1 **Divergent gene expression responses in two Baltic Sea heterotrophic model**
2 **bacteria to dinoflagellate dissolved organic matter**

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13 mRNA; RNAseq; phytoplankton exudation; ecological traits.

14 **Short title:** Marine bacteria responses to phytoplankton dissolved organic matter.

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16

17 **Abstract**

18 Phytoplankton release massive amounts of dissolved organic matter (DOM) into the water column
19 during recurring blooms in coastal waters and inland seas. The released DOM encompasses a
20 complex mixture of both known and unknown compounds, and is a rich nutrient source for
21 heterotrophic bacteria. The metabolic activity of bacteria during and after phytoplankton blooms can
22 hence be expected to reflect the characteristics of the released DOM. We therefore investigated if
23 bacterioplankton could be used as “living sensors” of phytoplankton DOM quantity and/or quality,
24 by applying gene expression analyses to identify bacterial metabolisms induced by DOM. We used
25 transcriptional analysis of two Baltic Sea bacterial isolates (*Polaribacter* sp. BAL334
26 [Flavobacteriia] and *Brevundimonas* sp. BAL450 [Alphaproteobacteria]) growing with DOM from
27 axenic cultures of the dinoflagellate *Prorocentrum minimum*. We observed pronounced differences
28 between the two bacteria both in bacterial growth and the expressed metabolic pathways in cultures
29 exposed to dinoflagellate DOM compared with controls. Differences in metabolic responses
30 between the two isolates were caused both by differences in gene repertoire between them (e.g. in
31 the SEED categories for membrane transport, motility and photoheterotrophy) and the regulation of
32 expression (e.g. fatty acid metabolism), emphasizing the importance of separating the responses of
33 different taxa in analyses of community sequence data. Similarities between the bacteria included
34 substantially increased expression of genes for Ton and Tol transport systems in both isolates, which
35 are commonly associated with uptake of complex organic molecules. *Polaribacter* sp. BAL334
36 showed stronger metabolic responses to DOM harvested from exponential than stationary phase
37 dinoflagellates (128 compared to 26 differentially expressed genes), whereas *Brevundimonas* sp.
38 BAL450 responded more to the DOM from stationary than exponential phase dinoflagellates (33
39 compared to 6 differentially expressed genes). These findings suggest that shifts in bacterial
40 metabolisms during different phases of phytoplankton blooms can be detected in individual bacterial
41 species and can provide insights into their involvement in DOM transformations.

42 **Introduction**

43 Dissolved organic matter (DOM) in seawater is estimated to represent one of the largest reservoirs
44 of organic carbon on earth (1). It consists of a complex mixture of compounds of different molecular
45 weights, solubility and volatility (2) and is traditionally classified according to bioavailability (i.e.
46 labile, semi-labile and refractory) with turnover times ranging from minutes to thousands of years
47 (3). DOM released by living and dying phytoplankton is an important source of organic carbon
48 available for heterotrophic bacteria (4). Up to 50% of the carbon fixed by primary producers in
49 marine and limnic ecosystems – bacterial and eukaryotic phytoplankton as well as multicellular
50 algae – is turned over by bacterioplankton in the microbial loop (5). This way, organic carbon is
51 degraded and transformed by the microbial community, with most eventually respired as CO₂. This
52 carbon turnover occurs at a rate that is orders of magnitude higher in the sea than in terrestrial
53 ecosystems (6, 7), particularly so in coastal environments and inland seas where nutrient
54 concentrations do not limit microbial activities to the same extent as in the open ocean. Given the
55 tight linkages between phytoplankton and bacteria, it is desirable to learn to what extent and by
56 which mechanisms the metabolic activity of heterotrophic bacteria regulate carbon and nutrient
57 cycling through the microbial loop.

58 Monitoring the actual rates of the plethora of metabolic pathways active in microbial communities
59 directly *in situ* is currently not feasible, but nucleotide sequencing-based methods, in particular
60 metatranscriptomics, can indicate which microbial metabolisms are actively transcribed.
61 Metatranscriptomics has hence become a widely used tool to provide detailed insights into the
62 genetic underpinnings of metabolic responses within communities both in natural environments and
63 controlled experiments (8-11). The complexity of gene regulation observed in communities
64 consisting of many thousands of individual populations is, however, daunting. For the purpose of
65 eventually using transcript profiling as a proxy for metabolic activity in complex natural
66 communities, zooming in to compare gene expression pattern in isolates of environmentally relevant
67 microbial taxa could be useful.

68 With the aim of charting the possibility of using transcriptional activity of bacterial isolates as living
69 sensors for the flow of nutrients in the ecosystem, we exposed two Baltic Sea model bacteria to
70 DOM derived from axenic cultures of *Prorocentrum minimum*, a dinoflagellate that forms major
71 blooms in the spring and autumn in the Baltic Sea. The bacterial isolates – *Polaribacter* sp. strain
72 BAL334 (*Flavobacteriaceae*, *Bacteroidetes*) and *Brevundimonas* sp. strain BAL450
73 (*Caulobacteraceae*, *Alphaproteobacteria*) – were selected to compare responses of bacteria with
74 different evolutionary histories. Furthermore, since previous studies of phytoplankton extracellular
75 DOM release have suggested that phytoplankton secrete different compounds during early and late
76 growth phases (12-16), the DOM was harvested both from dinoflagellates growing actively and in
77 stationary phase. This allowed characterization of potential differences in bacterial responses to
78 DOM released during exponential and senescence phases of phytoplankton blooms.

79

80 **Materials and Methods**

81 *Cultivation of axenic Prorocentrum minimum*

82 An axenic culture of the dinoflagellate *Prorocentrum minimum* strain CCMP1329 was obtained
83 from the Provasoli-Guillard National Center of Marine Algae and Microbiota (CCMP;
84 <https://ncma.bigelow.org/>). 5 mL of inoculum of *P. minimum* CCMP1329 was transferred and
85 cultivated in axenic conditions in 6 replicates using acid-washed Erlenmeyer flasks (2 L) containing
86 1.3 L of L1 medium (17), prepared using 0.2 µm membrane filters (Supor®, Pall Corporation) and
87 artificial seawater (30 practical salinity units, prepared from Sea Salts; Sigma). The cultures were
88 placed in 20°C with photosynthetically active radiation (PAR) of 83 – 101 µmol photon m⁻² s⁻¹ in
89 light:dark cycles of 13:11 h and bubbled with filtered air provided by an inhouse air system. To
90 follow the growth of the cultures, chlorophyll *a* concentrations were measured regularly by
91 collecting 1 mL of culture on 25 mm glass microfiber filters (GF/C, Glass Microfiber Binder Free,
92 Whatman), followed by chlorophyll *a* ethanol extraction according to (18).

93 *Collection of DOM*

94 DOM from *P. minimum* CCMP1329 was collected from three of the cultures in the exponential
95 growth phase (~15 days after inoculation) and from two of the cultures in stationary phase (~31
96 days), hereafter referred to as DOM_exp and DOM_sta, respectively. DOM from the exponential
97 growth phase was retrieved as follows. Phytoplankton cells were gently removed by first filtering
98 through an acid-washed 3.0 µm polycarbonate filter (GSV, Life Science) and then through an acid-
99 washed 0.22 µm polycarbonate filter (GSV, Life Science), using a Sterifil 47 mm filter holder
100 (Merck Millipore). DOM collected in stationary growth phase was obtained by first centrifuging the
101 cultures in acid-washed 50 mL Falcon tubes for 10 min at 3000 g (to prevent filters from clogging);
102 the supernatant was then filtered through an acid-washed 0.22 µm polycarbonate filter (GSV, Life
103 Science) using a Sterifil 47 mm filter holder (Merck Millipore). The flow-through liquid was
104 transferred into an acid-washed 10 L polycarbonate (PC) bottle and mixed before samples for
105 dissolved organic carbon (DOC) concentration and microscope samples were taken (see below for
106 detailed information of the sampling procedure). Finally, the DOM was aliquoted into 1 L acid-
107 washed PC bottles and stored at -80°C until further proceedings.

108 *Bacterial isolates and culture conditions*

109 The flavobacterium *Polaribacter* sp. strain BAL334 (hereafter referred to as *Polaribacter* BAL334)
110 and alphaproteobacterium *Brevundimonas* sp. strain BAL450 (hereafter referred to as
111 *Brevundimonas* BAL450) were isolated from surface water (2 m depth) at the Linnaeus Microbial
112 Observatory (LMO) in the Baltic Sea (N 56° 55.8540', E 17° 3.6420') during 2012. Seawater was
113 spread on Baltic Zobell agar plates containing a mixture of 5 g bacto peptone, 1 g yeast extract and
114 15 g bacto agar per L of sterile Baltic Sea water (i.e. a mix of 750 ml seawater and 250 ml MilliQ
115 water). Bacterial colonies were transferred into 1 mL of Baltic Zobell medium (i.e. mixture of 5 g
116 bacto peptone and 1 g yeast extract per L of sterile Baltic Seawater) and preserved in glycerol (25%,
117 final concentration) in -80°C.

118 *DNA extraction and genome sequencing*

119 To identify the bacteria, DNA from the isolates were extracted using the E.Z.N.A. Tissue DNA kit
120 (Omega bio-tek, USA) following the manufacturer's protocol for extraction of cultured cells in
121 suspension. For identification of the isolates, bacterial 16S rRNA genes were PCR amplified using
122 the primers 27F and 1492R at a final concentration of 10 picomole per μ l with the following PCR
123 thermal cycling program: 95°C for 2 min; 30 cycles of 95°C for 30 s, 50°C for 30 s, and 72°C for
124 45 s; and 72°C for 7 min. E.Z.N.A. Cycle-Pure Kit (Omega bio-tek, USA) were used for cleaning
125 the PCR product following the manufacturer's spin protocol instruction. Samples were sent for
126 Sanger sequencing at Macrogen Europe, Amsterdam, Netherlands. The partial 16S rRNA gene
127 sequences have been deposited in GenBank with the following accession numbers: KM586879
128 (*Polaribacter* BAL334) and KM586934 (*Brevundimonas* BAL450).

129 Genome sequences from the isolates *Polaribacter* BAL334 and *Brevundimonas* BAL450 were
130 obtained by sequencing the extracted genomic DNA using the Illumina HiSeq 2500 system (PE
131 2x125bp) at SciLifeLab, Solna, Sweden. The quality of sequences was checked with FastQC
132 (version 0.11.5) (19) and MultiQC (version 1.4) (20), adaptors were removed with cutadapt (version
133 1.12) (21) and Sickle (version 1.33) (22) was used to trim sequences based on quality score.
134 Assembly was performed using Megahit (version 1.1.2) (23) and annotation with the Rapid
135 Annotation using Subsystem Technology (RAST) server (24, 25). The genomes are available in the
136 RAST database SEED viewer (<https://rast.nmpdr.org/seedviewer.cgi>) with identities
137 6666666.325781
138 (<https://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=6666666.325781>) and
139 6666666.325780
140 (<https://rast.nmpdr.org/seedviewer.cgi?page=Organism&organism=6666666.325780>) for
141 *Polaribacter* BAL334 and *Brevundimonas* BAL450, respectively, using the guest account (user
142 login “guest”, password “guest”).

143 *Experimental setup and spiking of DOM*

144 Bacterial isolates were grown on Baltic Zobell agar plates for 3-4 days at room temperature after
145 being transferred from the -80°C freezer. Subsequently, they were inoculated into an acid-washed
146 100-mL glass bottle containing 20 mL Baltic Zobell medium, and were allowed to grow for 28 hours
147 (*Polaribacter* BAL334) and 54 hours (*Brevundimonas* BAL450) on a Unimax 2010 orbital shaker
148 (Heidolph) at 120 rpm. 2 mL of bacterial culture *Polaribacter* BAL334 (reaching an optical density
149 [OD₆₀₀] of 0.39) and 0.5 mL of bacterial culture *Brevundimonas* BAL450 (reaching OD₆₀₀ of 0.57)
150 were transferred to acid-washed 2 (L) glass bottles containing 300 mL fresh Baltic Zobell medium.
151 *Polaribacter* BAL334 was grown into early stationary phase at 80 rpm (38 hours; and OD₆₀₀ 0.20)
152 and *Brevundimonas* BAL450 bacterial cultures was grown into early stationary phase (40 hours;
153 OD₆₀₀ 1.3) at 120 rpm. To reduce nutrient concentrations and adapt bacterial cells to starvation,
154 bacterial cells were centrifuged at 4000 rpm for 7 min, supernatants were discarded and cell pellets
155 were washed by adding 1 volume of sterile artificial Baltic seawater (7 PSU, prepared from Sea
156 Salts; Sigma). Cells were resuspended in artificial Baltic seawater and the procedure was repeated
157 twice more.

158 To start the experiment, 30 mL (*Polaribacter* BAL334) and 14.5 mL (*Brevundimonas* BAL450) of
159 resuspended bacterial cells were divided into each of nine acid-washed (1 L) glass bottles containing
160 700 mL artificial seawater (7 practical salinity units, prepared from Sea Salts; Sigma). The different
161 volumes were to start the experiment with a similar bacterial biomass, and was based on OD
162 measures in the washed cells (see previous paragraph). Subsequently, after 1.5 hours, three of the
163 bottles were spiked with 67 mL DOM_exp and three with 14 mL DOM_sta to obtain an enrichment
164 with DOC corresponding to ~50 µM carbon (final concentration). To minimize potential effects
165 from the medium used for culturing *P. minimum* between the treatments, 53 mL of L1 medium were
166 added to the DOM_sta bottles. Three bottles serving as controls were spiked with 67 mL of L1
167 medium. Samples for determination of DOC concentrations and bacterial abundances were taken as
168 described below.

169 *Determination of DOC concentrations, bacterial abundance, OD and purity of cultures using*
170 *microscopy and cultivation*

171 Samples for DOC concentrations were collected 1 hour after DOM spiking by filtering 30 mL of
172 sample through 0.2 μ m acrodisc supor syringe filters 32 mm, into a 60 mL TC flask (Sarstedt) using
173 a 50 ml plastic syringe (NORM-JECT). Samples were then acidified by addition of 448 μ l 1.2 M
174 HCl. DOC concentrations were calculated as non purgeable organic carbon (using high temperature
175 catalytic oxidation followed by NDIR detection of the gaseous CO₂), analyzed on the high-
176 temperature carbon analyzer Shimadzu TOC-5000 at Umeå Marine Science Centre, Umeå, Sweden.
177 Bacterial abundance (BA) samples were taken in triplicates from each replicate 1 hour after DOM
178 spiking, by fixing the sample with paraformaldehyde at a final concentration of 1%. Samples were
179 then frozen at -80°C until determined by using the flow cytometer Cube 8 (CyFlow®) according to
180 protocol in (26). Optical density at 600 nanometer (OD₆₀₀) was measured with a Beckman DU®640
181 spectrophotometer. To ensure axenic conditions (i.e. exclusion of bacterial contamination), 1 mL of
182 algae cultures and samples from experiments were fixed with 1% paraformaldehyde, stained with
183 0.02% SYBR gold (final concentration) and filtered onto 0.2 μ m, 25 mm black polycarbonate filters
184 (Millipore). The filters were analyzed by epifluorescence microscope observation before and after
185 the cultivations/experiments. Additionally, aliquots from bacterial and phytoplankton cultures were
186 spread on Zobell agar plates for investigation of potential contamination.

187 *RNA sampling, extraction and sequencing*

188 One hour after DOM spiking, seawater samples for RNA were fixed by addition of an ethanol:phenol
189 mix (5 % phenol in absolute ethanol) in a 10:1 proportion (27). The fixed samples were then filtered
190 through Durapore 0.2 μ m, 47-mm membrane filters GV (Merck Millipore). Filters were folded and
191 transferred into clean nucleotide-free collection tubes and stored at -80°C until further procedure.
192 Extraction of RNA was performed using the RNeasy mini kit (Qiagen). Briefly, bacterial cells were
193 lysed by cutting membrane filters into smaller pieces and placing them in nucleic acid free microfuge
194 tubes containing RLT Buffer (with added β -Mercaptoethanol 1:100) and 1.5 gram 200 μ m Low

195 Binding Zirconium Beads (OPS diagnostics, USA). Thereafter, cell lysis, RNA purification, on
196 column DNase digestion and RNA elution were performed following the manufacturer's
197 instructions. Total RNA was DNase treated using the TURBO DNA-free Kit (ThermoFisher
198 Scientific) and quality checked on agarose gel. Ribosomal RNA was depleted using RiboMinus
199 Transcriptome Isolation Kit and RiboMinus Concentration Module (ThermoFisher Scientific) and
200 mRNA was amplified using the MeassageAmp II-Bacteria RNA Amplification Kit (ThermoFisher
201 Scientific) according to manufacturer's instructions. RNA sequencing was done at the at SciLifeLab,
202 Solna, Sweden. Raw sequence reads are available at NCBI's Sequence Read Archive under the
203 BioProject PRJNA678611 (<https://www.ncbi.nlm.nih.gov/bioproject/PRJNA678611>).

204 *Bioinformatics and statistical analysis*

205 RNA sequencing was done at the at SciLifeLab, Solna, Sweden, and RNA sequence reads were
206 quality trimmed with Sickle (version 1.33) (22) and mapped to the genomes with Bowtie 2 (28).
207 This resulted in between 92,655 and 329,457 mRNA sequence reads per sample. EdgeR (29) was
208 used to determine significantly differentially expressed genes (false discovery rate <5%) between
209 treatments and controls. EdgeR was also used to retrieve normalized counts per million (CPM)
210 estimates. Genes with an expression of less than five sequence reads were not included in the
211 analyses.

212

213 **Results**

214 *Growth of Prorocentrum minimum*

215 The dinoflagellate *Prorocentrum minimum* was grown as the DOM source for the experiments. It
216 followed a sigmoid growth curve with a lag phase of about 10 d. Thereafter, it entered an exponential
217 growth phase. DOM was collected during active growth (day 15) at a chlorophyll *a* concentration
218 of 629 ± 35 $\mu\text{g/L}$. After 31 d the samples for stationary phase DOM were retrieved at chl *a*
219 concentration of 2436 ± 130 $\mu\text{g/L}$ (**Fig. 1**).

Figure 1. Growth of axenic *P. minimum* cultures in L1 medium for collection of dissolved organic matter from different growth phases. Red line denotes growth of cultures harvested for DOM in exponential phase, and blue line shows growth of cultures harvested in stationary phase. Chlorophyll *a* concentrations were monitored as a proxy for biomass. Error bars denote the standard deviations of triplicates for exponential phase cultures and duplicates for stationary phase; when not visible, error bars are within symbols.

Bacterial abundance

In *Polaribacter* BAL334 cultures spiked with DOM from exponential and stationary phase dinoflagellate cultures, bacterial abundance reached 6.6 ± 0.6 and $6.4 \pm 0.6 \times 10^6$ cells ml⁻¹ (mean $\pm$ standard deviations, n=3), respectively. This was nearly a doubling compared to the control cultures where $3.8 \pm 0.7 \times 10^6$ cells ml⁻¹ were recorded. Bacterial abundance in the *Brevundimonas* BAL450 cultures spiked with exponential phase DOM increased to $3.7 \pm 0.4 \times 10^6$ cells ml⁻¹, and further increased to $4.6 \pm 0.4 \times 10^6$ cells ml⁻¹ with stationary phase DOM. Control culture cell abundance was $1.7 \pm 0.4 \times 10^6$ cells ml⁻¹.

Brief description of the Polaribacter sp. BAL334 and the Brevundimonas sp. BAL450 genomes

Polaribacter sp. BAL334 (Bacteroidetes) and *Brevundimonas* sp. BAL450 (Alphaproteobacteria) have similar genome sizes at ~3.3 Gb and ~3.2 Gb, respectively. The *Polaribacter* sp. BAL334 genome contains 2880 putative open reading frames (ORFs), of which 1021 (35.5%) have a SEED annotation, whereas 757 (26.3%) have a functional annotation but were not represented in SEED. The remaining 1102 ORFs (38.2%) were annotated as hypothetical proteins. The *Brevundimonas* sp. BAL450 genome encodes 3001 ORFs. Of these, 1269 (42.3%) have a SEED annotation, and 732 (24.4%) were not included in SEED but were functionally annotated; the remaining 1000 ORFs (33.3%) were annotated as hypothetical proteins.

As expected due to their role in central metabolism, dominant SEED categories (the highest level in the SEED hierarchy) in the genomes of both isolates were *Amino Acids and Derivatives* (up to 300 genes), *Carbohydrates*, *Protein Metabolism*, and the category *Cofactors, Vitamins, Prosthetic groups, Pigments* (**Fig. 2A**). Although the genome sizes of the two bacteria was comparable, *Polaribacter* BAL334 had a higher number of genomically encoded genes devoted to the categories *Carbohydrates* (222 genes, compared to 191 in *Brevundimonas* BAL450) and *Sulfur metabolism* (37 versus 18 genes) and *Photosynthesis*; the latter reflecting that only *Polaribacter* BAL334 has the proteorhodopsin gene encoded in its genome. The category *Motility and Chemotaxis* was only found in *Brevundimonas* BAL450 (84 genes), reflecting the lack of flagellar motility in *Polaribacter* BAL334 (**Fig. 2A**).

Figure 2. Comparison of number of genes in genomes and the relative expression levels in the two studied bacteria exposed to distinct DOM. A) Number of genomically encoded genes in top level SEED categories. B) Relative gene expression responses to enrichment with DOM collected from axenic exponential and stationary phase dinoflagellate cultures, and to control enrichments with only dinoflagellate L1 medium. “BAL334” and “BAL450” refers to *Polaribacter* BAL334 and *Brevundimonas* BAL450, respectively. Error bars in 2B indicate standard deviations for triplicates per treatment.

Messenger RNA sequencing outcome

Sequenced bacterial mRNAs sampled 1 h after addition to phytoplankton DOC treatments mapped to 2742 ORFs in *Polaribacter* BAL334 and 2984 ORFs in *Brevundimonas* BAL450, representing 95% and 99% of putative protein coding genes in the genomes of the two isolates, respectively. The SEED annotated genes (with full functional hierarchies allowing higher level metabolic analyses; 995 and 1228 respectively in *Polaribacter* BAL334 and *Brevundimonas* BAL450), attracted 36-38% of the mRNA reads in *Polaribacter* BAL334 and 56-58% of the reads in *Brevundimonas*

BAL450. The higher SEED annotation levels in the transcriptome of *Brevundimonas* BAL450 most likely reflects that knowledge of Proteobacteria genetics is generally higher than for Bacteroidetes.

Overview of transcriptional differences between the two bacteria

Upon comparison of the two isolates, it was interesting to note that in the category *Membrane Transport*, both the number of genes in the genome (**Fig. 2A**) and the relative expression level (**Fig. 2B**) were threefold higher in *Brevundimonas* BAL450 than in *Polaribacter* BAL334 (105 genes versus 31 expressed genes at ~97,000 versus ~26,000 CPM). *Brevundimonas* BAL450 also had higher expression than *Polaribacter* BAL334 in the two categories *Nitrogen Metabolism* and *Phosphorus Metabolism* (**Fig. 2B**). Despite both genomes encoding around 90 genes in the category *Fatty Acids, Lipids, and Isoprenoids* (**Fig. 2A**), expression in this category was approximately 3-fold higher in *Polaribacter* BAL334 than in *Brevundimonas* BAL450 (~52,000 versus ~15,000 CPM) (**Fig. 2B**).

The major differences between the isolates in expression levels of genes in the SEED categories *Membrane Transport* and *Fatty Acids, Lipids, and Isoprenoids* led us to do a closer inspection of the genes involved. In *Membrane Transport*, we identified many more expressed genes in *Brevundimonas* BAL450 (64 paralog groups) than in *Polaribacter* BAL334 (18 paralog groups). Moreover, these genes were distributed in a broader variety of transporter subsystems (the second level in the SEED hierarchy; roughly corresponding to “pathways” in other annotation databases) in *Brevundimonas* BAL450 (**Fig. 3**). In both isolates, transporter expression was dominated by the *Ton* and *Tol* transport subsystem, both in relative expression levels and the number of expressed genes. *TRAP* transporters and *Uni*-, *Sym*- and *Anti*porters were well represented in *Brevundimonas* BAL450 but were absent from *Polaribacter* BAL334 (**Fig. 3**).

Figure 3. Comparison of relative expression levels of genes in the SEED category *Membrane Transport* in the two studied bacteria. Colors of circles denote transporter types. The size of circles represents the number of paralogs (defined as ORFs with the same

name) in each of the genomes of the two bacteria. Y-axes shows the expression in counts per million (CPM) for the DOM treatments and the controls.

Regarding the category *Fatty Acids, Lipids, and Isoprenoids*, the higher expression in *Polaribacter* BAL334 compared to *Brevundimonas* BAL450 was primarily due to differences in *Fatty Acid metabolism* and *Polyhydroxybutyrate metabolism* (PHB) subsystems (**Fig. 4**). Both of the subsystems contained few expressed genes (five in both subsystems in *Polaribacter* BAL334; 11 and 15, respectively, in *Brevundimonas* BAL450; **Fig. 4**). In fact, two of the three most abundantly expressed genes in this category (*3-hydroxyacyl-CoA dehydrogenase* and *3-ketoacyl-CoA thiolase*) are shared between the subsystems, while the third (*Acetyl-CoA acetyltransferase*) occurred only in PHB. Still, we observed large differences between the isolates in the expression of these genes, with higher levels in *Polaribacter* BAL334 (**Table S1**). Moreover, *Polaribacter* BAL334 had substantially higher levels of expression of genes involved in isoprenoid synthesis (**Fig. 4**), in line with isoprenoids being precursors for carotenoids, which are likely responsible for the vividly orange color of *Polaribacter* BAL334 colonies.

Figure 4. Analysis of gene expression in SEED subsystems of the *Fatty Acid, Lipids and Isoprenoids* category. Colors of circles denote SEED subcategories. The size of symbols represents the number of genes expressed in each subsystem. Y-axes shows the expression in counts per million (CPM) for the two DOM treatments and the controls.

Significantly differentially expressed genes

To determine which expressed genes were significantly more (denoted “up”) or less (denoted “down”) abundant in the transcriptomes of the DOM-enriched samples than in controls, we performed a statistical analysis using EdgeR (29). The analysis was partitioned into four contrasts,

318 considering each of the bacterial isolates and each of the DOM pools from the dinoflagellate relative
319 to the control samples that did not receive any DOM. *Polaribacter* BAL334 enriched with DOM
320 from exponential phase dinoflagellates compared to the control (hereafter, BAL334Exp-Con
321 contrast) contained by far the highest number of differentially expressed genes (total of 128 genes;
322 106 up and 22 down; *false discovery rate* <5%) (**Fig. 5**). The treatment with DOM from stationary
323 phase dinoflagellates (hereafter, *Polaribacter* BAL334Sta-Con) resulted in many fewer
324 differentially expressed genes (24 genes up and two down). In *Brevundimonas* BAL450, on the other
325 hand, it was the DOM from stationary phase dinoflagellates (hereafter, *Brevundimonas* BAL450Sta-
326 Con) that induced more differentially expressed genes (23 genes up and ten down) than the
327 exponential phase DOM (hereafter, *Brevundimonas* BAL450Exp-Con): four genes up and two down
328 (**Fig. 5**). None of the significantly differentially expressed genes were shared between *Polaribacter*
329 BAL334 and *Brevundimonas* BAL450 (**Table S2**). In both bacterial isolates the majority of
330 expressed genes were not included in any SEED category (denoted “Not in SEED” in **Fig. 5**).

331 **Figure 5.** Influence of DOM from different growth phases of the dinoflagellate
332 *Prorocentrum minimum* on statistically significant differences in gene expression between
333 the two marine bacteria. Transcripts were defined as statistically significantly differentially
334 abundant based on EdgeR analyses with an $FDR \leq 0.05$. Each circle represents one gene and
335 the circle size shows the calculated expression in logCPM; note that a gene can occur in more
336 than one SEED category. Genes with significantly lower expression in controls compared to
337 treatments are indicated with negative logarithmic fold change (logFC) values. BAL334Exp-
338 Con and BAL334Sta-Con refers to *Polaribacter* BAL334 with DOM from exponential (Exp)
339 and stationary (Sta) phase DOM compared to controls (Con). BAL450Exp-Con and
340 BAL450Sta-Con refers to *Brevundimonas* BAL450 with DOM from exponential (Exp) and
341 stationary (Sta) phase DOM compared to controls (Con).

342

343 Out of the 128 differentially expressed genes, 104 genes were unique to the *Polaribacter* BAL334
344 exponential phase. Twelve SEED subsystems had at least two differentially expressed genes
345 compared to the control (**Fig. 6; Table S2**). Of these, only the *Proteorhodopsin* subsystem (in SEED
346 category *Photosynthesis*) had genes with relative expression levels that were significantly higher in
347 the controls (seen as negative value of differentially expressed genes in **Fig. 6**), one proteorhodopsin
348 gene and one phytoene dehydrogenase (the latter being involved in the synthesis of the rhodopsin
349 chromophore retinal). Both were moderately abundant with logCPM values of 11.1 and 9.6,
350 respectively (i.e. ~0.2% and 0.08% of total transcripts).

351 Furthermore, the *Polaribacter* BAL334Exp-Con contrast contained five subsystems in the
352 *Carbohydrates* category with at least two genes that increased in expression in the treated samples,
353 three of which were also highly expressed: *Trehalose Uptake and Utilization*, *Trehalose*
354 *Biosynthesis*, and *Cellulosome* (**Fig. 6; Table S2**). Several of the more abundant genes in these
355 subsystems encode enzymes involved in binding of starch (SusC; a component of the polysaccharide
356 utilization loci [PULs] widespread in Bacteroidetes (30), and eight genes involved in starch
357 degradation to trehalose/maltose and the potential modification of these sugars: for example, two
358 alpha-amylase genes the two enzymes in maltose to glucose degradation (maltose/trehalose
359 phosphorylase (**Fig. 6; Table S2**). This suggests that starch released by the dinoflagellate was an
360 important substrate fueling growth of *Polaribacter* BAL334.

361 Finally, the *Polaribacter* BAL334Exp-Con contrast included the *Amino Acids and Derivatives*
362 category, the *Branched-Chain Amino Acid Biosynthesis* subsystem which contained six genes
363 involved in isoleucine synthesis. In the *DNA Metabolism* category, three type I restriction-
364 modification system genes were detected, shared by two subsystems plus a type III restriction-
365 modification system gene found only in the *Restriction-Modification System* subsystem (**Fig. 6;**
366 **Table S2**). Only two significantly differentially abundant genes were unique to the contrast

367 *Polaribacter* BAL334Sta-Con, encoding 2-isopropylmalate synthase (*Amino Acids and Derivatives*)
368 and a SusC paralog, the outer membrane protein involved in starch binding (*Carbohydrates*).
369 Among the 32 significant genes that were unique in the *Brevundimonas* BAL450Sta-Con contrast,
370 it is interesting to note the *Ferric siderophore transport system, periplasmic binding protein TonB*
371 (**Table S2**). The Ton and Tol transport system (*Membrane Transport*) was the only subsystem in
372 *Brevundimonas* BAL450 that contained at least two differentially expressed genes (**Fig. 6; Table**
373 **S2**). Two of the three genes were annotated as *TonB-dependent receptors*, and were shared between
374 the exponential and stationary phase DOM contrasts with controls. In contrast, the *N-*
375 *acetylglucosamine-regulated TonB-dependent outer membrane receptor* was unique to the
376 BAL450Exp-Con contrast (**Fig. 6; Table S2**).

377 **Figure 6.** Subsystems in *Polaribacter* BAL334Exp-Con containing at least two significantly
378 differentially expressed genes. SEED subsystem is shown on Y-axis and SEED category is
379 shown in each plot title. The X-axis shows the number of genes whose expression is
380 significantly more (positive value) or less (negative value) abundant compared with controls.
381 Circle size represents the sum of logCPM.

383 Discussion

384 We investigated how representatives of two major taxa in the marine environment – *Flavobacteria*
385 and *Alphaproteobacteria* – react to DOM produced by dinoflagellates. These reactions partly
386 reflected differences in genomically encoded functional capacity, but also that each of the isolates
387 changed their allocation of relative transcriptional investment in certain metabolic functions. This
388 change in transcription coincided with roughly a doubling in abundance following a single hour of
389 DOM exposure. In agreement with the large phylogenetic distance between the two isolates, there
390 were striking differences in the gene expression responses to DOM between the two isolates. These
391 differences were noted even at the top level of the SEED classification system, with for example the

392 ~3-4-fold higher relative expression of *Membrane Transport* in *Brevundimonas* BAL450, and the
393 ~3-fold higher relative expression of *Fatty Acids, Lipids and Isoprenoids* in *Polaribacter* BAL334
394 compared to *Brevundimonas* BAL450. This suggests pronounced differences in resource utilization
395 between these marine bacteria, indicating a potential for resource partitioning at the level of major
396 metabolic categories.

397 As deduced from the number of genes that differed significantly in expression, we found that
398 *Polaribacter* BAL334 (total 154 genes; 5.6% of genome) showed a much stronger response to DOM
399 enrichment than *Brevundimonas* BAL450 (total 39 genes; 1.3% of genome). The responsiveness of
400 *Polaribacter* BAL334 is in line with findings from the marine environment that flavobacteria have
401 a pronounced ability to utilize organic matter produced during periods of phytoplankton blooms (31,
402 32). Moreover, it is noteworthy that the majority of significantly differentially expressed genes in
403 *Polaribacter* BAL334 were observed in treatments with DOM from exponential phase
404 dinoflagellates, whereas for *Brevundimonas* BAL450 most significantly expressed genes were found
405 with stationary phase DOM. The latter observation was consistent with the observation that BAL450
406 reached higher bacterial abundances when incubated with stationary phase DOM. These findings
407 imply that bacterial populations have diverged in their adaptation to utilize DOM produced and
408 released by dinoflagellates during active growth compared to stationary phase. Our findings are
409 encouraging for the future exploration of ecologically relevant patterns of how different bacterial
410 taxa respond to and/or transform DOM produced by different phytoplankton species, and in relation
411 to the physiological status of the phytoplankton as it differs across bloom development phases.

412 There were pronounced differences in which transporter genes the two bacteria expressed. For
413 example, *Brevundimonas* BAL450 uniquely expressed a number of *Na⁺ H⁺ antiporters* (involved
414 in pH and/or salinity adaptation (33)) and secretion system transporters (involved in adhesion, e.g.
415 to algal cells (34)). Curiously, one of the most highly expressed genes in *Polaribacter* BAL334 was
416 a transporter for phosphonate (also expressed in *Brevundimonas* BAL450 but at lower levels).

417 Phosphonate is an organic form of phosphorus which can be used as a sole source of phosphorus by
418 some microorganisms, allowing them higher fitness under phosphorus limiting conditions (35-37).
419 It is estimated that phosphonate constitutes a large fraction (5-25%) of the dissolved organic
420 phosphorus (DOP) in the oceans (38). During phosphate depletion in phytoplankton blooms, ABC-
421 type phosphonate transporters proteins typically increase in abundance in some bacterial taxa (32).
422 Genes involved in phosphonate utilization are thus candidates to act as sensors for phosphate status
423 in marine environments, complementing genes involved in phosphate utilization (e.g. phosphate
424 membrane transporters and alkaline phosphatase) (39).

425 In the two model bacteria studied here, we found that the expression of *Ton and Tol transport*
426 *systems* were dominant in both transcript abundance and in number of expressed genes. This class
427 of transporters is found in the outer membrane of gram-negative bacteria and is involved in the
428 uptake of a broad set of macromolecules, such as siderophores for iron, vitamin B₁₂, nickel
429 complexes and poly- or oligomeric carbohydrates (40). Since DOM produced by phytoplankton can
430 be rich in polysaccharides amenable to utilization by bacteria (41), expression of transporters for
431 this type of compounds can be expected. It is particularly intriguing that we found so many different
432 genes involved in the Ton and Tol systems expressed, as this is consistent with the uptake of not just
433 a few preferred molecules, but the simultaneous uptake of a wide array of compounds exuded by
434 phytoplankton. The characterization of sets of transporters in greater detail thus has the potential to
435 provide deeper understanding of bacterial DOM metabolism along the progression of phytoplankton
436 blooms.

437 Interestingly, *Polaribacter* BAL334 also had higher relative expression of genes involved in the
438 subsystems *Fatty acid metabolism cluster* and *Polyhydroxybutyrate metabolism* (**Fig. 3; Table S1**).
439 PHB is produced by diverse bacteria in response to physiological stress or carbon excess (42). The
440 carbon stored in PHB can be used later as an energy source or as anabolic building blocks in times
441 of low availability of DOM (42). Interestingly, another flavobacterium, *Dokdonia* sp. MED134, has

earlier been seen to express genes for a different carbon storage molecule – glycogen – under conditions where two strains of proteobacteria expressed genes for PHB synthesis (43). *Polaribacter* BAL334 encodes both pathways and the carbon storage strategy hence appears to not only reflect phylogenetic relatedness but also temporary ecological factors such as the composition of available substrates.

Even at the highest level of the SEED hierarchy, two categories stood out being exclusively expressed by just one of the two isolates: *Motility and Chemotaxis* in *Brevundimonas* BAL450 and *Photosynthesis* in *Polaribacter* BAL334 (**Fig. 2B**). Genome analysis showed that *Polaribacter* BAL334 lacks the full complement of flagellar motility (it uses gliding motility for movement) (44). In contrast, the flagellar motility system is present in *Brevundimonas* BAL450 where it was highly expressed. At the top level SEED (**Fig. 2B**), motility and chemotaxis gene expression was particularly high in the treatments with dinoflagellate DOM as compared to controls. This could potentially relate to the cells sensing increased nutrient availability in the DOM treatments or that the DOM provided energy that fueled increased swimming (45). The *Photosynthesis* genes expressed by *Polaribacter* BAL334 are those encoding the energy-generating proteorhodopsin photosystem, which *Brevundimonas* BAL450 lacks. Strikingly, the two differentially abundant genes in proteorhodopsin synthesis were expressed at higher relative values in the controls than in the treatments with DOM. Proteorhodopsin is known to help bacterial cells to survive during starvation (46) or even contribute to growth at low DOC availability (47). This suggests that *Polaribacter* BAL334 used the proteorhodopsin for surviving in the no-substrate controls, and when provided with DOM in the treated samples preferentially utilized DOM rather than photoheterotrophy for its energy demand.

The relative expression responses we observed between bacterial species, and between DOM from two different dinoflagellate growth phases, helped identify genes potentially involved in shaping the ecology of heterotrophic marine microbes. Our findings emphasize the potential usefulness of

467 experimental approaches for identifying indicator genes for different environmental conditions that
468 are informative of mechanisms underlying important dynamics of carbon and nutrient fluxes in
469 marine ecosystems. Our findings have implications for metatranscriptome analysis, since sequences
470 taken from a community of phylogenetically diverse populations will likely blur signals of
471 biogeochemical relevance because of differences in functional capacity and lifestyles between
472 species. Separation of taxa based on taxonomic annotation before analysis of differentially abundant
473 genes has been proposed to resolve this issue (48). Attaining sufficient precision in the identification
474 of species – for example through the use of metagenomic assembled genomes (MAGs) (49, 50) –
475 would allow the use of the genetic responses of particular species of marine bacteria sampled in
476 natural environments as “living sensors”.

477

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491

492 **Author contributions**

493 JP, MAM and CMGO conceived the study. CMGO and CK performed the experiment. Laboratory
494 work was done by CMGO and CK with help from JET. DL and CMGO did the bioinformatics.
495 CMGO, DL, MAM and JP wrote the article.

496

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624

625 **Supplementary table legends**

626 **Table S1.** Expression of genes in the SEED category *Fatty Acids, Lipids, and Isoprenoids* in
627 *Polaribacter* BAL334 and *Brevundimonas* BAL450. Both the SEED subcategory and subsystem are
628 shown together with the expression abundance of the gene in counts per million (CPM) and standard
629 deviation (CPM) in treatments and control. Note that a gene can occur in more than one SEED
630 category.

631 **Table S2.** Significantly differentially abundant genes in *Polaribacter* BAL334 and *Brevundimonas*
632 BAL450 with SEED categories and SEED subsystems. Expressed genes were determined to be
633 significantly more (denoted “up”) or less (denoted “down”) abundant in the transcriptomes of the
634 DOM-enriched samples compared to controls, using EdgeR statistical analysis. Contrast indicates
635 whether the gene occurs only in DOM from exponential phase (i.e. Exp-Con) or only in the
636 stationary phase (i.e. Sta-Con) or is in both (i.e. shared genes). Note that a gene can occur in more
637 than one SEED category.

638

Prorocentrum minimum growth

Figure 1

Figure 2

Figure 4

SEED category

Figure 5

Figure 6