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3 **Living on the edge: biofilms developing in oscillating environmental conditions**

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20 **Abstract**

21 For the first time, densities and diversity of microorganisms developed on the ocean glider  
22 were investigated using flow cytometry and Illumina MiSeq sequencing of 16S and 18S rRNA  
23 genes. Ocean gliders are autonomous buoyancy-driven underwater vehicles, equipped with  
24 sensors continuously recording physical, chemical, and biological parameters. Biofilms on  
25 the glider were exposed to periodical oscillations of salinity, oxygen, temperature, pressure,  
26 depth and light, due to periodic ascending and descending of the vehicle. Among the  
27 unpainted surfaces, the highest microbial abundance was observed on the bottom of the  
28 glider's body, while the lowest density was recorded on the glider's nose. Antifouling paints  
29 had the lowest densities of microorganisms. Multidimensional analysis showed that  
30 microbial communities formed on unpainted parts of the glider shared some similarity with  
31 non-toxic paint but they were significantly different from ones on toxic antifouling paint and  
32 seawater.

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34 Keywords: biofilm, antifouling, chitosan, next generation sequencing, ocean glider, Indian  
35 Ocean.

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## 38 **Introduction**

39 Ocean gliders are a relatively recent tool in oceanography, which allow autonomous  
40 collection of long-term oceanographic data over long distances (Figure 1A). Ocean gliders  
41 are autonomous buoyancy-driven autonomous underwater vehicles (AUV), equipped with  
42 sensors continuously recording physical, chemical, and biological parameters. Compare to  
43 other AUV, the lithium sulfur chloride battery enables ocean gliders to be operational for  
44 up to 10 months and powers different sensors that can be directly controlled by an  
45 operator. The examples of sensors typically installed on ocean gliders include temperature,  
46 salinity, oxygen and chlorophyll sensors (Eriksen 2001). During the collection of the data,  
47 ocean gliders can dive from the surface to the sea bottom through changes in their  
48 buoyancy (Figure 1B). At such times, the glider and, in particular, its sensors may be  
49 vulnerable to transient biofouling. Intensity of biofouling on ocean gliders depends on the  
50 environment (Lobe et al. 2010). Generally, more organisms accumulate on surfaces in  
51 tropical waters compare to temperal ones (Moline and Wendt 2011).

52 Marine biofouling is the undesirable growth of organisms on submerged surfaces  
53 (Wahl 1989). Any clean artificial substrata will be colonized by bacteria and later by diatoms  
54 and other microscopic unicellular eukaryotes within hours after submersion (Salta et al.  
55 2013). At this stage, a well-developed biofilm will be formed composed of multiple species  
56 of prokaryotic and eukaryotic organisms with dominance of bacteria and diatoms  
57 (Dobretsov 2010).

58 Biofouling has huge economic impacts on maritime industries. Worldwide, countries  
59 spend billions of dollars in order to manage and prevent this problem (Callow and Callow  
60 2011). Biofouling may significantly increase vehicle drag of the ocean glider, interfere with  
61 the stability of the scientific sensors and limit good data collection (Davis et al. 2003;  
62 Medeot et al. 2011). In order to prevent biofouling, maritime industries use biocides or  
63 other toxic compounds applied as antifouling paints (Yebra et al. 2004; Lobe et al. 2010).  
64 Biocidal paints kill marine organisms and cause undesirable environmental impacts, hence  
65 new low toxic and non-toxic antifouling paints are urgently needed.

66 In this regard, chitosan has been proposed as a promising non-toxic antifouling agent  
67 (Pelletier et al. 2009) due to its antimicrobial properties (Kim and Rajapakse 2005). Chitosan  
68 is a naturally occurring linear polysaccharide composed of D-glucosamine and N-acetyl-D-

69 glucosamine obtained by deacetylation of crustacean waste (Xiao 2012). Our previous  
70 laboratory and mesocosm experiments showed that chitosan led to a reduction of densities  
71 of diatoms and bacteria on experimental paints (Al-Naamani et al. 2017). Another study  
72 demonstrated that chitosan-based paints reduced growth of bacteria for 4 days and  
73 inhibited densities of photosynthetic organisms for 14 days in northern estuarine waters  
74 (Pelletier et al. 2009). Antifouling properties of chitosan have not been studied in long-term  
75 field marine experiments in tropical waters.

76 The Sea of Oman, previously known as the Gulf of Oman, is situated between the  
77 shallow, (less 50 m) high salinity waters of the Arabian Gulf and the deeper (>1000 m)  
78 Arabian Sea, and hence possesses a unique hydrological regime (Al-Hashmi et al. 2010;  
79 Banse 1997; Vic et al. 2015). One of the most intensive coastal upwelling phenomena in the  
80 world characterizes Oman coastal waters (Reynolds 1993; Al-Hashmi et al. 2010). The  
81 circulation is driven by reversing summer and winter monsoons, impacting the depth of high  
82 salinity Persian (Arabian) Gulf outflow water and exchanging at the eastern boundary with  
83 the Arabian Sea (Vic et al. 2015). The unique circulation and high production in the Sea of  
84 Oman create an oxygen minimum zone where there is almost no oxygen in the water  
85 column ( $< 2 \mu\text{mol kg}^{-1}$ , Banse and Piontkovski 2006; Piontkovski et al. 2017). The Sea of  
86 Oman provides a unique opportunity to investigate formation of microbial biofilms on ocean  
87 gliders at the gradients of salinity, temperature, oxygen and pressure.

88 In this study, we investigated the formation of microbial biofilms on coated and  
89 uncoated parts of an ocean glider during its deployment in the Sea of Oman. Biofilms  
90 developed on the glider over 3 months and were exposed to continuous variations of  
91 salinity, oxygen, light, temperature and pressure. The main objectives of this study were to  
92 investigate: 1) the composition of prokaryotic and eukaryotic communities formed on  
93 painted and unpainted parts of the glider, and 2) the antifouling effect of commercial and  
94 non-toxic experimental paints.

95

## 96 **Material and methods**

### 97 *Ocean glider's deployment*

98 A Kongsberg ocean glider was deployed 5km off the coast of Muscat, Oman, at 23°41.66'N,  
99 58°40.7'W (Figure 1C) on 4<sup>th</sup> of March 2015 and was retrieved on 3<sup>d</sup> of June 2015 (at  
100 23°43.01'N, 58°39.7'W). The intention was to collect data throughout the end of the North-

101 East monsoon and the onset of the spring inter-monsoon period. The ocean glider consisted  
102 of an aluminium pressure hull surrounded by a flooded fiberglass fairing. The body of the  
103 instrument was not coated with any specific antifouling agents. Yellow coloured fiberglass  
104 maximises durability and visibility of the glider at the sea. A total of 712 dives with 1424  
105 vertical profiles of environmental parameters covering over 2080 km and repeating the  
106 survey transects 24 times out over a period of 91 days were carried out. The ocean glider  
107 was equipped with a Seabird free-flushing CT sail, an Aanderaa 4330F oxygen optode, a  
108 Biospherical QSP-2150 PAR sensor (spectral region - 400-700 nm), a Wetlabs Triplet ECO  
109 sensor measuring chlorophyll *a* (based on fluorescence intensity) and backscatter at 470 and  
110 700 nm (Piontkovski et al. 2017). Satellite communication was used for retrieval of the data  
111 in near real time after every dive at a speed of about 25 cm s<sup>-1</sup>.

112

### 113 *Paints*

114 In total, five different coatings were tested (Table 1). These include two types of biocidal  
115 antifouling paints (International Micron Extra YBA 920 and Hempel Olympic 86950, later  
116 “paint”, PIn and PHe), one experimental non-biocidal chitosan paint (later “paint”, PCh) and  
117 two primer base (Intershield 300 and Hempel primer, later “base”, BIn and BHe). Base did  
118 not contain biocides and served as controls for antifouling paints. Chitosan paint was  
119 prepared according to Al-Naamani et al. (2007). Briefly, chitosan paints were made using  
120 1.5% chitosan (Sigma Aldrich, UK) solution in 1% acetic acid (Sigma Aldrich, UK). The solution  
121 was mixed for 10 min and then sonicated for 15 min. The fibreglass surface of the glider was  
122 not protected with any specific antifouling agents (later “unprotected”, U). Before  
123 application of paints, the surface of the ocean glider was cleaned with ethanol (96%, Sigma,  
124 USA). All paints were applied in strips (10 x 70 cm) at the top and bottom of the ocean glider  
125 using brushes. The paints were air dried at room temperature for 24h before the glider was  
126 deployed into the sea.

127

### 128 *Sampling*

129 On 3<sup>d</sup> of June 2015, the ocean glider was gently lifted to the surface of the Research Vessel  
130 Al Jamiya, Muscat, Oman. Biofilms from the painted area ~700 cm<sup>2</sup> (paint and base, Figure  
131 1D) were scraped off using sterilized microscope slides and collected into individual sterile  
132 tubes. Remaining biofilms were washed with sterilized seawater and collected into the same

133 tube. Replicated samples of undisturbed biofilms were scraped off as described above from  
134 unprotected parts of the ocean glider covering an area of ~500 cm<sup>2</sup>: top (UGT) and bottom  
135 (UGB) of the body, and the top of the wings (UWT) (Figure 1D, Table 1). Biofilms from the  
136 bottom parts of the wings were disturbed during withdraw of the ocean glider and, thus,  
137 were not sampled. The smaller areas (~100 cm<sup>2</sup>) were also sampled as described above from  
138 the glider's nose (UN), the top tail wing (UTT) and the bottom tail wing (UTB) (Figure 1D).  
139 Additionally, one-litre seawater samples (seawater) were collected on 3<sup>d</sup> of June 2015 from  
140 the area of the ocean glider retrieval with Niskin bottles (volume 5L) from the depth 15m  
141 (SW1), 25m (SW2), 35m (SW3) and 50 m (SW4). Biofilm and water samples were  
142 immediately brought on ice to the Sultan Qaboos University laboratories and processed (see  
143 below).

144

#### 145 *Sample analysis*

##### 146 *Abundance of microbes*

147 Abundances of phytoplankton eukaryotes in water were determined by direct count in the  
148 Niskin bottle samples using a Zeiss inverted microscope (Germany, 50× and 100×  
149 magnification). The taxonomic composition of the phytoplankton eukaryotic community was  
150 characterised according to Piontkovski et al. (2017). Abundance of prokaryotes in each  
151 sample was estimated using flow cytometry (FC). FC measurements were performed using  
152 BD FACSAria™ III (BD Biosciences, Franklin Lakes, NJ, USA). Before the analysis, each  
153 sample was filtered through 40 µm nylon cell strainer Falcon™ (Fischer Scientific, USA) to  
154 exclude large cells, cell clumps and detritus particles. Samples were stained with SYBR green  
155 I stain (Molecular Probes, Invitrogen, Carlsbad, CA, USA, excitation/emission wavelengths:  
156 497 nm/520 nm; dilution 1:10,000) and incubated for 10 minutes in the dark. Each sample  
157 was divided onto three independent fractions. Thus, three independent FC readings were  
158 recorded for each sample. The average number of cells ml<sup>-1</sup> for each sample was calculated.  
159 The density of microorganisms on the surface of the ocean glider was calculated taking into  
160 account the size of sampled area and the amount of liquid used to wash it. Densities of  
161 prokaryotes in the water column at different sampling depths and on different parts of the  
162 glider were compared using factorial analysis of variance (ANOVA) using Statistica 11  
163 (Statsoft, USA). Normality of the data was verified using the Shapiro-Wilk's W test. Post-hoc

164 Tukey's HSD test was used to test significance of differences between microbial  
165 abundances. In all cases, a  $p$  value  $< 0.05$  was considered statistically significant.

166

#### 167 *DNA extraction and MiSeq analyses*

168 The scraped samples from painted and unprotected surfaces of the glider were frozen and  
169 kept at  $-80^{\circ}$  C until the analysis of microbial community composition using next generation  
170 sequencing. Prior to DNA extraction, water samples were filtrated through  $0.2 \mu\text{m}$  Whatman  
171 (USA) filter. DNA from each sample from the glider and water column was extracted using a  
172 Power Biofilm (MoBio, USA) kit following the manufacturer's instructions. Purified DNAs  
173 were analysed at the Molecular Research (MRDNA) company (Shallowater, TX, USA).  
174 Illumina MiSeq was used to sequence the 16S and 18S rRNA genes. Bacterial V3-V4 regions  
175 of 16S rRNA genes were sequenced using the primers 515F (5'-GTGCCAGCMGCCGCGGTAA-  
176 3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Eukaryotic 18S rRNA genes were  
177 sequenced using the primers Euk7F (5'- AACCTGGTTGATCCTGCCAGT -3') and Euk570R (5'-  
178 GCTATTGGAGCTGGAATTAC-3').

179 Sequence data was processed using MRDNA analysis pipeline (MRDNA, Shallowater, TX,  
180 USA). In summary, sequences were joined, barcodes were deleted. Then, sequences  $<150\text{bp}$   
181 and sequences with ambiguous base calls were removed. Sequences were de-noised. OTUs  
182 generated and chimeras were removed. Operational taxonomic units (OTUs) were defined  
183 by clustering at 3% divergence and 97% similarity. Final OTUs were taxonomically classified  
184 using BLASTn against a curated database derived from RDPII and NCBI  
185 (<http://rdp.cme.msu.edu>, <http://ncbi.nlm.nih.gov>).

186 Rarefaction curves and diversity indices (OTU richness, Chao-1 and ACE) were  
187 calculated using the Mothur software (Schloss et al. 2009). Statistical analysis of sequencing  
188 data was carried out using the PAST program (Paleontological Statistics, ver. 1.47,  
189 <http://folk.uio.no/ohammer/past>) and the R v.2.15.0 statistical platform using the *Vegan*  
190 package. The table containing samples by OTUs was used to calculate pairwise similarities  
191 among samples based on Bray-Curtis dissimilarity index (Clarke 1993). A multivariate  
192 analysis of all samples was performed using multidimensional scaling (MDS) based on Bray-  
193 Curtis dissimilarities as between biofouling communities developed on paint, base,  
194 unprotected and present in seawater. Ordination of the Bray-Curtis dissimilarities was  
195 performed using non-metric MDS, with 100 random restarts, taking into account the

196 presence/absence, as well as the relative abundance of OTUs in all samples. The MDS  
197 results were plotted in two dimensions. Analysis of similarities (ANOSIM) with Bonferroni  
198 corrected P values was carried out to test for significant differences between the defined  
199 sample groupings. ANOSIM produces a sample statistic *R*, which represents the degree of  
200 separation between test groups (Clarke 1993). Similarity percentage (SIMPER) analysis was  
201 performed using the PRIMER® software to compare microbial communities from seawater,  
202 coated with paint and base, as well as unprotected parts of the glider. OTU partitioning was  
203 used to find out the number of OTUs that are specific for each dataset in the MDS analysis  
204 and the number of shared OTUs between different datasets. This was done on OTUs  
205 datasets using Microsoft Excel and a custom R script.

206

## 207 **Results**

### 208 *Environmental parameters*

209 During the study, the ocean glider dove from the surface to a depth of 1015m. Vertical  
210 profiles of physical, chemical and biological characteristics recorded by the glider showed  
211 high variability during the study period (Supplementary Figure S1). This variation was  
212 attributed to the movement of the ocean glider and of mesoscale eddies, as well as seasonal  
213 changes.

214 The average seawater temperature during the period of investigation varied from  
215 27.8° to 12.2° C (Figure S1A). As expected, the highest temperatures were recorded at the  
216 surface, while the lowest ones were measured at depth > 560m. The averaged vertical  
217 temperature profile implied that the surface mixed layer extended to ~25m and followed by  
218 the seasonal thermocline (Figure S1A). In turn, the thermocline layer was underlined by the  
219 Persian (Arabian) Gulf waters, with the core at 250m. This water mass, located between 150  
220 and 350 m, flowed eastward.

221 The vertical profile of salinity differed from that of temperature (Figure S1B). The  
222 highest salinity of 36.9 ppt was recorded at the depth of ~260m, co-occurring with the Gulf  
223 outflow, while the lowest salinity was found at depth. The average salinity varied from 35.7  
224 to 36.9 ppt during this study.

225 The photosynthetically active radiation (PAR) varied from 0 to 2400  $\mu\text{E m}^{-2} \text{sec}^{-1}$ ,  
226 during this study. A characteristic feature in the vertical distribution of the fluorescence  
227 intensity was the fluorescence peak observed at ~30-40m deep, persisting throughout the



228 deployment period (Figure S1C). This peak was formed by the phytoplankton community  
229 dominated by the dinoflagellate *Noctiluca scintillans* (see abundance of microbes).

230 Vertical distribution of the dissolved oxygen concentration showed a decline from  
231 saturated surface water ( $\sim 220 \mu\text{mol kg}^{-1}$ ) to near anoxic conditions in the oxygen minimum  
232 zone ( $< 2 \mu\text{mol kg}^{-1}$ ; below 400m). This pattern was interrupted by the outflow of Gulf  
233 water, injecting high salinity oxygenated water ( $\sim 140 \mu\text{mol kg}^{-1}$ ) between 150 and 350 m  
234 (Figure S1D).

235

### 236 *Abundance of microbes*

237 Taxonomic analysis of Niskin seawater samples collected within the layer of fluorescence  
238 peak (see Figure S1C) showed the presence of the dinoflagellate *Noctiluca scintillans*. This  
239 species made up about 90% of the total phytoplankton abundance (data are not shown). As  
240 for the upper mixed layer, processed samples showed that *Noctiluca* abundance was equal  
241 to  $\sim 120,000 \text{ cell L}^{-1}$  at the beginning of the experiment. At the end of the study, *Noctiluca*  
242 abundance became  $\sim 20,000 \text{ cell L}^{-1}$ .

243 The highest densities of microorganisms were recorded at a depth of 35 m, which  
244 correlated well with the presence of *N. scintillans* (Figure 2). The densities of  
245 microorganisms at the other depths were 14-fold to 23-fold lower. Densities of  
246 microorganisms on the ocean glider were significantly (ANOVA  $p < 0.05$ ) different (Figure 2).  
247 Among unprotected surfaces, the highest density of prokaryotes was observed on the ocean  
248 glider bottom (UGB) followed by the glider's wing (UWT) and the glider's top (UGT). The  
249 lowest density (HSD  $p < 0.05$ ) was recorded on the glider nose (UN). Among paints, the  
250 lowest density (HSD  $p < 0.05$ ) of microorganisms was found on PChT. The densities of  
251 microbes on the top and bottom surfaces were different (ANOVA  $p < 0.05$ ; Figure 2). There  
252 were significant differences (HSD  $p < 0.05$ ) in the densities of microbes on different bases  
253 tested; 3-fold higher densities were observed on BIn in comparison to that on BHe. The  
254 density on BIn was 1.5–2-fold lower (HSD  $p < 0.05$ ) than on the unprotected glider (UG)  
255 surfaces. The densities of microbes on antifouling paints (PInB) were 1.3-fold lower than on  
256 UGB (Figure 2). There was a significant difference between densities of microorganisms in  
257 biofilms collected from the top and the bottom of the unprotected parts of the ocean  
258 gliders (HSD  $p < 0.05$ ). Generally, the density of microbes was lower on the top of the glider

259 than on the bottom (Figure 2). Similarly, the density of microbes on PlnT was 9-fold lower  
260 (HSD  $p < 0.05$ ) than the density on the same paint located at the bottom of the glider (PlnB).

261

### 262 *Microbial diversity*

263 A total of 1,259,486 and 584,473 of 16S and 18S rDNA sequences, respectively, were  
264 obtained by Illumina MiSeq sequencing (Table 2). The lowest number of OTUs was observed  
265 on the ocean glider wing (UWT) and the highest number – on the primer BHeT (Table 2). A  
266 similar pattern was observed for Chao-1 and ACE indices. For eukaryotic communities, the  
267 highest number of sequences was found on the bottom of the ocean glider top tail (UTT),  
268 while the lowest number – on the bottom of the tail (UTB). The highest number of OTUs and  
269 the highest diversity Chao-1 and ACE indices were observed on the antifouling paint PlnB  
270 and the lowest at the bottom of the glider's top tail (UTT) (Table 2).

271 The bacterial communities that developed on paints, base and uncoated surfaces  
272 had 1158 OTUs in common. The eukaryotic communities shared 355 OTUs (Figure 3). The  
273 highest number of unique bacterial OTUs was observed for paints, while the lowest one was  
274 found in biofilms on base. For eukaryotic communities, biofilms developed on unprotected  
275 parts of the glider had 138 unique OTUs, while the numbers of OTUs on base and antifouling  
276 paints were lower (Figure 3).

277 Multidimensional analysis (MDS) showed that bacterial communities formed on the  
278 ocean glider and present in seawater were different (Figure 4), which was supported by  
279 ANOSIM analysis ( $r = 0.94$ ,  $p = 0.006$ ). While bacterial communities formed on unprotected  
280 parts of the glider (U) shared some similarity with the base (Bln and BHe) and the  
281 experimental chitosan paint (PCh), they were different from the copper-based paints (Pln  
282 and PHe) (Figure 4). Similar tendency was observed for eukaryotic communities. MDS  
283 analysis showed that eukaryotic communities in the seawater and on the ocean glider were  
284 significantly different (ANOSIM  $r = 0.62$ ,  $p > 0.05$ ). Eukaryotic communities formed on  
285 unprotected and base surfaces shared some similarities, as well as communities from the  
286 base and paints.

287 SIMPER analysis demonstrated that bacteria belonging to genera *Dasania*, *Pantoea*  
288 and *Vibrio* contributed from 14% to 22% of dissimilarity between communities developed  
289 on the ocean glider and existing in seawater (Table 3). Additionally, bacterial communities  
290 formed on unprotected and base surfaces differed by the presence of the genera

291 *Exiguobacterium*. Eukaryotes belonging to Bacillariophyceae and Hydrozoa accounted for  
292 more than 21% each between communities developed on the glider and existing in  
293 seawater (Table 4). Additionally, Holozoa (Ichthyosporea) and different fungi  
294 (Agaricomycetes and Dothideomycetes) contributed for more than 10% each for  
295 dissimilarities between eukaryotic communities.

296 Bacteria belonging to the classes Gamma- and Alpha-proteobacteria dominated  
297 biofilms developed on the ocean glider during the experiment (Figure 5A). These were  
298 mainly represented by the genera *Vibrio*, *Pseudomonas*, *Alteromonas*, *Marinobacter*,  
299 *Dasania*, *Teredinibacter* and *Cycloclasticus* (Gamma-proteobacteria), *Pseudoruegeria*,  
300 *Parvibaculum*, *Sphingomonas*, *Hyphomonas*, *Erythrobacter* and *Tateyamaria* (Alpha-  
301 proteobacteria). The class Bacilli (mainly genus *Exiguobacterium*) was abundant on all  
302 unprotected parts of the ocean glider. Sequences belonging to chloroplasts of diatoms  
303 (Bacillariophyceae and Fragillariophyceae) were detected. There were clear difference  
304 between the compositions of bacterial communities developed on antifouling paints, base  
305 and unprotected parts of the ocean glider (Figure 5A). Copper-based antifouling paints had  
306 high (51-66%) relative abundance of Gamma-proteobacteria. Among antifouling paints, the  
307 chitosan paint (PChT and PChB) were characterized by the lower relative abundance of  
308 Gamma-proteobacteria (14-33%) and the higher relative abundance of Beta-proteobacteria  
309 (28-29%). Bacteria belonging to the genera *Dasania*, *Erythrobacter* and *Cycloclasticus* were  
310 the most common on paints containing copper. In contrast, *Ralstonia* sp. was dominant on  
311 chitosan experimental paint (PCh) and base. Among base, the high relative abundance of  
312 Beta-proteobacteria was observed on BHeT and BHeB, while the high abundance of Bacilli  
313 was observed on BInT and BInB. The bacterial genera *Pantoea* and *Exiguobacterium* were  
314 dominant on base and unprotected parts of the ocean glider. Different unprotected parts of  
315 the ocean glider had distinct communities. For example, biofilms on the wing (UWT)  
316 dominated with Gamma-proteobacteria (85%), while the lower relative abundance of Alpha-  
317 proteobacteria (7%) and Bacilli (7%) was also recorded. On the other hand, there were no  
318 Bacilli in the biofilms on the glider's nose (UN).

319 Eukaryotic communities on the ocean glider were highly diverse and represented by  
320 different groups of fungi, microalgae, nematodes, arthropods and hydrozoans (Figure 5B).  
321 Sequences of some macrofouling genera, such as *Megabalanus*, *Hydractinia*, *Actinostola*,  
322 *Dicoryne* were found. Additionally, sequences belonging to some planktonic species, like

323 *Nectopyramis* sp. (Siphonopora) were detected. Hydrozoa had high relative abundance on  
324 base (BlNB 58%; BHeT 79%), the glider's nose (UN 79%) and the antifouling paint (PlNB 53%).  
325 High fungal diversity (represented by 5 different classes) in marine biofilms was detected  
326 (Figure 5B). The fungal class Agaricomycetes was highly abundant (relative abundance 72%)  
327 on paint PHeT, while the class Dothideomycetes (relative abundance 46%) and  
328 Eurotiomycetes (relative abundance 14%) were highly abundant on unprotected parts  
329 (UTB). The relative abundance of the fungal class Sordariomycetes on the antifouling paint  
330 PHeB was 7%. The highest relative abundance (89%) of the class Ichtyosporea  
331 (Mesomycetozoea, Holozoa) on PlNT was recorded (Figure 5B). Diatoms (Bacillariophyceae),  
332 mainly *Amphora* and *Cylindrotheca* species, dominated biofilms on the ocean glider  
333 unprotected surfaces (UWT 82% and UTT 94%), chitosan paint (PChB 71%; PChT 59%) and  
334 base (BlNT 73%). More than half of the sequences (54%) obtained from the unprotected  
335 bottom of the ocean glider (UGB) belonged to Chlorophyta (*Pycnococcus* sp.).

336

### 337 **Discussion**

338 This is the first study that investigated microbial fouling on the ocean glider by next  
339 generation sequencing. Previous studies reported presence of macrofouling organisms on  
340 ocean gliders (Nicholson et al. 2008; Moline and Wendt 2011), but neglected microbial  
341 biofilms, which may also affect the performance of the glider and its sensors (Davis et al.  
342 2003; Cetinic et al. 2009).

343 During the study period, vertical profiles of temperature, oxygen, chlorophyll and  
344 salinity showed high variability. The study area is highly influenced by three hydro-  
345 dynamical processes: (1) the outflow from the Persian (Arabian) Gulf, (2) the inflow from the  
346 northern Arabian Sea, and (3) the mesoscale (cyclonic and anti-cyclonic) eddies persisting in  
347 the region (across the Gulf) and connecting the northern-banked inflow and the southern-  
348 banked outflow (Al-Hashmi et al. 2010; Vic et al. 2015). Thus, we assume that variations of  
349 the physical and biological parameters in the study region could be attributed to the spatial  
350 shifts in the location of the mesoscale eddy affecting regional circulation over the shelf in  
351 Muscat region. Additionally, seasonal changes (i.e. increase of temperature from March to  
352 June) have affected vertical profiles of the physical and biological parameters (Piontkovski et  
353 al. 2017).

354 Biofilms on the surface of the ocean glider were exposed to continuous  
355 fluctuations of oxygen (0 – 287  $\mu\text{mol kg}^{-1}$ ), temperature (12.2- 32.3° C), salinity (35.4-38.0  
356 ppt), depth (0- 1015 m), pressure (0.04- 102 Bar) and light intensity (0- 2400  $\mu\text{E m}^{-2} \text{sec}^{-1}$ )  
357 for more than 3 months. We expected that microbes on the glider would differ from those  
358 in the water column. Indeed, as shown by the MDS plots, the composition of bacterial and  
359 eukaryotic organisms on the ocean glider and in the water column was very different.  
360 Previously it was reported that attached and particle-bind bacteria are more abundant and  
361 more metabolically active than unattached bacteria (Kirchman and Mitchell 1982; Dang and  
362 Lovell 2016). The densities of microorganisms on the glider's surface varied from 8,820 to  
363 228,000 cell/ $\text{mm}^2$ . Among unprotected surfaces the lowest densities of microbes were  
364 found on the nose of the ocean glider (UN). Probably, it is due to the higher dynamic  
365 pressure and velocities on that part of the glider (Isa et al. 2014; Chen et al. 2015).  
366 Additionally, the densities of microorganisms on the top of the ocean glider were generally  
367 lower than on the bottom. The orientation of different parts of the ocean glider could affect  
368 the densities of microbes (Bellou et al. 2012).

369 Ocean glider's parts painted with antifouling paints had lower densities of  
370 microorganisms than unprotected parts of the glider. This is not surprising, as antifouling  
371 paints contain chemical compounds that kill or prevent growth of microfouling organisms  
372 (Casse and Swain 2006; Mollino et al. 2009; Briand et al. 2012; Briand et al. 2017). The  
373 lowest densities of microorganisms were observed on the antifouling paints PHeT and PHeB  
374 that contained biocides cuprous oxide and zineb. In previous studies, both biocides were  
375 recognised as effective antifouling agents (Hunter and Evans 1991). Among 11 commercial  
376 antifouling paints tested during a 1 year study in Oman coastal waters, the lowest microbial  
377 biomass was recorded on the paint with these biocides (Muthukrishnan et al. 2014).

378 Compared to other studies of marine biofilms on artificial surfaces utilizing the  
379 Illumina MiSeq technique, the diversity of communities formed on the ocean glider was  
380 similar to that found in Australia (Tan et al. 2015) and Swedish (Oberbeckmann et al. 2016)  
381 coastal waters. Bacteria belonging to the classes Gamma- and Alpha-proteobacteria, mostly  
382 *Vibrio*, *Pseudomonas*, *Teredinibacter*, *Cycloclasticus*, *Pseudoruegeria*, *Parvibaculum*,  
383 *Sphingomonas*, *Erythrobacter* and *Tateyamaria*, dominated in biofilms. Similarly, previous  
384 investigations demonstrated the dominance of Alpha- and Gamma-proteobacteria in marine  
385 biofilms on various artificial substrata (Dobretsov et al. 2013; Tan et al. 2015; Sathe et al.

2016; Flach et al. 2017; Hunsucker et al. 2018). Differences between bacterial communities developed on the ocean glider were due to the genera *Dasania*, *Pantoea*, *Exiguobacterium* and *Vibrio* as indicated by SIMPER analysis. *Dasania* are obligately aerobic bacteria (Lee et al. 2007), which previously found associated with the deep sea tubeworm (Forget and Juniper 2013). *Exiguobacterium profundum* was previously isolated from a deep sea hydrothermal vent (Crapart et al. 2007). This might suggest possible adaptations of these bacteria to high pressure and low oxygen conditions. Archaea dominate deep sea waters (De Long 1992; Jensen et al. 2012) but this group was not detected in this study. While the universal 16S RNA 515F/806R primers used in this research have been used to study both archaea and bacteria (Bates et al. 2010; Walters et al. 2011), it is possible that the absence of archaea in this study was due to poor amplification of this group of microorganisms (Eloe-Fadrosh et al. 2016).

Eukaryotic communities on the ocean glider were predominantly represented by fungi, hydrozoans and arthropods. While only biofilms were observed on the glider, sequences of macrofouling organisms, such as the barnacle *Megabalanus* sp. and the hydrozoan *Hydractinia* sp., might indicate recruitment of these species on the ocean glider. Barnacles are reported as the main fouling species on ocean gliders (Lobe et al. 2010). Additionally, sequences of photosynthetic species belonging to the classes Bacillariophyta, Dinophyceae, Chlorophyta and Mediophyceae were recorded. This might indicate that some photosynthetic species on the ocean glider, like *Amphora* sp. and *Cylindrotheca* sp., can sustain some time without light. It has been shown that the benthic diatoms *Amphora coffeaeformis* and *Cylindrotheca closterium* can survive in the dark, anoxic conditions for 6-28 weeks (Kamp et al. 2011). The researchers have found that these diatoms accumulated nitrate and used it for their respiration in the absence of oxygen and light.

The composition of microbial communities developed on paints, primer and unprotected parts of the glider was different. This could be explained by different chemical (chemical composition) and physical (wettability) properties of unprotected and coated surfaces. For example, biologically and physically inert substrates, like glass, fouled quicker and had more diverse communities than active substrates, like copper-nickel alloys (Marszalek et al. 1979). 454 pyrosequencing of 16S genes revealed the presence of different microbial communities on different antifouling paints (Muthukrishnan et al. 2014; Briand et al. 2017). The copper antifouling paint resulted in significant changes in both bacterial and

418 eukaryotic communities in New Zealand waters (von Ammon et al. 2018). Similar results  
419 were obtained in the experiments with plastic panels painted and not painted with  
420 antifouling paints in Swedish waters (Flach et al. 2017). Bacteria belonging to  
421 Cryomorpaceae and Alcanivoraceaea were exclusively present on polyethylene  
422 terephthalate but not on glass surfaces in another study in the North Sea (Oberbeckmann et  
423 al. 2016). In our study, bacteria belonging to the genera *Dasania*, *Erythrobacter* and  
424 *Cycloclasticus* were common on antifouling paints containing cuprous oxide. While the  
425 genus *Dasania* was previously detected on antifouling paints, the genus *Erythrobacter* was  
426 observed in biofilms on cuprous oxide antifouling paints (Muthukrishnan et al. 2014).  
427 *Cycloclasticus* was one of the two most abundant genera in biofilms on antifouling paints  
428 exposed to fouling in Swedish coastal waters (Flach et al. 2017). This could suggest that  
429 bacteria belonging to *Dasania*, *Erythrobacter* and *Cycloclasticus* are commonly associated  
430 with antifouling paints.

431 Bacterial and eukaryotic communities on the chitosan paint were different from  
432 other antifouling paints. Differences in antifouling mechanisms can explain differences  
433 between community composition of chitosan and copper-based paints. Copper-based paints  
434 kill microorganisms due to the displacement of essential metals in proteins (Thurman et al.  
435 1989). Copper ions may alter enzyme and nucleic acids structure and function, facilitate  
436 their hydrolysis and have an adverse effect on oxidative phosphorylation and osmotic  
437 balance (Borkow and Gabbay 2005). On the other hand, chitosan inhibits biofouling due to  
438 its cationic nature and interactions with positively charged microbial cell membranes  
439 (Alisashi and Aider 2012).

440 The current study was conducted using one ocean glider. While the replicated  
441 samples were collected, these cannot be treated as true replicates. It is partially difficult to  
442 replicate naval structures. There are several similar studies of biofilms on ship hulls that did  
443 not have true replicates (Hunsucker et al. 2014; Inbakandan et al. 2010; Zargiel et al. 2011).  
444 Gliders are expensive autonomous vehicles and cannot be easily replicated. In fact, we tried  
445 to have two independent replicates but the second ocean glider was lost during the  
446 experiment. Thus, conclusions of this study need to be treated with caution.

447 In conclusion, for the first time the presence of diverse microbial biofilms formed  
448 on the surface of the ocean glider exposed to oscillating environmental conditions was  
449 demonstrated using next generation sequencing techniques. Densities and compositions of

450 microbial communities on different parts of the glider were different, which could be  
451 explained by differences in hydrodynamic conditions on different parts of the glider.  
452 Additionally, chemical composition of unprotected surfaces and coated with base and paint  
453 shaped the composition of microbial communities on the surface of the ocean glider. This is  
454 the first attempt to investigate of biofouling on ocean gliders and much work is required in  
455 the future to confirm our findings. Differential antifouling performance of paints, suggested  
456 that proper antifouling solutions for long endurance autonomous underwater vehicles need  
457 to be developed.

458

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465

### 466 **Disclosure statement**

467 The authors declare that they have no conflicts of interest related to this work.

468

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- 597
- 598

599 **Figure legends**

600 **Figure 1. A.** Ocean glider on the boat of the research vessel. **B.** The scheme of a glider's dive  
601 (from <http://www.kronberg.com> and <http://www.ueaseaglider.uea.ac.uk/DIVES/>). **C.**  
602 Google Map showing the sampling area. **Insert:** Location of ocean glider's transects in the  
603 Sea of Oman. **D.** The sampled locations on the glider (modified from  
604 <http://auvac.org/platforms/view/160>).

605

606 **Figure 2.** Total microbial abundance of microbial cells in seawater samples, on unprotected  
607 ocean glider surface and coated with paints and base. Data are the mean + standard  
608 deviation (SD). For the sample abbreviations, see Table 1.

609

610 **Figure 3.** Vienn diagram showing the number of shared and unique OTUs in bacterial and  
611 eukaryotic communities developed on paints, base and the glider's unprotected surface.

612

613 **Figure 4.** Multidimensional scaling (MDS) plots of bacterial and eukaryotic microbial  
614 communities obtained from seawater and developed on the ocean glider. For the codes, see  
615 Table 1.

616

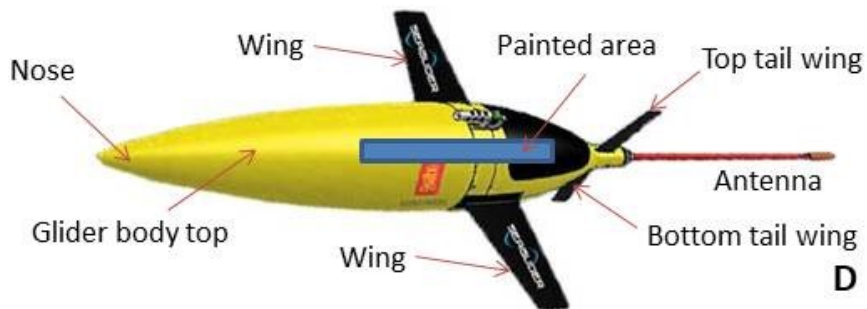
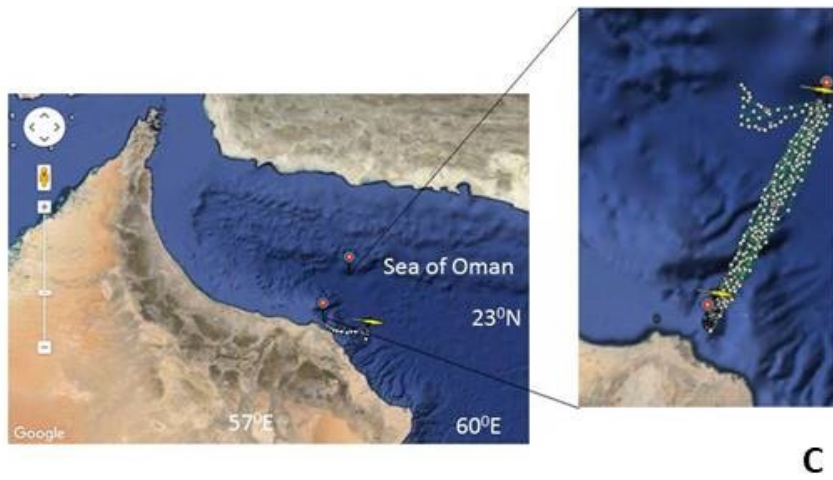
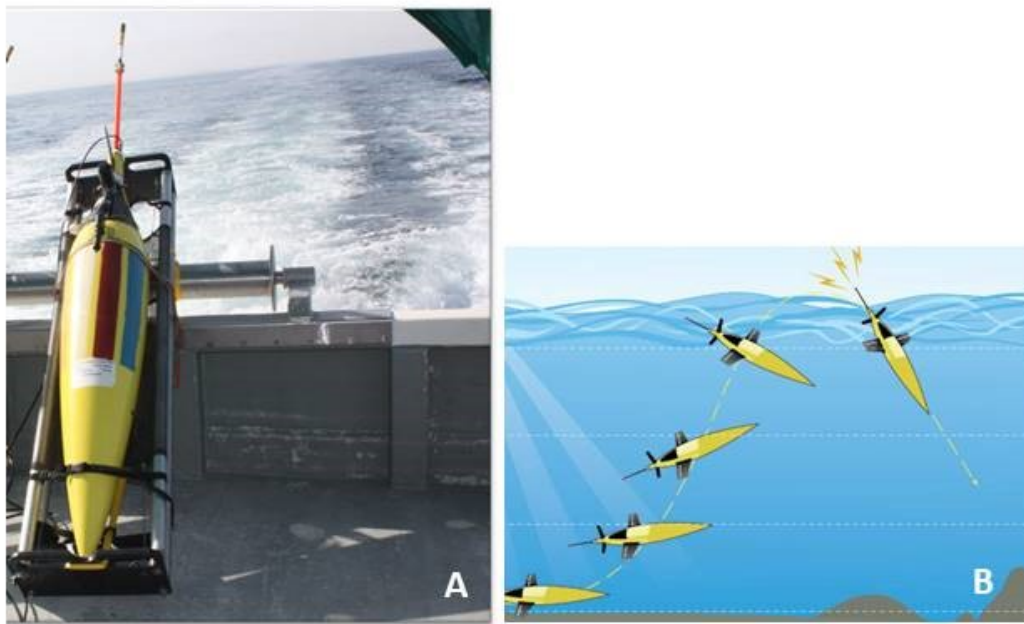
617 **Figure 5.** Heat map showing the relative abundance (%) of the main **A:** prokaryotic and **B:**  
618 eukaryotic classes present in biofilms developed on the ocean glider. For the codes, see  
619 Table 1.

620

621

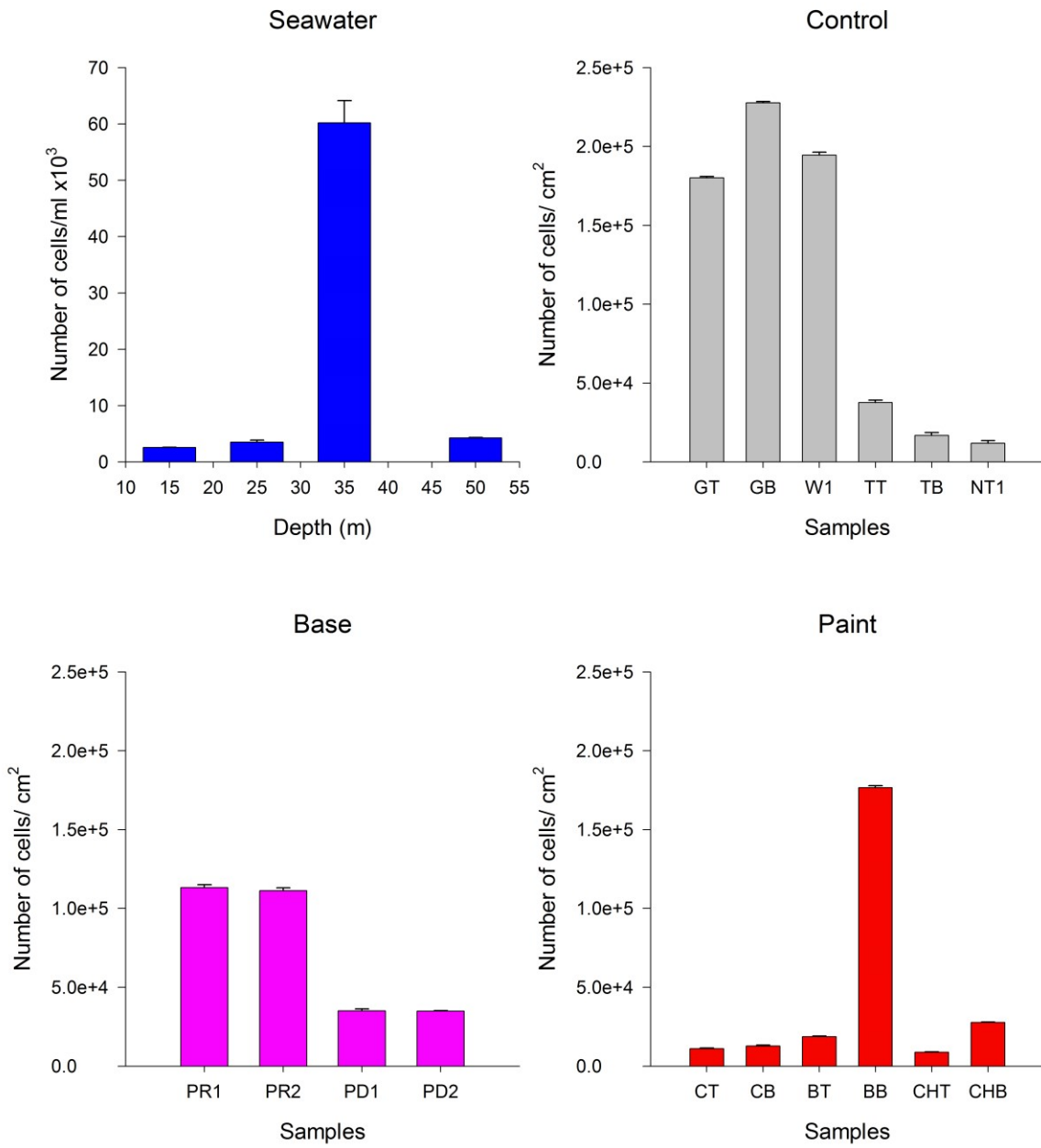
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623 **Figure 1**



624

625



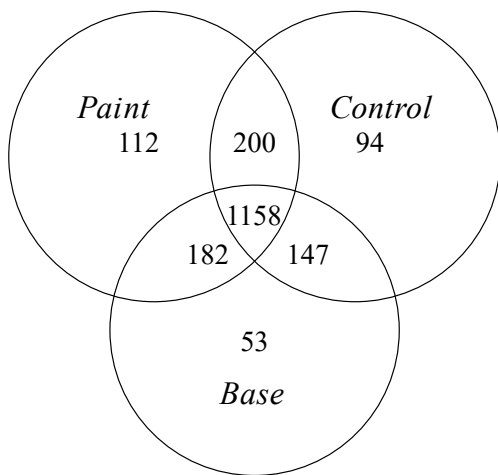
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628 **Figure 2**

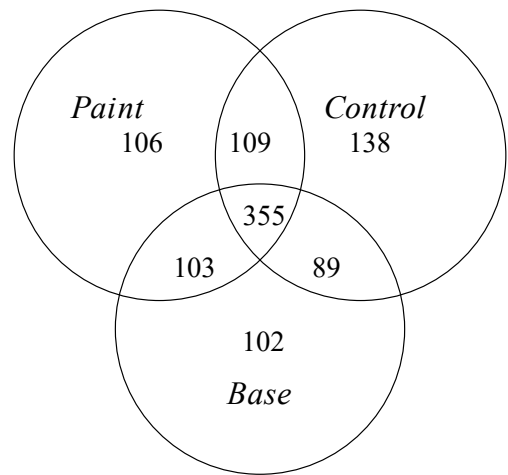
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### Bacterial communities



### Eukaryotic communities



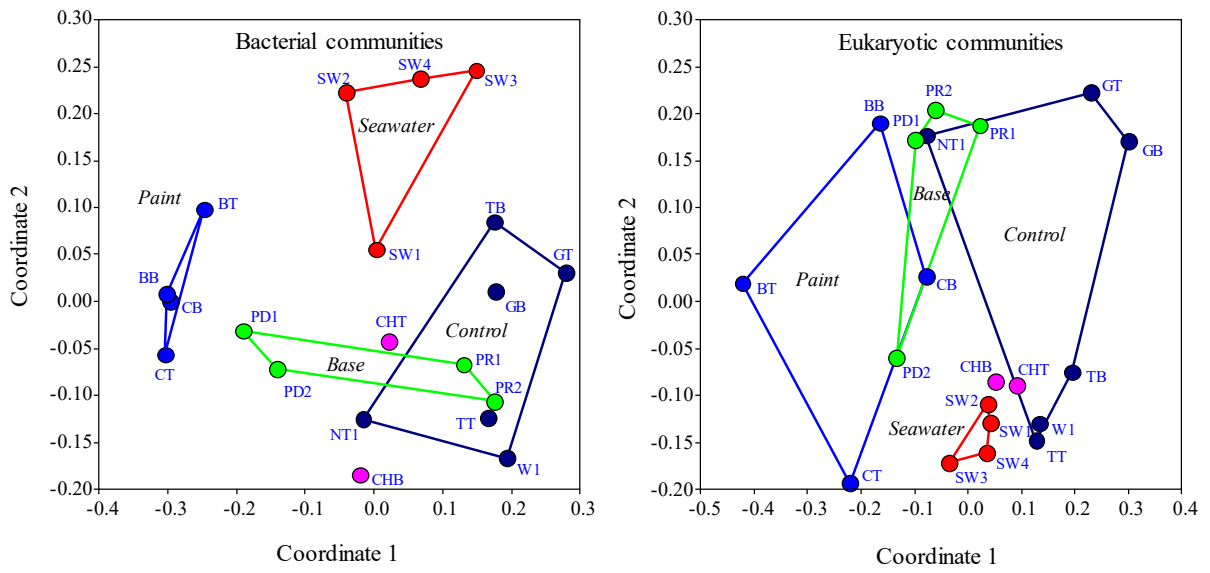
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633 **Figure 3**

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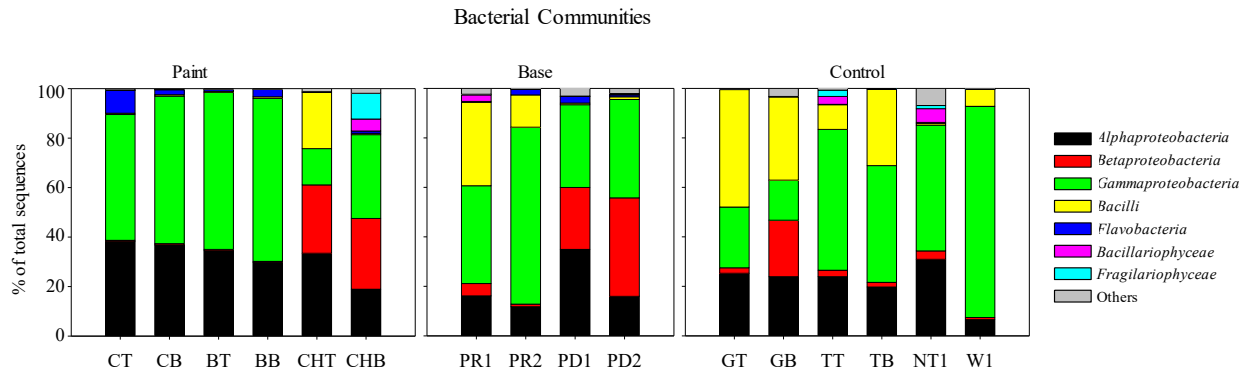
638 **Figure 4**

639



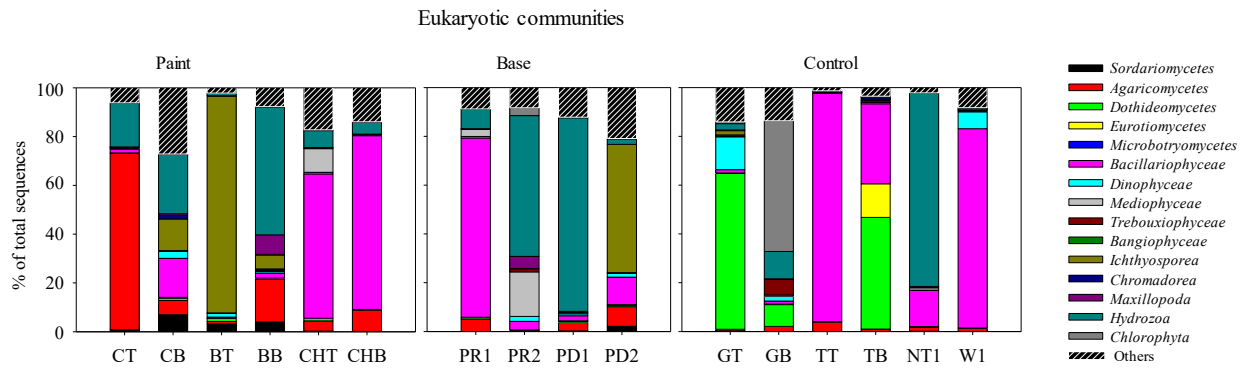
640

641 **A**



642

643 **B**



644

645

646 **Figure 5**

647

648 **Tables**

649 **Table 1.** Samples taken from the ocean glider and characteristics of paints used in this study

650

<b>Code</b>	<b>Treatment</b>	<b>Location</b>	<b>Paint</b>	<b>Type of paint</b>	<b>Active ingredient</b>
PHeT	Paint	Glider top	Hempel Olympic 86950	Biocidal	Cuprouse oxide and Zineb
PHeB	Paint	Glider bottom	Hempel Olympic 86950	Biocidal	Cuprouse oxide and Zineb
PInT	Paint	Glider top	International micron extra YBA920	Biocidal	Cuprous oxide Dichlofluanid
PInB	Paint	Glider bottom	International micron extra YBA920	Biocidal	Cuprous oxide Dichlofluanid
PChT	Paint	Glider top	Experimental chitosan	Non-biocidal	Chitosan
PChB	Paint	Glider bottom	Experimental chitosan	Non-biocidal	Chitosan
BInT	Base	Glider top	Primer Intershield 300	Non-biocidal	No
BInB	Base	Glider bottom	Primer Intershield 300	Non-biocidal	No
BHeT	Base	Glider top	Hempel Primer 26050	Non-biocidal	No
BHeB	Base	Glider bottom	Hempel Primer 26050	Non-biocidal	No
UGT	Unprotected	Glider top	No	No	No
UGB	Unprotected	Glider bottom	No	No	No
UTT	Unprotected	Glider's tail wingtop	No	No	No
UTB	Unprotected	Glider's tail wing bottom	No	No	No
UWT	Unprotected	Glider's wings top	No	No	No
UN	Unprotected	Glider's nose	No	No	No

651

652

653 **Table 2.** Amplicon library size and diversity estimators for bacterial and eukaryotic  
654 communities of the samples using MiSeq. Operational taxonomic units (OTUs) at 3%  
655 sequence dissimilarity were calculated based on equal subsets of sequences for all samples.  
656 For the codes see Table 1.

Sample ID	<i>Bacterial communities</i>				<i>Eukaryotic communities</i>			
	No. of sequences	No. of OTUs <sub>0.03</sub>	Chao-1	ACE	No. of sequences	No. of OTUs <sub>0.03</sub>	Chao-1	ACE
CT	98674	762	1093	1088	36456	195	304	308
CB	83526	707	1015	975	30834	281	404	414
BT	82157	703	1047	1024	46547	207	313	311
BB	93168	727	1059	1031	43754	313	439	449
CHT	77260	701	1059	1055	30718	241	361	396
CHB	44662	657	937	991	34850	205	325	338
GT	100276	593	931	917	30879	289	395	361
GB	101425	645	978	985	29418	228	363	344
TT	110038	605	906	904	47224	156	297	286
TB	69545	619	945	949	29047	188	283	277
NT1	62828	840	1171	1240	40020	235	345	352
W1	64661	561	946	912	36698	261	343	330
PR1	81755	660	977	986	30779	226	307	320
PR2	64634	628	957	984	45861	286	359	352
PD1	63290	879	1142	1166	36206	245	364	364
PD2	61587	766	1148	1134	35182	263	346	342

657

658

659

660 **Table 3.** The contribution of particular bacterial genera towards the total dissimilarity (in per  
661 cent) between the bacterial communities using similarity percentage (SIMPER) analysis.  
662 Groups with contribution  $\geq 2\%$  are shown.  
663

Paint vs. Control		Paint vs. Base		Paint vs. Seawater		Control vs. Base		Control vs. Seawater		Base vs. Seawater	
Taxon	Con trb. %	Taxon	Con trb. %	Taxon	Con trb. %	Taxon	Con trb. %	Taxon	Con trb. %	Taxon	Con trb. %
<i>Dasania</i>	21.7	<i>Dasania</i>	21.4	<i>Dasania</i>	22.8	<i>Pantoea</i>	22.6	<i>Vibrio</i>	21.1	<i>Vibrio</i>	21.4
<i>Pantoea</i>	14.6	<i>Pantoea</i>	16.7	<i>Vibrio</i>	20.3	<i>Exiguoba cterium</i>	14.7	<i>Pantoea</i>	16	<i>Pantoea</i>	14.4
<i>Exiguoba cterium</i>	12.7	<i>Ralstonia</i>	13.6	<i>Erythrobac ter</i>	11.4	<i>Ralstonia</i>	13.2	<i>Exiguobact erium</i>	11.9	<i>Ralstonia</i>	11.3
<i>Erythrob acter</i>	10.6	<i>Erythrob acter</i>	11.1	<i>Alteromon as</i>	8.77	<i>Pseudom onas</i>	9.29	<i>Alteromon as</i>	9.75	<i>Alteromon as</i>	10.0
<i>Pseudom onas</i>	6.99	<i>Exiguoba cterium</i>	8.86	<i>Cycloclastic us</i>	5.26	<i>Dasania</i>	6.40	<i>Pseudomo nas</i>	7.5	<i>Exiguobact erium</i>	7.60
<i>Cycloclas ticus</i>	4.72	<i>Cycloclas ticus</i>	4.66	<i>Idiomarina</i>	4.40	<i>Sphingob ium</i>	3.97	<i>Idiomarina</i>	4.63	<i>Dasania</i>	5.41
<i>Pseudoru egeria</i>	3.77	<i>Pseudoru egeria</i>	3.68	<i>Pseudoalte romonas</i>	4.10	<i>Erythrob acter</i>	3.90	<i>Sphingobiu m</i>	4.52	<i>Idiomarina</i>	4.67
<i>Sphingob ium</i>	3.70	<i>Alteromo nas</i>	3.48	<i>Pseudorue geria</i>	3.78	<i>Cycloclas ticus</i>	3.49	<i>Pseudoalte romonas</i>	4.31	<i>Pseudoalte romonas</i>	4.35
<i>Alteromo nas</i>	2.96	<i>Caulobac ter</i>	3.08	<i>Exiguobact erium</i>	3.59	<i>Caulobac ter</i>	3.26	<i>Brevundim onas</i>	2.72	<i>Erythrobac ter</i>	3.33
<i>Brevundi monas</i>	2.44	<i>Marinob acter</i>	2.32	<i>Pantoea</i>	3.33	<i>Brevundi monas</i>	3.18	<i>Sphingomo nas</i>	2.07	<i>Caulobacte r</i>	2.75
<i>Gilvibact er</i>	2.19	<i>Gilvibact er</i>	2.27	<i>Gilvibacter</i>	2.16	<i>Enhydrob acter</i>	2.49			<i>Cycloclastic us</i>	2.49
<i>Marinob acter</i>	2.10					<i>Sphingo monas</i>	2.41			<i>Sphingobiu m</i>	2.06
						<i>Massilia</i>	2.04				

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**Table 4.** The contribution of particular eukaryotic taxa towards the total dissimilarity (in per cent) between the bacterial communities using similarity percentage (SIMPER) analysis. Groups with contribution  $\geq 2\%$  are shown.

Paint vs. Control		Paint vs. Base		Paint vs. Seawater		Control vs. Base		Control vs. Seawater		Base vs. Seawater	
Taxon	Contrib. %	Taxon	Contrib. %	Taxon	Contrib. %	Taxon	Contrib. %	Taxon	Contrib. %	Taxon	Contrib. %
<i>Bacillariophyceae</i>	21.5	<i>Hydrozoa</i>	25.05	<i>Bacillariophyceae</i>	22.56	<i>Bacillariophyceae</i>	26.43	<i>Bacillariophyceae</i>	26.43	<i>Hydrozoa</i>	25.49
<i>Hydrozoa</i>	16.81	<i>Ichthyosporea</i>	23.77	<i>Ichthyosporea</i>	18.18	<i>Hydrozoa</i>	25.54	<i>Dothideomycetes</i>	15.02	<i>Bacillariophyceae</i>	25.41
<i>Ichthyosporea</i>	16.25	<i>Agaricomycetes</i>	16.74	<i>Agaricomycetes</i>	14.51	<i>Dothideomycetes</i>	13.89	<i>Hydrozoa</i>	12.18	<i>Ichthyosporea</i>	10.02
<i>Agaricomycetes</i>	13.92	<i>Bacillariophyceae</i>	16.39	<i>Hydrozoa</i>	14.31	<i>Ichthyosporea</i>	9.358	<i>Agaricomycetes</i>	8.322	<i>Agaricomycetes</i>	6.903
<i>Dothideomycetes</i>	12	<i>Mediophyceae</i>	4.042	<i>Chlorophyta</i>	6.706	<i>Chlorophyta</i>	6.921	<i>Dinophyceae</i>	5.238	<i>Maxillopoda</i>	4.522
<i>Chlorophyta</i>	5.508	<i>Sordariomycetes</i>	2.538	<i>Dinophyceae</i>	4.657	<i>Mediophyceae</i>	3.704	<i>Trebouxiophyceae</i>	4.607	<i>Trebouxiophyceae</i>	4.271
<i>Dinophyceae</i>	2.245	<i>Maxillopoda</i>	2.063	<i>Maxillopoda</i>	4.316	<i>Dinophyceae</i>	2.537	<i>Maxillopoda</i>	4.035	<i>Mediophyceae</i>	4.04
<i>Sordariomycetes</i>	2.242			<i>Trebouxiophyceae</i>	3.754	<i>Agaricomycetes</i>	2.293	<i>Bangiophyceae</i>	2.299	<i>Bangiophyceae</i>	2.305
				<i>Sordariomycetes</i>	2.52			<i>Arachnida</i>	2.027	<i>Arachnida</i>	2.083
				<i>Bangiophyceae</i>	2.049						

## Supplementary

**Figure S1.** Vertical profiles of mean values of **A:** temperature, **B:** salinity, **C:** fluorescence intensity ( $\lambda=695\text{nm}$ ) and **D:** oxygen concentrations ( $\mu\text{mol kg}^{-1}$ ) during the period of investigations. The data plotted until the depth of 650m, as measured parameters did not change after that depth. Dashed curves represented minimal and maximal values.

