishes migrate daily between the surface and the so-called deep

69 scattering layer (DSL) located at 400-650 m in the mesopelagic zone (Klevjer et al. 2012, Røstad  
70 et al. 2016). DVM fishes have been recently suggested to generate hotspots for heterotrophic  
71 prokaryotes, yielding significantly higher bacterial growth efficiencies compared with shallower  
72 layers (Calleja et al. 2018). An analysis of a 24 h intensive sampling at the same location has  
73 supported the existence of diel inputs of labile DOM fueling the HP community at the depths  
74 occupied by mesopelagic fishes during daytime (García et al. 2018). Both DOC concentrations  
75 and the contribution of high nucleic acid content (HNA) bacteria, usually made up of  
76 copiotrophic taxa (Schattenhofer et al. 2011, Vila-Costa et al. 2012) more active than the low  
77 nucleic acid content (LNA) group (e.g. (Bouvier et al. 2007, Gasol et al. 1999, Morán et al. 2011),  
78 fluctuated as widely in waters below 200 m as in the upper layers. However, for the hypothesis  
79 of the mesopelagic labile DOM hotspots to be true, we should be able to demonstrate that the  
80 presence or absence of fishes in the twilight zone does make a difference.

81 Here, we report on the results of two short-term incubations with water collected from  
82 the epi- and mesopelagic layers (surface and 550 m, respectively) of the central Red Sea at  
83 midnight and at the following midday. After removing protistan grazers and other larger  
84 organisms by filtration, we followed the dynamics of DOM-heterotrophic prokaryotes  
85 interactions for 8 days. In parallel, we conducted a high frequency (every 2 h for a full 24 h  
86 starting at noon) characterization of the same depths focusing on the response of  
87 heterotrophic prokaryotes abundance, cell size and biomass to changes in DOM concentrations  
88 including its fluorescent properties, previously unreported for this basin. The specific objectives  
89 of this study were: i) to assess the diurnal scales of variability in the standing stocks of HP and  
90 DOM in epipelagic and mesopelagic waters of the central Red Sea, and ii) to test for differences  
91 in the specific growth rate, maximum biomass and growth efficiency of HP between nighttime  
92 and daytime in both layers. Our hypothesis is that DOM supplied by DVM fishes in the  
93 mesopelagic zone during the day had a commensurable effect on the above-mentioned  
94 variables.

95

## 96 MATERIALS AND METHODS

97

98           **Environmental sampling**

99           We occupied one station located 13.4 km offshore to the north of King Abdullah  
100 Economic City, Saudi Arabia (lat 22.46°N, lon 39.02°E) between noons of March 6<sup>th</sup> and 7<sup>th</sup> 2016  
101 (Calleja et al. 2018, García et al. 2018). Sampling was conducted on board of RV Thuwal.  
102 Continuous acoustic measurements in order to locate the position of the vertically migrating  
103 mesopelagic fishes were recorded with a Simrad EK60 38 kHz echosounder mounted on the  
104 ship's hull. From noon on March 6<sup>th</sup> until the same time on the following day we conducted CTD  
105 casts every 2 hours. At each cast we sampled discrete depths in the water column with Niskin  
106 bottles mounted on a Rosette sampler, ranging from the surface to 650 m depth. Water filtered  
107 through pre-combusted Whatman GF/F filters was collected for analyzing DOC bulk  
108 concentrations and fluorescent DOM (FDOM) properties (40 mL pre-combusted glass vials).  
109 Unfiltered water was collected for characterizing the community of heterotrophic prokaryotes  
110 (2 mL cryovials).

111           Hourly apparent DOC production and consumption rates were estimated as the largest  
112 difference between DOC concentration in consecutive sampling times consistently increasing  
113 and decreasing, respectively. The same approach was used for estimating the apparent biomass  
114 production of heterotrophic prokaryotes over the diel cycle.

115

116           **Experimental incubations**

117           10 L of seawater from the surface and 550 m depth were collected in the midnight and  
118 noon casts on March 7<sup>th</sup> for conducting the experimental incubations of DOC consumption,  
119 change in FDOM and heterotrophic prokaryotes biomass response. In order of remove  
120 protistan grazers and planktonic organisms larger than bacteria and archaea, water was gently  
121 filtered through pre-combusted Whatman GF/C filters (142 mm, nominal pore size 1.2 µm) and  
122 used to fill 3 x 2 L acid-cleaned polycarbonate bottles, which were subsequently incubated at in  
123 situ temperature and light regime (darkness for 550 m samples). Removal of prokaryotic cells  
124 by filtration was minor (83% ± 7% SE of the initial abundance was retrieved in the water used  
125 for the incubations) and mean cell size was virtually unaffected (2.6% ± 1.0% smaller biovolume  
126 than in the unfiltered water). Filtration virtually eliminated all protistan grazers of heterotrophic

127 prokaryotes, since the mean abundance of heterotrophic nanoflagellates in the GF/C filtrate  
128 was 1.5% (E. I. Sabbagh, pers. comm.) Subsamples were taken twice per day on the first 2 days,  
129 then daily until day 6 and finally at day 8. DOC and FDOM subsamples from the incubations  
130 were filtered through 0.2 Millipore polycarbonate filters. We will occasionally use the codes S  
131 and F to refer to the incubations made with water from the Surface (0 m) and the Fish layer  
132 (550 m), respectively, followed by D or N to refer to the period of sampling (Day or Night): SD,  
133 SN, FD, FN.

134

### 135 **DOC analysis**

136 Samples for DOC were acidified with  $H_3PO_4$  and kept in the dark at 4 °C until analysis by  
137 high temperature catalytic oxidation at the laboratory. All glass material used was acid cleaned  
138 and burned (450°C, 4.5 h). Consensus reference material of deep sea carbon (42–45  $\mu\text{mol C L}^{-1}$   
139 and 31-33  $\mu\text{mol N L}^{-1}$ ) and low carbon water (1-2  $\mu\text{mol C L}^{-1}$ ), provided by D. A. Hansell and W.  
140 Chen (Univ. of Miami) was used to monitor the accuracy of our DOC concentration  
141 measurements. The analytical error of DOC concentration was 1.4  $\mu\text{mol L}^{-1}$ .

142

### 143 **DOM fluorescence measurements and PARAFAC modeling**

144 UV-VIS fluorescence spectroscopy was measured using a HORIBA Jobin Yvon AquaLog  
145 spectrofluorometer with a 1 cm path length quartz cuvette. Three dimensional fluorescence  
146 excitation emission matrices (EEMs) were recorded by scanning with an excitation wavelength  
147 range of 240- 600 nm and emission of 250-600 nm, both at 3 nm increments and integrating at  
148 8 seconds. To correct and calibrate the fluorescence spectra post-processing steps were  
149 followed according to Murphy et al. (Murphy et al. 2010). Briefly, fluorescence spectra were  
150 Raman area (RA) normalized by subtracting daily blanks that were performed using Ultra-Pure  
151 Milli-Q sealed water (Certified Reference, Starna Cells). Inner-filter correction (IFC) was also  
152 applied according to McKnight et al. (2001) RA normalization, blank subtraction, IFC and  
153 generation of EEMs were performed using MATLAB (version R2015b).

154 A total of 165 samples for DOM fluorescence were collected (81 from 7 vertical profiles  
155 and 84 from the experimental incubations). The EEMs obtained were subjected to PARAFAC

156 modeling using DOMFluor Toolbox (Stedmon and Bro 2008). Before the analysis, Rayleigh  
157 scatter bands were trimmed. A four-component model was validated using split-half validation  
158 and random initialization (Stedmon and Bro 2008): peak C1 at Ex/Em 240(325)/ 407 nm, peak  
159 C2 at Ex/Em 258(390)/492 nm, peak C3 at Ex/Em 240/337 and peak C4 at Ex/Em 276/312 nm.  
160 C1 corresponds to peak M (Coble 2007) and is comparable to component 2 identified by Català  
161 et al. (Catalá et al. 2015). C2 represents a combination of peaks A and C (Coble 2007) and is  
162 comparable to component 1 in (Catalá et al. 2015). C3 corresponds to peak T (Coble 2007),  
163 attributed to tryptophane, and is comparable to component 3 in (Catalá et al. 2015). C4  
164 corresponds to peak B (Coble 2007), attributed to tyrosine, and is comparable to component 4  
165 in (Catalá et al. 2015). The maximum fluorescence (F<sub>max</sub>) is reported in Raman units (RU).

166

#### 167 **Heterotrophic prokaryotes abundance and biomass**

168 Triplicate samples (1.8 mL) for estimating the abundance of heterotrophic bacteria and  
169 archaea in situ and in the experimental incubations were fixed with 1% paraformaldehyde and  
170 0.05% glutaraldehyde, deep frozen in liquid nitrogen and stored at -80°C until analysis. Once  
171 thawed, 400 µL aliquots were stained with SYBR-Green run in a BD FACSCanto II flow cytometer  
172 for estimating the abundance of low (LNA) and high (HNA) nucleic acid content cells as detailed  
173 in Gasol and Morán (Gasol and Morán 2015). The Abundances were estimated based on time  
174 and the actual flow rates, which were calibrated daily using the gravimetric method. The right  
175 angle light scatter or side scatter (SSC) signal relative to the value of 1 µm fluorescent latex  
176 beads added to each sample was used to estimate the cell diameter according to Calvo-Díaz  
177 and Morán (Calvo-Díaz and Morán 2006). LNA and HNA cell numbers were summed to estimate  
178 the total abundance and their specific cell sizes averaged to obtain the mean cell size of the  
179 heterotrophic prokaryote community at both depths and different times. Assuming spherical  
180 shape, the mean cell size (biovolume in µm<sup>3</sup>) was converted into cellular carbon content  
181 following Gundersen et al. (Gundersen et al. 2001). Heterotrophic prokaryotes biomass was  
182 then calculated as the product of cell abundance and mean cellular carbon content.

183

#### 184 **Growth rate estimates**

185 In situ apparent or net growth rates of the heterotrophic prokaryote assemblage at the  
186 surface and the mesopelagic fish layer were estimated from changes in biomass ( $\mu\text{g C L}^{-1}$ )  
187 resulting from changes in abundance and mean cell size over 24 h. Net growth rates ( $\mu$ , in units  
188  $\text{h}^{-1}$ ) were calculated as:

189

$$190 \quad \mu = \ln (N_1/N_0) / \Delta t \quad (1)$$

191

192 where  $N_1$  is the final biomass,  $N_0$  is the initial biomass and  $\Delta t$  is the time interval (2 h).  
193 We modeled the overall daily growth rate from Eq. (1) using the size distribution of the  
194 organisms with the R package *ssPopModel*, which included a modified version of the size-  
195 structured matrix population model originally developed by Sosik et al. (Sosik et al. 2003).  
196 Matrix population model assumption is that changes in size distribution are only related to  
197 growth and division of the cells. We adapted and simplified the application of this function as  
198 described by Hunter-Cevera et al. (Hunter-Cevera et al. 2014) for *Synechococcus* cyanobacteria  
199 to be used with heterotrophic prokaryote cells.

200 Specific growth rates in the incubations were calculated as the slope of the ln-  
201 transformed total abundance vs. time for the linear response period, equivalent to the phase of  
202 exponential growth (usually lasting between 2 and 3 days).

203

#### 204 **Prokaryotic growth efficiency**

205 Prokaryote heterotrophic production (PHP) in the midnight and midday incubations was  
206 estimated as the rate of increase in bacterial biomass during the exponential phase of growth.  
207 Prokaryotic carbon demand (PCD, i.e. the sum of heterotrophic prokaryotes production and  
208 respiration) was approached by the consumption rate of DOC during the same period.  
209 Prokaryotic growth efficiency (PGE) was therefore calculated as the ratio of PHP to PCD.

210

#### 211 **Statistical analyses**

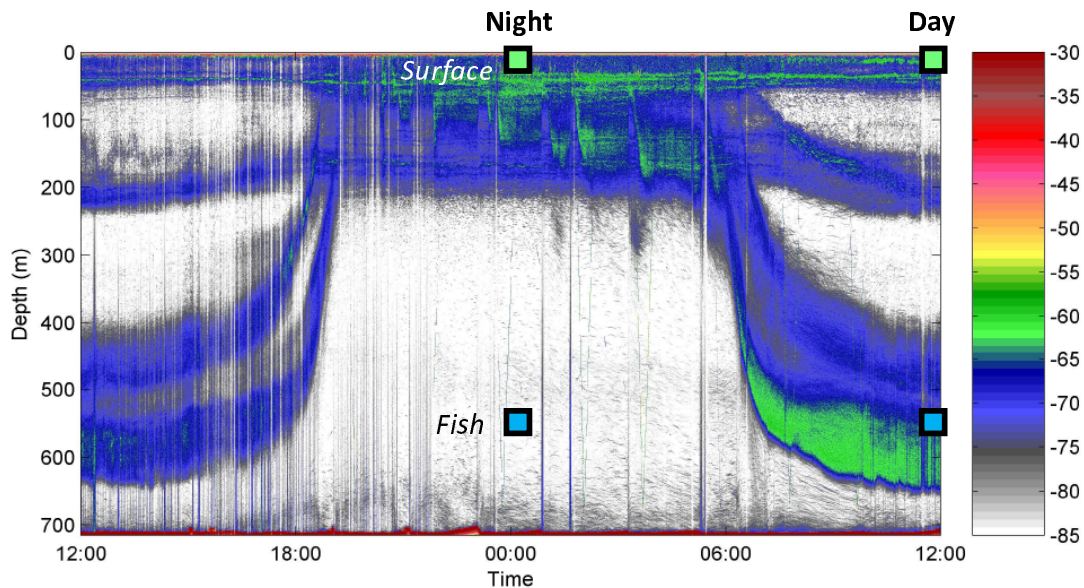
212 Model I or ordinary least squares (OLS) linear regressions for estimating specific growth  
213 rates were done separately for each replicate, using a common period for each experiment.



214 Differences between treatments and/or depths were assessed with one way ANOVAs and  
215 Fisher least significance (LSD) post-hoc tests. General relationships between variables were  
216 represented by Pearson's correlation coefficients. Statistical analyses were done with JMP and  
217 STATISTICA software packages.

218

## 219 RESULTS



220

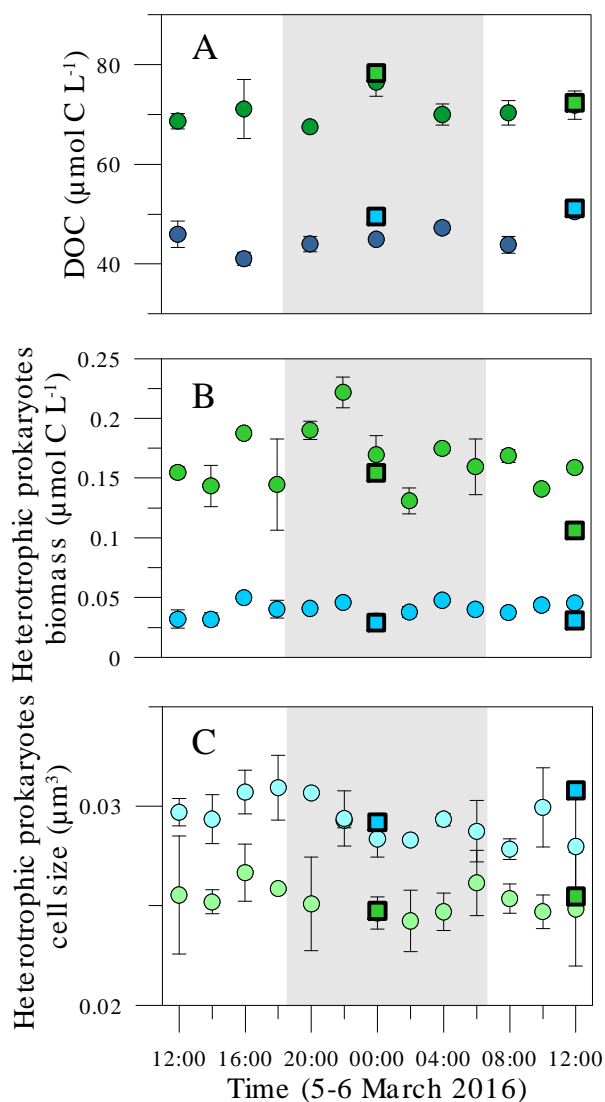
221 **Fig. 1.** Echogram from March 5<sup>th</sup> to 6<sup>th</sup> 2016 at the study site showing 2 scattering layers of mesopelagic fish  
222 performing diel vertical migration: up to the surface at night and down to deep waters during daytime. Symbols  
223 indicate the depth and time of water collection for the incubation experiments.

224

### 225 Environmental variability of DOC and heterotrophic prokaryotes

226 The complete diel vertical migration of the mesopelagic fishes present at the study site  
227 can be clearly seen in the echogram of **Fig. 1**, with the deeper, more intense layer (dominated  
228 by *Benthosema pterotum*) occupying the depths between ca. 520 and 630 m during daytime on  
229 March 6<sup>th</sup> 2016. **Fig. 2** shows the diel variability of DOC concentrations and the biomass of HP at  
230 the station's surface and 550 m depth. Mean DOC values were almost 50% higher at 0 than at  
231 550 m ( $71.0 \pm 1.6$  SE and  $45.6 \pm 1.5 \mu\text{mol C L}^{-1}$ , respectively). Both depths showed similar  
232 dynamics, with two relative maxima of DOC at around midnight and noon (**Fig. 2A**). The

233 midnight peak was higher and more conspicuous at the surface than at the fish layer. However,  
234 both depths displayed a similar diel variability with slightly higher CV in the fish layer (8.1% vs.  
235 5.7%). From these values we were able to estimate apparent production and consumption  
236 rates. The hourly rates of DOC production (from 8 am to 12 pm) and consumption (from 12 am  
237 to 8 am at the surface and from 12 pm to 4 pm at 550 m) were similar within each layer: ca. 1.3  
238  $\mu\text{mol C L}^{-1} \text{h}^{-1}$  at the surface and 2.0  $\mu\text{mol C L}^{-1} \text{h}^{-1}$  at the fish layer. The protein (Tyrosine)-like  
239 fluorescent DOM component C4 was on average one order of magnitude higher at the surface  
240 than at 550 m, although it showed more variability at depth (**Table S1**).

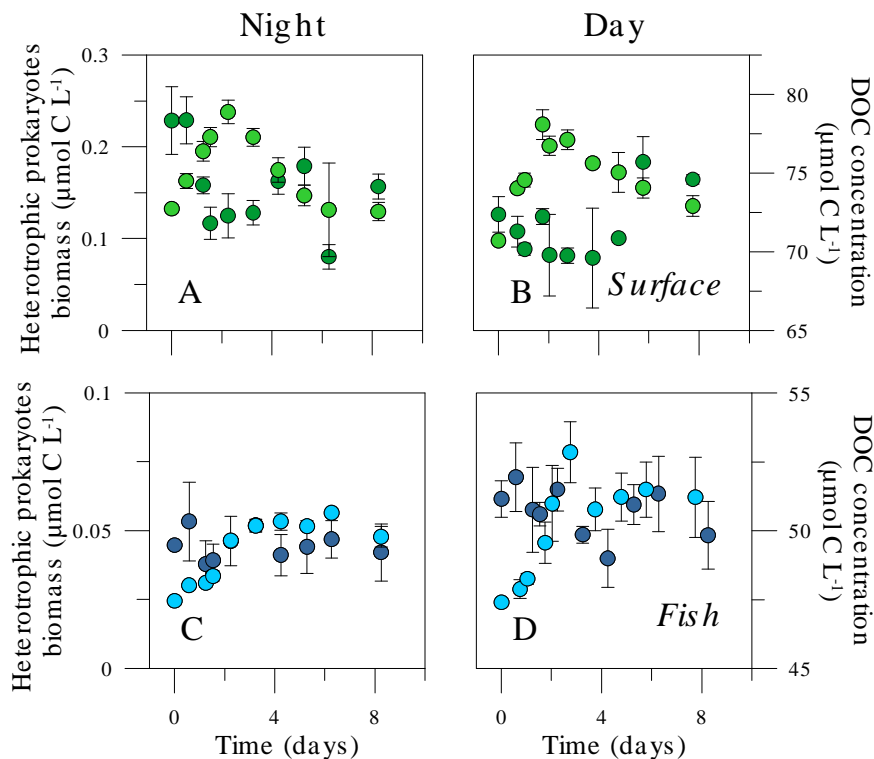


241

242 **Fig. 2.** Variability of mean DOC concentration (A) and heterotrophic prokaryoplankton biomass (B) and cell size (C)  
243 in two layers of the study site: upper (0-25 m) and mesopelagic occupied by fish during daytime (450-600 m)  
244 during the 24 h sampling. Squares indicate initial values at the onset of the experimental incubations. The gray

245 area represents nighttime hours at the date of sampling. Error bars represent the standard error of the mean  
246 (average of 0 and 25 m in the upper layer and 450, 550 and 600 m in the mesopelagic one).  
247

248 The abundance of HP at the surface (mean  $4.31 \pm 0.17 \times 10^5$  cells  $\text{mL}^{-1}$ ) was also one  
249 order of magnitude higher than at 550 m (mean  $9.43 \pm 0.39 \times 10^4$  cells  $\text{mL}^{-1}$ ), but varied similarly  
250 with no clear diel patterns. Although their size was  $22\% \pm 8\%$  larger in the mesopelagic (mean  
251 values of  $0.034$  and  $0.028 \mu\text{m}^3$  at the fish and surface layers, respectively, **Fig. 2C**), the  
252 corresponding biomass was driven mostly by changes in abundance, averaging  $1.91 \pm 0.09 \mu\text{g C}$   
253  $\text{L}^{-1}$  at the surface and  $0.50 \pm 0.02 \mu\text{g C L}^{-1}$  at 550 m. **Fig. 2B** (in  $\mu\text{mol C L}^{-1}$  for comparison with  
254 **Fig. 2C**) shows that HP biomass was equally variable at both layers (CV 16.0 vs. 16.3%). In situ  
255 apparent or net growth rates based on changes in HP cell size were of  $0.15$  and  $0.10 \text{ d}^{-1}$  at 0 and  
256 550 m, respectively. HP cell specific growth rates changed cyclically over the 24 h cycle in both  
257 depths (**Fig. S2**). Two maxima, at 20:00 and 8:00, were found at the surface layer while the  
258 maximum at 550 m was observed at 16:00



259

260 **Fig. 3.** Dynamics of heterotrophic prokaryoplankton biomass and DOC concentration in the predator-free  
261 experimental incubations of samples taken at noon (**A, C**) and at midnight (**B, D**) from the surface and 550 m  
262 depth. Error bars are standard errors of 3 replicates.  
263

264 **Table 1.** Mean  $\pm$  SE values of specific growth rates ( $\mu$ ), DOC consumption rates or prokaryotic carbon demand  
 265 (PCD, see the text), prokaryotic heterotrophic production rates (PHP) and prokaryotic growth efficiency (PGE) in  
 266 the surface and fish layer incubation experiments performed at noon (Day) and midnight (Night). Rates were  
 267 calculated for each period of exponential growth, also indicated in days. The same period was used for DOC  
 268 consumption and biomass production rates. Also indicated are the maximum heterotrophic prokaryotes biomass  
 269 reached within the incubation and the corresponding ratio of maximum to initial biomass (Max:t0 biomass ratio).  
 270

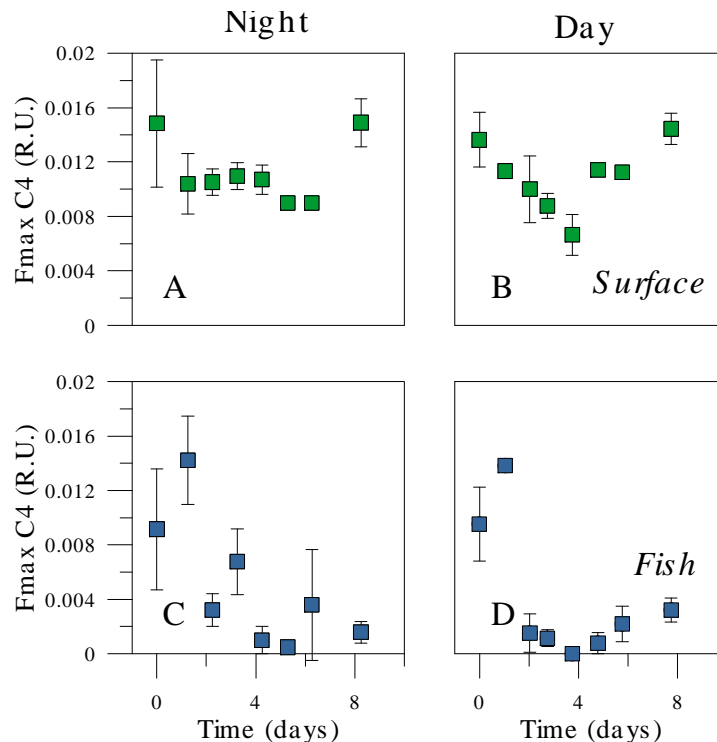
Layer	Time	Period (d)	$\mu$ ( $d^{-1}$ )	DOC consumption rate ( $\mu\text{mol C L}^{-1} d^{-1}$ )	PHP rate ( $\mu\text{mol C L}^{-1} d^{-1}$ )	PGE (%)	Maximum HP biomass ( $\mu\text{g C L}^{-1}$ )	Max:t0 HP biomass ratio
Surface	Day	0-1.75	$0.34 \pm 0.07$	$1.0 \pm 0.5$	$0.040 \pm 0.004$	4.2	$2.69 \pm 0.20$	2.30
	Night	0-2.25	$0.18 \pm 0.02$	$2.7 \pm 1.1$	$0.047 \pm 0.006$	1.8	$2.85 \pm 0.16$	1.80
Fish	Day	0-2.75	$0.16 \pm 0.04$	$0.5 \pm 0.3$	$0.020 \pm 0.004$	4.2	$0.94 \pm 0.13$	3.27
	Night	0-2.25	$0.09 \pm 0.01$	$0.3 \pm 0.8$	$0.010 \pm 0.001$	3.1	$0.68 \pm 0.02$	2.31

271  
 272  
 273  
 274

### Experimental incubations of surface and deep samples

275 With initial concentrations similar to ambient values (**Fig. 2**), DOC was consumed in the  
 276 first 2-3 days in the predator-free experiments (**Fig. 3**), albeit at different daily rates (**Table 1**),  
 277 followed by net production after day 4 especially in the Surface incubations. Minimum and  
 278 maximum consumption rates were  $0.32$  and  $2.69 \mu\text{mol C L}^{-1} d^{-1}$ , found in the Fish and Surface  
 279 Night experiments, respectively (FN and SN). Values in the other two experiments carried out  
 280 with noon samples were below  $1 \mu\text{mol C L}^{-1} d^{-1}$  ( $0.47$  and  $0.95 \mu\text{mol C L}^{-1} d^{-1}$ , respectively in in  
 281 FS and SD). The initial fluorescence intensity values of the component C4 in the experiments  
 282 was higher but reflected the values measured concurrently in the water column (paired *t*-test,  
 283  $p > 0.05$ ,  $n = 4$ ). C4 showed a very consistent consumption pattern regardless of the layer and  
 284 treatment. Heterotrophic prokaryotes in the Surface incubations consumed in 4 days 40 and  
 285 50% of the initial values during Night and Day respectively, while bacteria inhabiting deep  
 286 waters consumed almost all of it (95-100 %) within the same time frame regardless of the

287 sampling time (**Fig. 4**). Hereinafter we consider changes in C4 as representative of labile DOM  
 288 dynamics. Day and night C4 consumption patterns did not show any significant differences  
 289 within the same layer but they displayed significantly higher consumption rates in the Fish layer  
 290 (ANOVA,  $p=0.004$ , post-hoc Fisher LSD test, **Figure 4** and **Table S1**).



291  
 292 **Fig. 4.** Dynamics of the concentration of the FDOM protein-like C4 component in the predator-free experimental  
 293 incubations of samples taken at noon (**A, C**) and at midnight (**B, D**) from the surface and 550 m depth. Error bars  
 294 are standard errors of 3 replicates.

295  
 296 Heterotrophic prokaryotes responses in the incubation experiments differed between  
 297 depths and sampling times (**Fig. 3**). Consistent differences were found between the specific  
 298 growth rates at both depths (**Table 1**), with  $\mu$  being double in the Surface than in the Fish  
 299 experiments. Within each layer, the Day  $\mu$  values were also higher than the Night ones (t-tests,  
 300  $p=0.020$  and  $p=0.060$  at the Surface and Fish experiment, respectively,  $n=6$ ). HNA cells always  
 301 grew faster than their LNA counterparts resulting in increases in their relative contribution from  
 302 43-55% to 55-62%, more noticeable in the FD experiment. The mean size of the cells also  
 303 increased substantially in the Fish experiments, from  $0.027$  to  $0.060 \mu\text{m}^3$  in the FD incubation  
 304 and from  $0.028$  to  $0.047 \mu\text{m}^3$  in the FN one, while changes in cell size were much smaller in the

305 Surface experiments, and virtually the same in both periods, from 0.026 to 0.037  $\mu\text{m}^3$  (SD) and  
306 from 0.025 to 0.035  $\mu\text{m}^3$  (SN) (**Fig. S2**). Consequently, cell size played an important role in the  
307 increase in biomass, especially in both Fish experiments (**Fig. 3C, D**). The biomass production  
308 rates of heterotrophic prokaryotes for the same periods of DOC consumption ranged 4-fold,  
309 from 0.010 to 0.047  $\mu\text{mol C L}^{-1} \text{d}^{-1}$ , mirroring the changes in the latter variable (**Table 1**). The  
310 rates of heterotrophic prokaryotes biomass production and DOC consumption were used for  
311 estimating prokaryotic growth efficiencies (PGE) in the four experimental incubations. PGE was  
312 uniformly below 5%, ranging from 1.8% (SN) to 4.2% (both SD and FD, **Table 1**). Following the  
313 pattern of in situ values, maximum HP biomass measured in the incubations was higher in the  
314 Surface than in the Fish experiments (**Fig. 3, Table 1**), although the increase ratios (i.e. the ratio  
315 of maximum to initial biomass, **Table 1**) were significantly higher in the Fish experiments with  
316 all data pooled (t-test,  $p=0.048$ ,  $n=12$ ).

317

## 318 DISCUSSION

319 There is consensus that marine biota biomass and activity peak in the upper layers and  
320 decrease exponentially with depth, following the strong vertical gradients in physico-chemical  
321 properties (Aristegui et al. 2009). Heterotrophic prokaryotes inhabiting the Red Sea seem to  
322 challenge this view. Together with diel variations of standing stocks (García et al. 2018), this  
323 study), night- and day-initiated incubations of predator-free ambient assemblages with the  
324 DOM pool available at the time of sampling yielded surprising similarities in epipelagic and  
325 mesopelagic waters. A method for estimating division rates in cyanobacteria based in changes  
326 in cell size (Hunter-Cevera et al. 2014, Sosik et al. 2003) was adapted to obtain independent  
327 estimates of in situ net growth rates (**Fig. S2**). The diel variability in heterotrophic prokaryotes  
328 cell size showed the same pattern at the surface and the mesopelagic fish layer (**Fig. 2**), yielding  
329 low but comparable net growth estimates at the surface than at 550 m depth (0.10-0.15  $\text{d}^{-1}$ ).  
330 However, it must be noted here that the already large prokaryotes inhabiting the mesopelagic  
331 fish layer were able to grow much bigger in the absence of protistan grazers (**Fig. S1**),  
332 confirming the results of a previous study conducted at the same site (Calleja et al. 2018).

333           Diel cycles in biogeochemical properties and plankton biomass and activity in the upper  
334 ocean layers are reasonably well known, following diurnal changes in photosynthesis and food  
335 web processes (Gasol et al. 1998, Ruiz-González et al. 2012). No clear patterns were observed  
336 for in situ heterotrophic prokaryotes abundance or biomass, probably due to strong coupling  
337 between growth and mortality due to protistan grazing (Calbet et al. 2015, Silva et al.) and/or  
338 viral lysis in the Red Sea (E. Sabbagh et al., in prep.), and in tropical waters in general (Morán et  
339 al. 2017). However, likewise early observations in the Caribbean (Johnson et al. 1981) recently  
340 confirmed for this site (García et al. 2018), DOM concentrations displayed a coherent diel  
341 pattern suggesting different timing of production and consumption (**Fig. 1**). Confirming previous  
342 experiments (Calleja et al. 2018), DOC consistently decreased in the first 2-3 days in both  
343 Surface and Fish samples, ranging from 0.7 to 6.1  $\mu\text{mol L}^{-1}$ , followed by net production after  
344 day 4 especially in the Surface incubations, coincident with a sharp decay in prokaryotic  
345 biomass (**Fig. 3A, B**). Indeed, the strong apparent uptake of surface DOC in situ during nighttime  
346 coincided with the highest DOC consumption experimental result, pointing to its labile nature,  
347 although the buildup of HP biomass was similar in both SD and SN experiments, resulting in  
348 lower specific growth rates and PGE values in the Night (**Table 1**). Labile DOC incorporation  
349 does not automatically inform us of its subsequent partitioning between metabolism  
350 (respiration) and growth (biomass production), as shown by Condon et al. (Condon et al. 2011)  
351 for DOM originated by jellyfish blooms. Since the alternation between light and dark periods  
352 within the incubator used for Surface seawater was common for SD and SN experiments, if  
353 photoheterotrophy (Béjà et al. 2000, Ruiz-González et al. 2013) affected DOM dynamics, it  
354 should have been equally apparent in both Night and Day incubations, which were initiated  
355 with only 12 h difference (**Fig. 1**). We therefore ruled out photoheterotrophic processes  
356 explaining the differences observed after 8 days of incubation. Rather, the quality of labile DOM  
357 at midday (including recent photosynthate) was probably higher than at midnight (perhaps with  
358 a greater contribution of DOM coming from sloppy feeding, (Nagata 2000), causing the  
359 significantly higher  $\mu$  values (**Table 1**). That the quality of labile DOC at noon could have been  
360 higher was supported by a faster increase in bacterial cell size (**Fig. S1B**) and the contribution of  
361 HNA cells, which increased by 35% in SD compared with 15% in SN. The decrease in the protein-

362 like C4 component was also more sustained in the SD experiment compared to SN after 4 days  
363 (13.2% vs. 5.3% was consumed daily during that period, **Fig. 4**) although the rates were virtually  
364 the same in the initial periods (2.75 and 2.25 days, respectively, **Table S1**). Changes in  
365 mesopelagic DOC concentrations and lability at diel ((García et al. 2018), this study) and  
366 seasonal scales (Calleja et al. 2019) in the central Red Sea support the recent claim that the  
367 diverse pool of DOM in the deep ocean fluctuates at timescales much shorter than previously  
368 thought (Follett et al. 2014). Since the conditions at the study site were hypoxic in most of the  
369 mesopelagic realm (Calleja et al. 2019), the low in situ oxygen concentrations in the Fish water  
370 samples, consistent from 300 m downwards ( $0.69 \pm 0.03 \text{ mg L}^{-1}$ ) might have been  
371 supplemented by pre-filtration and sampling from the experimental bottles. Although we did  
372 not control for this potential artefact, the same protocol was followed for the FN and FD  
373 experiments. Therefore, the consistently higher values of DOM consumption, prokaryotic cell  
374 size, growth rates and efficiency in the Day compared with the Night incubation strongly  
375 support that the presence of fish indeed had a major impact on the microbial community.

376         Surface specific growth rate in the Day experiment was nevertheless notably higher  
377 than the  $0.08 \text{ d}^{-1}$  measured in a previous study carried out in November 2015 at the same  
378 location (Calleja et al. 2018). The discrepancy cannot be explained by total DOC or chlorophyll *a*  
379 concentrations, but could instead be related to the availability of labile DOM compounds since  
380 C4 concentrations were 61% higher in March than in November (M. L. Calleja, pers. comm.). At  
381 a shallower, nearby site characterized by higher total and labile DOC concentrations, specific  
382 growth rates were still considerably higher, ranging from  $0.79$  to  $1.75 \text{ d}^{-1}$  (Silva et al.).

383         The daily rates of apparent DOC production and consumption based on changes in in  
384 situ concentration were virtually the same within each of the two layers compared, indicating  
385 no net accumulation. This is the expected result in oligotrophic regions at the short time scale  
386 of one day (Johnson et al. 1981, Wright 1984). However, these rates were still ca. 50% higher in  
387 the mesopelagic zone resulting in apparent turnover of labile DOC of  $23.6\% \text{ d}^{-1}$  in the  
388 mesopelagic compared with  $14.7\% \text{ d}^{-1}$  at the surface, if we consider the measured diel  
389 variability and maximum concentrations in each layer. This finding conflicts with the contention  
390 that DOM is largely of refractory nature within the mesopelagic waters of the global ocean (Jiao



391 et al. 2010). The role of vertically migrating animals, zooplankton and fishes, as vectors of  
392 organic matter to deep layers complementary to the biological pump (Herndl and Reinthaler  
393 2013) has been recently recognized (Bianchi et al. 2013, Bianchi et al. 2013, Isla et al. 2015)). In  
394 this regard, work at the study site has suggested DVM fishes as a transport mechanism  
395 supplying labile DOM that does not accumulate but fuels heterotrophic bacterial activity in  
396 mesopelagic waters (Calleja et al. 2018). Here we tested this hypothesis by further examining  
397 the fluorescence properties of DOM and its transformation by heterotrophic prokaryotes in  
398 experimental incubations with and without the fishes present. FDOM are useful tracers for  
399 biogeochemical processes in the dark ocean (Catalá et al. 2015, Nelson and Siegel 2013).  
400 Fluorescence intensity of the two aminoacid-like fluorophores C3 and C4 decreased with depth  
401 (data not shown), indicating that these fluorophores were mainly produced autochthonously in  
402 surface waters. Both phytoplankton and bacteria are sources of tryptophan and tyrosine  
403 (Determann et al. 1998), while Urban-Rich et al. (Urban-Rich et al. 2006) have reported that  
404 grazing and excretion by zooplankton can also release material with amino acid-like  
405 fluorescence signals. Our results strongly suggest that DVM fishes can also provide C4 in the  
406 mesopelagic realm.

407         Contrary to the epipelagic zone, very few studies on the diel variability of DOM-  
408 heterotrophic prokaryotes interactions are available for deep waters (Carlucci et al. 1986,  
409 García et al. 2018). Gasol et al. (Gasol et al. 2009) suggested that mesopelagic prokaryotes in  
410 the subtropical NE Atlantic were as active as in the epipelagic. We demonstrate here not only  
411 that heterotrophic prokaryotes specific growth rates at 550 m were of the same order of  
412 magnitude than in surface waters, clearly challenging the most accepted view (Arístegui et al.  
413 2009, Baltar et al. 2010), but that those rates were almost double at noon conditions, when the  
414 mesopelagic fishes were present, than at midnight, when the entire population was closer to  
415 the surface (Klevjer et al. 2012). From **Fig. 1** it is clear that the fishes were absent at midnight in  
416 the entire mesopelagic zone but their presence at 550 m had been established for ca. 4 h when  
417 the noon sampling took place. Specific growth rates were nevertheless lower than in the  
418 previous study ( $0.24 \text{ d}^{-1}$ , (Calleja et al. 2018)). Although seasonality of C4 in the deep scattering  
419 layer was less marked than at the surface, November 2015 was characterized by 82% higher C4

420 concentrations than in March 2016 (M. Calleja, pers. comm.). Altogether, these results point  
421 out to a major role of protein-like substances in determining the specific growth rates of  
422 heterotrophic prokaryotes throughout the water column, as recently found for nearby shallow  
423 waters in a seasonal study (Silva et al.). C4 fluctuated widely in the 24 h monitoring at 550 m  
424 depth (**Table S1**) and was also actively consumed in all our incubations, thus revealing a clearly  
425 labile nature. C4 was consumed faster in the Fish experiments, at 42.1% and 25.8% d<sup>-1</sup> in FD and  
426 FN, respectively (**Fig. 4, Table S1**), than in the Surface ones (ca. 13% d<sup>-1</sup> in both SD and SN). A  
427 similar relative consumption of protein-like FDOM (12% d<sup>-1</sup>), mostly occurring during the first 5  
428 days, was measured by (Yamashita and Tanoue 2004) in experiments conducted with marine  
429 surface waters. Our explanation is that fishes released DOM directly or it leaked from particles  
430 associated to the fish presence (e.g. fecal pellets). That DOM could have been delivered by  
431 sinking particles (Smith et al. 1992) not related to vertical migration would not explain the  
432 difference between the FD and FN experiments.

433 Cell size has been used as an indicator of the activity of heterotrophic prokaryotes  
434 (Gasol et al. 1995). While in the Surface experiment growing HP cells were only slightly larger  
435 than at time 0 (11% larger size for both the SD and SN experiments), the cell size increase in the  
436 Fish experiment was dramatic, especially in the Day incubations (118% vs. 68% larger, **Fig. S2**).  
437 The contribution of bigger cells to the observed increase in HP biomass is not at all minor: had  
438 we used, as in many studies, a fixed cellular carbon content of 4 fg C cell<sup>-1</sup> (corresponding to the  
439 initial mean cell size of 0.027 μm<sup>3</sup> for the two depths and periods, **Fig. 2C**), maximum HP  
440 biomass in **Table 1** would have become 2.10 (SD), 2.32, (SN), 0.43 (FD) and 0.39 (FN) μg C L<sup>-1</sup>,  
441 i.e. between 42 and 54% lower than the actual values for the mesopelagic prokaryotes. We  
442 conclude that the presence of fishes in the mesopelagic zone resulted in significantly higher  
443 growth rates of markedly larger cells, exacerbating the changes in biomass relative to those in  
444 abundance. The maximum biomass of heterotrophic prokaryotes that could be sustained by  
445 extant DOM concentrations was significantly higher than the initial value in both Fish  
446 experiments (**Table 1**). Altogether, these results point out to substantial inputs of labile DOM  
447 during daytime at the mesopelagic fish layer that are rapidly mobilized by large bacterial taxa.  
448 The archaea *Nitrosopulimus maritimus*, which makes up much of the heterotrophic

449 prokaryoplankton biomass at these depths (Ngugi et al. 2012), were apparently not able to  
450 respond to these DOM hotspots, since their contribution to total numbers at the end of a  
451 similar incubation dropped from 50% to 3% (Calleja et al. 2018). The typical size of  
452 Chrenoarcheota is small (Konneke et al. 2005), so it is unlikely that they were the dominant  
453 groups growing in our Fish incubations after 2 days (**Fig. S2C, D**). Besides excreting ammonium  
454 that boosts anammox by Chrenarcheota (Bianchi et al. 2014), mesopelagic fishes thus seem  
455 capable to fuel the metabolism of large, copiotrophic bacteria.

456 Prokaryotic growth efficiencies are typically low in open ocean, oligotrophic  
457 environments (del Giorgio and Cole 1998, Reinthaler et al. 2006). The recently reported low  
458 PGE values at this Red Sea site (1.6-3.4%, (Calleja et al. 2018) are confirmed by this new study,  
459 while higher values(2.5-12.8%) were recorded in a shallow, richer bay located a few km south  
460 (Silva et al.). Few studies have estimated the vertical variability in PGE values, but those that  
461 have usually depict lower values with depth (Lemée et al. 2002, Reinthaler et al. 2006), related  
462 to the increased presence of refractory DOM compounds (Jiao et al. 2010) or to the higher  
463 dilution of the labile ones (Arrieta et al. 2015). Notably, the estimated growth efficiency of  
464 heterotrophic prokaryotes in our Day experiments was exactly the same in Surface and Fish  
465 water (4.2%), which can only be explained by the existence of labile DOC of similar quality  
466 within both layers. PGE in the mesopelagic fish layer was significantly higher than at shallower  
467 depths in the experiment conducted at noon in November 2015 (Calleja et al. 2018). However,  
468 when averaging our new two estimates, the mean PGE value at 550 m (3.6%) was still 22%  
469 higher than at the surface, strongly supporting the presence of high quality DOM hotspots  
470 (Calleja et al. 2018) in the deep scattering layer.

471 In conclusion, this study confirms that the Red Sea mesopelagic zone is not a  
472 permanently impoverished environment but subject to daily inputs of labile DOM compounds  
473 similarly to the epipelagic layers. This novel process driven by mesopelagic fishes, which  
474 complements other recently discovered sources of deep organic carbon (Boeuf et al. 2019,  
475 Dall'Olmo et al. 2016, Giering et al. 2014, Herndl and Reinthaler 2013) seems to have been  
476 overlooked due to the tight coupling between the components of microbial food webs  
477 (Pernthaler 2005). If vertically migrating fishes are able to fuel an active and distinct community

478 (T. Huete-Stauffer et al., in prep.) of heterotrophic prokaryotes in the mesopelagic layer of the  
479 Red Sea, we might expect this fast DOM flux to be widespread. The mesopelagic Red Sea has an  
480 unusually high temperature, therefore the effect of colder conditions on fish DOM-microbial  
481 interactions remain to be explored. The implications for global biogeochemical cycling would  
482 also vary depending on the actual biomass of mesopelagic fishes and the fraction performing  
483 DVM (Klevjer et al. 2016), yet its impact may increase as deep waters warm up (Luna et al.  
484 2012). That these small fishes seem able to sustain the microbial communities inhabiting the  
485 twilight zone also may help reconcile current discrepancies between carbon pools and fluxes in  
486 the global ocean.

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X.A.G.M. conceived the research, led the experiment design, data analysis and wrote the paper. F.C.G. modeled in situ growth rates. A.R. performed the acoustic research. L.S. and N. A-O. analyzed the heterotrophic prokaryotes. X.I. contributed to the interpretation of results. M.L.C. was responsible for DOC and FDOM measurements, contributed to experimental design and data analysis. F.C.G., A.R., X.I. and M.L.C. also contributed to writing.

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