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The transcriptional response of prokaryotes to phytoplankton-derived dissolved organic matter in seawater

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Summary

To better understand the functional responses in prokaryotes to dissolved organic matter (DOM), we compared the transcriptional pattern of natural prokaryotic communities grown in continuous cultures on seawater amended with phytoplankton-derived DOM. Metatranscriptomic reads were classified taxonomically (by genomic binning) and functionally (using Kyoto Encyclopedia of Genes and Genomes), and the relative gene expression of individual taxa (genome bins) was compared with the total community response. In the first experiment comparing seawater and seawater amended with diatom-derived DOM, metatranscriptomes revealed pronounced differences in pathways involved in carbohydrate and lipid metabolism. In the second experiment comparing seawater amended with cyanobacteria- and diatom-derived DOM, metatranscriptomes had similar functional profiles, likely reflecting more similar DOM regimes in this experimental setup. Among the five most abundant taxa investigated in more detail, two featured pronounced differences in transcript abundance between treatments suggesting that they were specialized in the use of only one of the two DOM regimes. However, these two taxa were less involved in carbohydrate metabolism than others and had few genes that were significantly regulated in response to the DOM source. Our results indicate that both substrate composition and the competitive interplay of community members were decisive for the functional response of a microbial system.

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Introduction

Prokaryotes are main processors of marine dissolved organic matter (DOM), much of which is derived from phytoplankton exudates (Baines and Pace, 1991; Benner, 2002). Taxonomically distinct phytoplankton species produce DOM with different chemical composition (Biersmith and Benner, 1998; Sarmento *et al*., 2013). Thus, the question of whether prokaryotic community composition changes in response to phytoplankton DOM of different origins has been investigated. Outcomes of these studies have differed, however, with some indicating pronounced compositional responses and others only weak effects (Van Hannen *et al*., 1999; Lekunberri *et al*., 2012; Sarmento and Gasol, 2012; Landa *et al*., 2013b). One explanation for these contrasting results could be that both specialist and generalist strategies exist among prokaryotic taxa (Langenheder *et al*., 2005; Gómez-Consarnau *et al*., 2012; Dinasquet *et al*., 2013). Depending on the degree of selectivity for specific organic substrates, specialist or generalist behaviour could dominate a community.

Recently developed methodologies such as metatranscriptomics allow insights into the genes that underlie the functional response of microbes, and therefore can provide a new perspective on the taxonomic response (Jansson *et al*., 2012). Early metatranscriptomic studies found microbial taxa, transporters, enzymes and metabolic pathways that specifically responded to the addition of phytoplankton and vascular plant-derived DOM (Poretsky *et al*., 2010) or marine high-molecular-weight DOM (McCarren *et al*., 2010; Shi *et al*., 2012). Other metatranscriptomic studies described the temporal succession of taxa and expressed functions in response to phytoplankton blooms (Rinta-Kanto *et al*., 2012; Teeling *et al*., 2012).

Most previous experimental studies are based on single additions of DOM, thereby exploring short-term responses to pulsed amendments or successional changes associated with shifts in the quality or quantity of the remaining DOM over time. In contrast, little attention has been paid to how DOM supplied under steady-state conditions affects bacterial taxonomy, gene composition or ecological strategies. Continuous cultures allow temporally sustained inputs of DOM, more closely mimicking the gradual release of phytoplankton exudates.

However, they select for organisms with growth rates that exceed cell loss due to the dilution. Continuous cultures typically have high reproducibility (Hoskisson and Hobbs, 2005) and limit succession of the bacterial community thereby providing a better opportunity to observe direct interactions between taxa. In combination with high-throughput metatranscriptomics, continuous cultures are well suited to untangle the complex processes occurring during DOM degradation by focusing on a subset of organisms and interactions while simultaneously mimicking natural conditions.

The objective of the present study was to investigate concurrently the response at the taxon and the community level of a natural prokaryotic assemblage supplied with different DOM sources under steady state conditions. We focused on the gene expression pattern of a few abundant taxa and characterized their transcriptional behaviour in comparison to the functional patterns in the bulk community, with particular interest in specialist versus generalist behaviour of individual taxa.

Results

Inorganic nutrients, DOM and bulk prokaryotic abundances in continuous cultures

We compared the transcriptional response of prokaryotic marine communities to different DOM sources in two independent continuous culturing experiments (Exp_SwDi and Exp_CyDi). During the 15-day experimental period, nutrient conditions remained stable in the continuous cultures (Supporting Information Table S1). After an initial growth phase of 24 h in Exp_SwDi, prokaryotic abundances stabilized in three out of four continuous cultures. Cell abundances decreased in one of the control cultures, and this replicate was therefore discarded from further analyses. Prokaryotic abundances were $3.9 \pm 0.9 \times 10^6$ cells ml^{-1} and $4.6 \pm 0.7 \times 10^6$ cells ml^{-1} in the two replicates of the diatom treatment (mean ± SD, $n = 15$) and $2.6 \pm 0.8 \times 10^6$ cells ml^{-1} in the control (Landa *et al.*, 2013a). In Exp_CyDi, prokaryotic abundances remained stable at 5.1 ± 0.6 and $4.2 \pm 0.7 \times 10^6$ cells ml^{-1} (mean ± SD, $n = 5$) in the diatom and cyano treatment respectively until day 8. A decrease in prokaryotic abundances was then detectable until day 11, followed by a second stable phase at $7.5 \pm 0.5 \times 10^5$ cells ml^{-1} in the diatom treatment and at $5.2 \pm 0.7 \times 10^5$ cells ml^{-1} in the cyano treatment. We observed an increase in heterotrophic nanoflagellates that coincided with the decrease in prokaryotic abundance (Landa *et al.*, 2013b). Chemical characteristics of the two DOM sources were published previously (Landa *et al.*, 2013b) and indicate differences in the relative concentrations of total hydrolyzable neutral sugars (Supporting Information Fig. S1A) and amino acids (Supporting Information Fig. S1B). Ultra-high resolution mass spectrometry (FT-ICR MS) points furthermore to compositional differences of other DOM molecules including i.e. lipids (Supporting Information Fig. S1C). Samples for metatranscriptomic analyses were collected at the end of the experiments on day 15.

Characterization of the most active community members

In total, we obtained > 17 million potential prokaryotic protein encoding reads for downstream analysis, of which 71% could further be assigned to a function (Table 1). The most commonly sampled taxonomic class was *Gammaproteobacteria*, representing 64% of the total

Table 1. Summary of sequenced transcripts.

Technical replicates	# taxonomic assignments	# functional assignments	% functional assignments	# taxa	# genes	mRNA copies ml^{-1}	mRNA copies cell^{-1}
Exp_SwDi: control1a	1 383 864	1 058 036	76%	1650	4241	1.5×10^9	620
Exp_SwDi: diatom1a	4 602 232	3 091 085	67%	1822	6095	1.4×10^9	316
Exp_SwDi: diatom1b	1 942 427	1 311 716	68%	1783	5251	1.9×10^9	432
Exp_SwDi: diatom2a	2 560 303	1 601 515	63%	1796	5234	1.2×10^9	230
Exp_SwDi: diatom2b	1 385 098	988 978	71%	1740	4817	1.5×10^9	290
Exp_CyDi: cyano1a	734 343	548 611	75%	1699	4790	1.4×10^8	442
Exp_CyDi: cyano1b	809 356	615 961	76%	1700	4806	1.8×10^8	557
Exp_CyDi: cyano2a	553 250	414 514	75%	1626	4362	1.4×10^8	551
Exp_CyDi: diatom1a	740 665	531 561	72%	1731	4804	1.9×10^8	516
Exp_CyDi: diatom1b	1 336 344	913 605	68%	1774	5272	1.6×10^8	450
Exp_CyDi: diatom2b	1 176 335	841 247	72%	1758	5170	1.6×10^8	426
Total	17 224 217	11 502 315	71%	1868	7876		

control = aged seawater control; diatom = addition of DOM derived from *Phaeodactylum tricornutum*; cyano = addition of DOM derived from *Synechococcus* sp. WH7803; 1,2 = suffixes for the biologic replicates; a,b = suffixes for the technical replicates; mRNA-tax = number of mRNA reads with prokaryotic taxonomic assignment; mRNA-fun = number of prokaryotic reads with assigned function (KEGG orthologs); %fun = per cent of prokaryotic reads assigned to a function; taxa = number of genome bins; genes = number of KEGGorthologs.

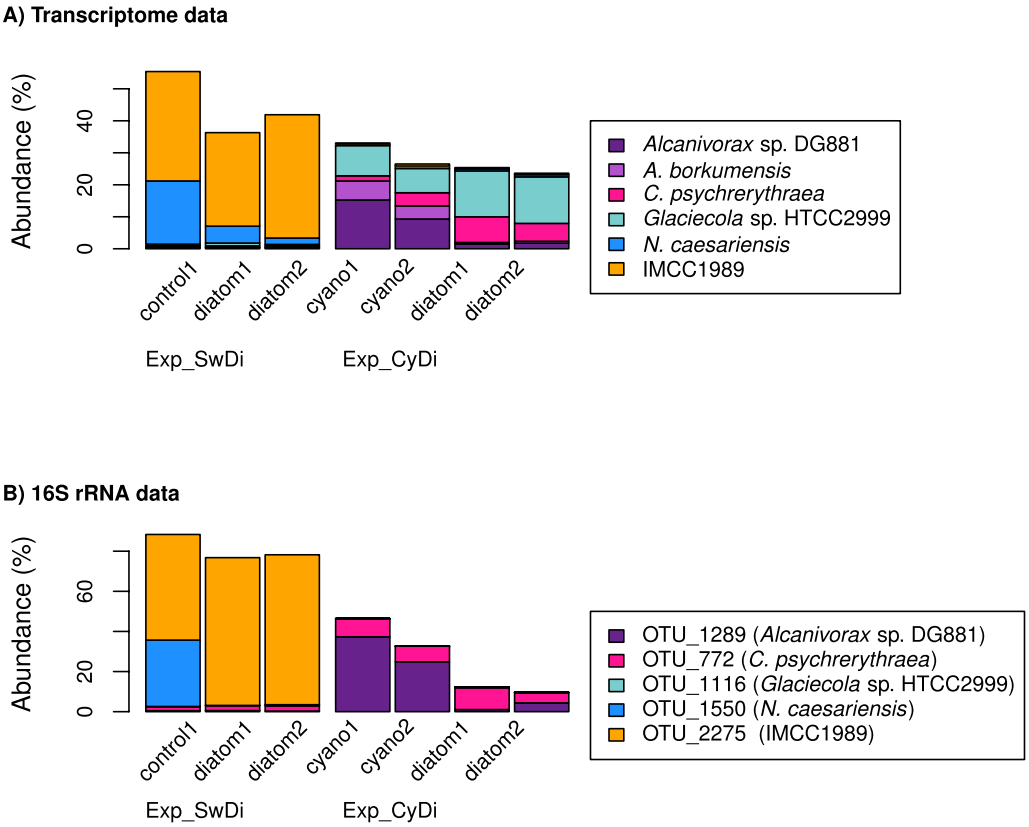


Fig. 1. Relative abundance of mRNA reads assigned to the key taxa (≥ 5% of metatranscriptome in at least one of the cultures) (A), and relative abundance of the corresponding OTUs as determined by 454 pyrosequencing of the 16S rRNA gene (Landa *et al*., 2013a,b) (B). IMCC1989: *gammaproteobacterium* IMCC1989.

metatranscriptome, followed by *Alphaproteobacteria*, *Flavobacteria* and *Betaproteobacteria* with 20%, 3% and 2% respectively. We could detect transcriptomes assigned to five taxa that accounted for > 5% of the metatranscriptome in at least one of the continuous cultures, hereafter referred to as key taxa: IMCC1989, *Neptuniibacter caesariensis*, *Colwellia psychrerythraea*, *Glaciecola* sp. HTCC2999 and *Alcanivorax* sp. These taxa are all *Gammaproteobacteria* and together made up between 24% and 55% of the total metatranscriptomes (Fig. 1A). Four of the taxa could be assigned with 93–100% similarity to 16S rRNA gene operational taxonomic units (OTUs) obtained from 454 pyrosequencing of the communities from the same experiment (Table 2), and had similar relative abundance in both 16S rRNA libraries and transcriptomes (Fig. 1). We also identified an OTU sequence with satisfactory 16S rRNA gene sequence similarity (96%; Table 2) to *Glaciecola* sp. HTCC2999, but it was detected only at low abundance in the pyrosequencing data sets (Fig. 1B). The transcriptome of *Alcanivorax borkumensis* was pooled with *Alcanivorax* sp. DG881 in all downstream analyses (see Methods).

Table 2. BLASTN query output of 16S rRNA gene OTU representative sequences from pyrosequencing analysis Landa *et al*., (2013a,b) versus 16S rRNA sequences of reference genomes.

strain	OTU	% id	length	# mismatches	gap size	e-value
gammaproteobacterium IMCC1989	2275	97.6	250	6	0	4^{-128}
Neptuniibacter caesariensis	1550	92.6	349	26	0	2^{-139}
Alcanivorax borkumensis	–	–	–	–	–	–
Alcanivorax sp. DG881	1289	99.8	398	0	1	0
Glaciecola sp. HTCC2999	1116	95.6	362	14	2	3^{-166}
Colwellia psychrerythraea	3504	97.0	394	6	5	0

OTU = OTU identifier for 16S rRNA; %id = per cent identity; length = alignment length; # mismatches = number of mismatches.

In Exp_SwDi, gammaproteobacterium IMCC1989 and *N. caesariensis* were the two key taxa. IMCC1989 displayed similar transcript abundances in both DOM regimes, while *N. caesariensis* had much lower transcript abundance in the diatom treatment compared with the control treatment. In Exp_CyDi, *Glaciecola* sp. *HTCC2999, C. psychrerythraea* and *Alcanivorax* sp. were the key taxa. *Glaciecola* sp. HTCC2999 and *C. psychrerythraea* had similar transcript abundances in the diatom and the cyano treatment; in contrast, *Alcanivorax* showed pronounced abundance differences between treatments and was the most abundant taxon in the cyano treatment (Fig. 1). Within each experiment, the taxonomic distribution of absolute mRNA copy numbers was similar to that of the relative values because mRNA copies ml^{-1} were roughly equal in all treatments (Table 1).

A dendrogram based on functional assignments of transcripts was constructed to check whether treatment or taxonomic assignment correlated better with expression patterns for the key taxa. Within an experiment, transcripts assigned to the same reference genome bin were more similar to each other than they were to different genome bins, and the influence of treatment was subordinate to taxonomy (Fig. 2). However, if considering treatments from both experiments, reads from all key taxa except *Glaciecola* sp. HTCC2999 separated into two distinct clusters for each taxon (Fig. 2).

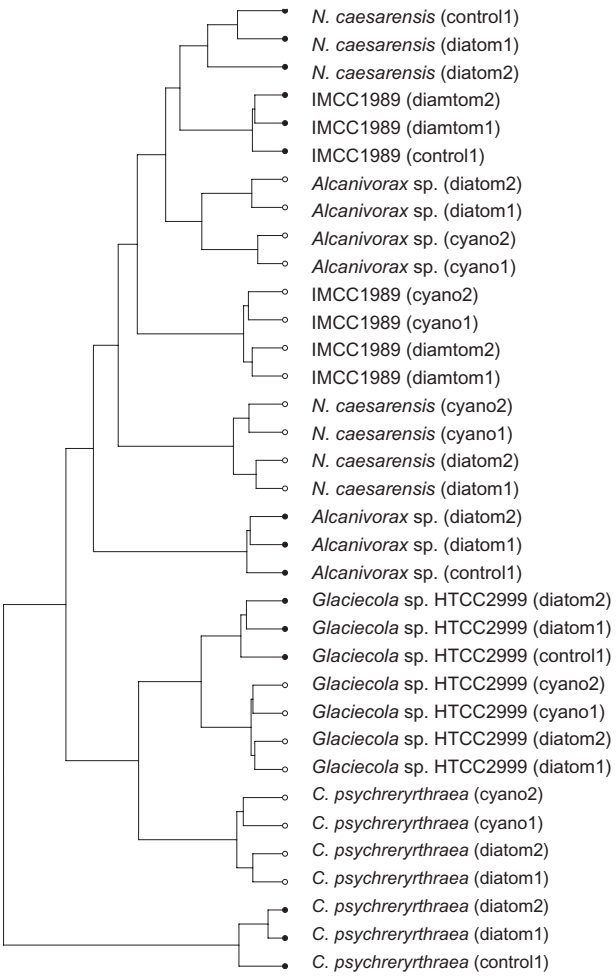


Fig. 2. Dendrogram displaying similarities between the genome bins of the key taxa in different cultures. The clustering was based on normalized count data after functional binning (Bray–Curtis dissimilarity, Ward's linkage). Transcriptome bins from Exp_SwDi are labelled with filled circles, whereas those from Exp_CyDi are labelled with open circles. IMCC1989: *gammaproteobacterium* IMCC1989; abbreviation for cultures: see Table 3.

Functional changes of the bulk community

Pathways were assigned to a variety of Kyoto Encyclopedia of Genes and Genomes (KEGG) categories, and we used the path-index (PI) values (see Methods) to investigate the change of a pathway's importance between treatments. We designed the calculation so that positive PIs indicate an enrichment in the diatom treatment (Exp_SwDi and Exp_CyDi), while negative PIs indicate an enrichment in the control (Exp_SwDi) or cyano treatment (Exp_CyDi). Higher absolute values of PI indicate that the treatment had a larger effect on the expression of a pathway. Among the pathways considered here (KEGG classes 'metabolism' and transporter proteins in pathways 'ko02010' and 'ko02060'), we found more pathways with PIs close to zero in Exp_CyDi compared with Exp_SwDi, including a narrower spread and a lower mean absolute value of the PI in Exp_CyDi (variance = 0.14; mean absolute = 0.22) than in Exp_SwDi (variance = 0.32, mean absolute = 0.44).

For subsequent evaluations, we considered only those pathways that contained at least one significantly different gene (Fig. 3). In Exp_SwDi, all pathways involved in carbohydrate and lipid metabolism had PIs that indicated enrichment in the diatom treatment (Fig. 3A). Pathways related to nucleotide metabolism were also diatom biased, but mostly in a range closer to zero. In contrast, pathways related to amino acid metabolism were spread over a wide range of PIs (Fig. 3A). Pathways encoding the metabolism of vitamins and cofactors were mostly enriched in the control, often with PIs in the outer range (Fig. 3A). Transporters were usually assigned to PIs in the outer range, although which treatment was enriched depended on the substrate, e.g. cobalt/nickel transport was enriched in the control treatment, while iron and sugar transport was enriched in the diatom treatment (Fig. 3A).

In Exp_CyDi, pathways involved in nucleotide metabolism, lipid metabolism and amino acid metabolism generally had PIs close to zero (Fig. 3B). In contrast, pathways involved in carbohydrate metabolism were distributed

Fig. 3. A list of KEGG pathways sorted according to their path indices (PI) and organized by functional category. *P*-values from differential gene expression analysis of the total metatranscriptome (all transcripts) or key taxa (IMCC1989: *gammaproteobacterium* IMCC1989) are highlighted in red if significantly increased in the control and in green if significantly increased in the diatom treatment (significance level: $P < 0.1$). Indicator genes for the key taxa are indicated in black. If modules subordinate to KEGG pathways were considered, the KEGG pathway name is given before the module. Only pathways with at least one significantly different gene in selected categories are shown, along with their indicator genes and significantly different genes. Each row in the *P*-value and indicator gene columns represents an individual gene. Different row heights serve only to facilitate reading of pathway information. The complete list of pathways and genes is provided in Supporting Information Table S2.

over a wider range of positive and negative PIs (Fig. 3B). As in Exp_SwDi, transporters had strongly positive or negative PIs, with the direction of the trend depending on the specific substrate. Here, for example, sulfate transport was enriched in the cyano treatment, whereas sulfur in the diatom treatment was possibly incorporated in its organic form (sulfonate or taurine) as indicated by an enhanced sulfonate/nitrite/taurine transport signal.

Functional differences in key taxa

Neptuniibacter caesariensis (Exp_SwDi) and *Alcanivorax* sp. (Exp_CyDi) featured pronounced fluctuations in relative activity and abundance within an experiment based on mRNA and 16S rRNA abundances (Fig. 1). This was in contrast to IMCC1989, *Glaciecola* sp. HTCC2999 and *C. psychrerythraea*, which had similar relative abundances between treatments within an experiment. To better understand the fluctuation patterns in mRNA abundance of *N. caesariensis* and *Alcanivorax* sp., we compared individual gene expression patterns between treatments using taxon-specific differential expression analyses, as well as the function of indicator genes compared with those of the other taxa.

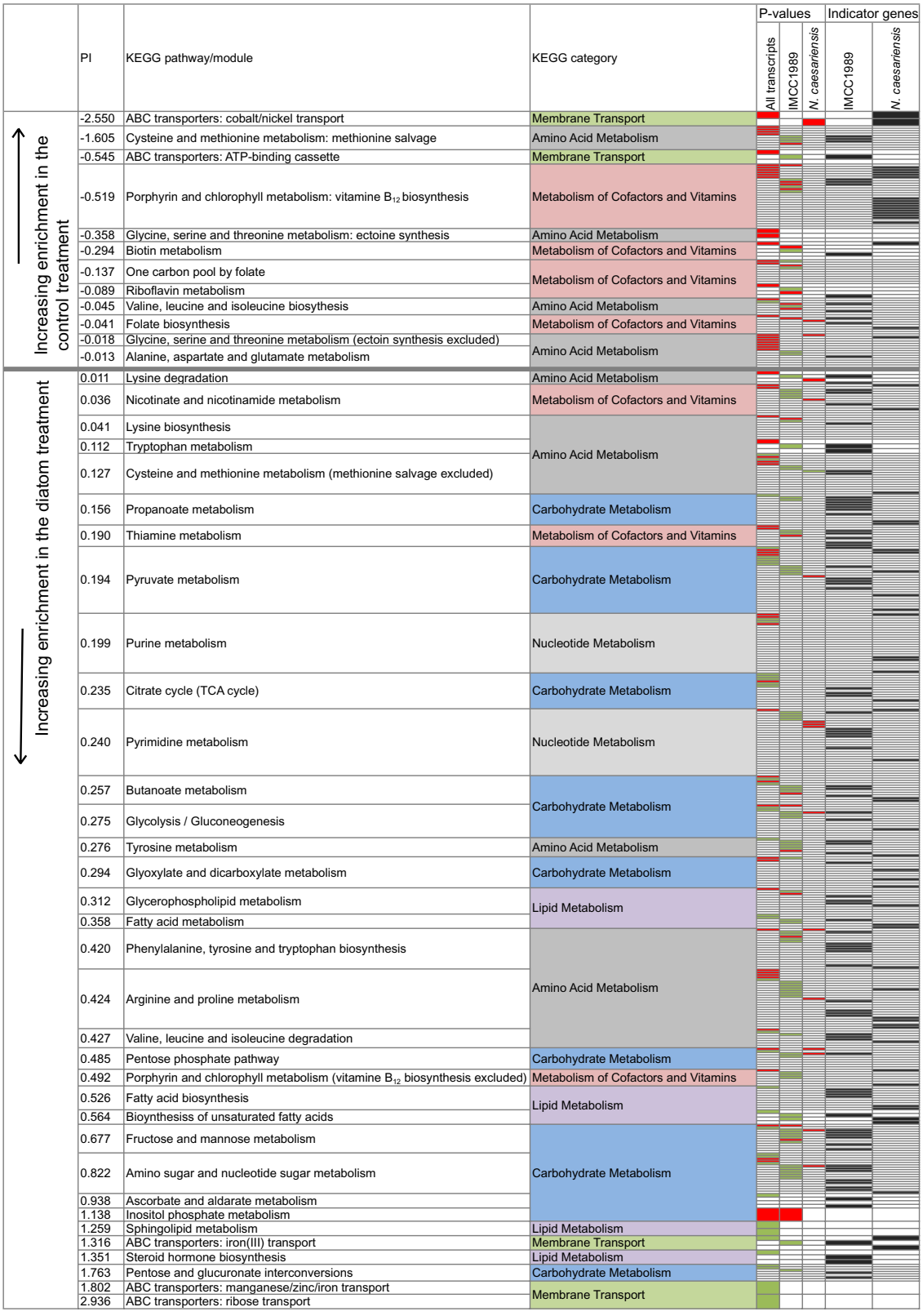
In the *N. caesariensis* transcriptome bin in Exp_SwDi, only a few of the metabolic and transporter genes were significantly different between treatments (34 genes; Supporting Information Table S2), indicating only subtle metabolic regulation in this taxon. This was in contrast to IMCC1989, which was characterized by minor abundance differences but for which many genes showed differential regulation (188 genes, Supporting Information Table S2). Among the few genes that were significantly changed in the *N. caesariensis* transcriptome, some were in pathways that were relevant to the observed community level differences i.e. genes in the pathways for amino sugar and nucleotide sugar metabolism and for cobalt transport, the central atom of vitamin B_{12}. However, *N. caesariensis* did not feature significant changes of genes within the pathway itself. *Neptuniibacter caesariensis* also had many indicator genes in the pathways involving cobalt transport and vitamin B_{12} biosynthesis (Fig. 3A). IMCC1989 expressed indicator genes within the vitamin B_{12} biosynthesis pathway as well, and several of these genes were significantly different between treatments (Fig. 3A). IMCC1989 expressed many other indicator genes in the carbohydrate metabolism pathways, but this was not the case for *N. caesariensis* (Fig. 3A).

In Exp_CyDi, both *Alcanivorax* sp. and *C. psychrerythraea* had limited intra-specific expression differences; only 11 metabolic and transporter genes were differentially expressed in *Alcanivorax* sp. and only four in *C. psychrerythraea* (Supporting Information Table S2). By contrast, *Glaciecola* sp. HTCC 2999 had 37 genes with significantly different relative expression (Supporting Information Table S2). Several indicator genes for *Alcanivorax* sp. were found in pathways associated with sulfate transport. For both pathways, there were large absolute PI values and significantly enriched genes in the cyano treatment. In contrast, there were few cases of indicator genes of *Alcanivorax* sp. in pathways associated with carbohydrate metabolism (Fig. 3B). Instead, carbohydrate metabolism pathways were dominated by indicator genes of the *Glaciecola* sp. HTCC2999 transcriptome (Fig. 3B).

Discussion

Characterizing functional changes in the bulk community

In Exp_SwDi we compared the response of prokaryotes to aged seawater (control) and seawater amended with diatom-derived DOM, the latter of which contained slightly higher concentrations of DOM (13 μM) that was likely rich in labile compounds in the form of amino acids and carbohydrates (Landa *et al*., 2013a,b). The culture with diatom-derived DOM showed an enrichment of transcripts for both carbohydrate and lipid metabolism. The enhanced expression of genes for lipid metabolism was found in both anabolic and catabolic pathways, and therefore might reflect degradation of lipids present in the diatom DOM (Landa *et al*., 2013b) and/or a general increase in membrane synthesis. Previous studies have similarly shown that the addition of high molecular weight DOM from surface seawater to microbial communities induced the enrichment of genes involved in carbohydrate and lipid metabolism (McCarren *et al*., 2010). The increased expression of enzymes involved in vitamin synthesis in the control treatment (Fig. 3A) likely reflects a lower vitamin concentration compared with the diatom



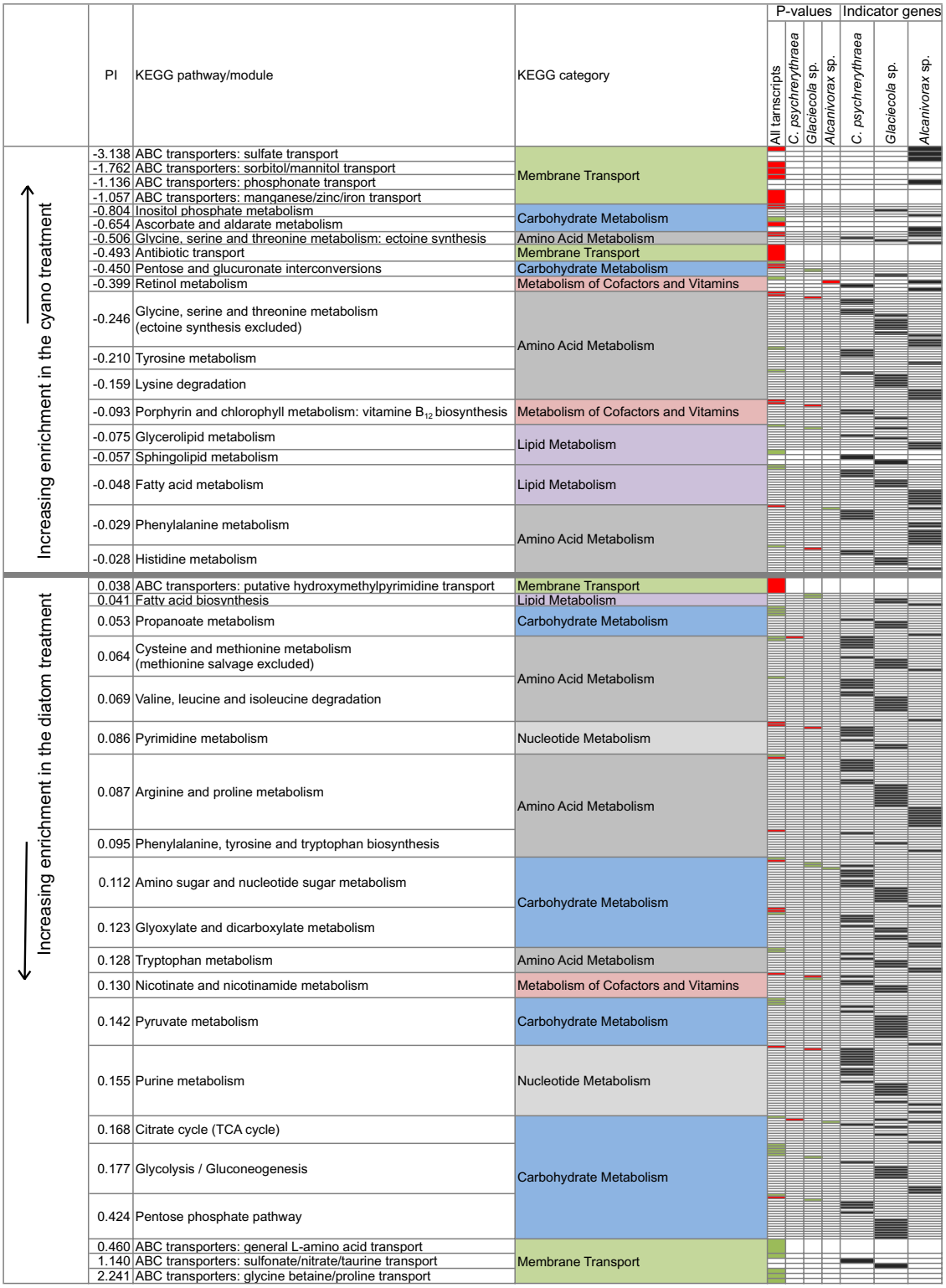


Fig. 3. *cont.*

treatment, since vitamins added to the phytoplankton culture growth medium would have been carried over into the diatom DOM preparation. The maximum carry-over (i.e. if phytoplankton cells did not use any of the added B_{12}) would result in 57 pmol l^{-1} vitamin B_{12} in the continuous culture medium, which is in the same range as vitamin B_{12} concentrations in the ocean that reach up to 30 pmol l^{-1} (Sañudo-Wilhelmy *et al*., 2012). Pathways in the nucleotide metabolism class were slightly increased in the diatom treatment; this is likely due to greater prokaryotic growth. A less distinct pattern was observed for amino acid metabolism, and this may be due to the central role of amino acids in prokaryotic metabolism: if amino acids are scarce in a treatment, bacteria may express genes for amino acid biosynthesis, and if plentiful, they may induce the expression of catabolic enzymes.

In Exp_CyDi, we compared the prokaryotic response to two different phytoplankton-derived DOM sources that both contained labile compounds but had several distinct chemical differences, including differences in the composition of neutral sugars and amino acids (Supporting Information Fig. S1). Differences in amino acid composition of phytoplankton exudates that could be linked to differences in bacterial community composition have been observed previously (Sarmento *et al*., 2013). Further, some marine bacteria are auxotrophic for specific amino acids (Tripp *et al*., 2009) and therefore may respond strongly to different amino acid composition in the exudates.

We found little correspondence between the analysed amino acids or neutral sugars (Supporting Information Fig. S1) and the enrichment or depletion of specific pathways that could be involved in their processing in Exp_CyDi (Fig. 3B). However, neutral sugars and amino acids made up between 2–6% of the carbon in DOM in the media (Landa *et al*., 2013b), leaving open the question of whether overall chemical changes correlated with transcriptional patterns. Nonetheless, none of the major KEGG metabolic categories displayed any clear trend in response to the treatments (Fig. 3B) even though some individual amino acid and carbohydrate pathways were clearly increased in the cyano or diatom treatment. Moreover, PIs calculated for Exp_CyDi tended to be close to zero. These results suggest that the communities responded functionally in a similar way to the addition of diatom-derived and cyanobacteria-derived DOM, and thus that the labile components of these DOM sources were of similar quality. Our results accordingly imply that two phytoplankton derived DOM sources (Exp_CyDi) triggered more similar transcriptional responses by prokaryotes than did phytoplankton derived DOM compared with unamended seawater (control, Exp_SwDi). Similarly, a study focusing on taxonomic data showed that different phytoplankton blooms induced the development of similar prokaryotic communities that were nonetheless quite distinct from the unamended control community (Lekunberri *et al*., 2012).

Characterizing functional changes in the key taxa

The majority of prokaryotes growing in our continuous cultures were *Gammaproteobacteria* (Landa *et al*., 2013a,b) which, based on transcript abundance, were also the most active community members. Members of *Alphaproteobacteria* and *Bacteroidetes* are often detected in association with the occurrence of marine phytoplankton blooms (Lekunberri *et al*., 2012; Sarmento and Gasol, 2012; Teeling *et al*., 2012) but were not abundant here. The continuous cultures possibly favoured *Gammaproteobacteria* by selecting for faster growing organisms. Indeed, a recent study demonstrated that *Alphaproteobacteria* and *Bacteroidetes*, although actively incorporating phytoplankton exudates, proliferated less rapidly than *Gammaproteobacteria* (Sarmento and Gasol, 2012). For the key taxa in both experiments, transcript and 16S rRNA abundances matched well for four of the five dominant *Gammaproteobacteria* taxa. However, *Glaciecola* sp. HTCC2999 appeared disproportionately active given the low relative abundance of 16S rRNA (Fig. 1).

Transcriptome bins of the key taxa clustered within an experiment according to their taxonomic origin and only on a subordinate level according to the treatment (Fig. 2). Consequently, at least within each experiment, the taxonomic identity was more predictive of gene expression patterns than the DOM regime. Pronounced functional differences in the transcripts that were binned to the same taxon but derived from two different experiments (Fig. 2) suggest different gene regulation by these taxa across experiments. However, this observation could also indicate that the transcripts were derived from genetically different populations present in the inocula used for the two independent experiments, despite the fact that they binned to the same reference genome. Nonetheless, population-level variation does not affect our analyses because direct comparisons were only made within the same experiment.

The abundance of *Gammaproteobacteria* as a group remained unchanged between treatments (Landa *et al*., 2013a), but some individual members of this class exhibited abundance and expression changes in response to the treatments (Fig. 1). We were especially interested in why *N. caesariensis* and *Alcanivorax* sp. had higher fluctuations in relative transcript abundance in response to different DOM sources. The observed changes in transcript abundance, which correlated well with abundance changes of corresponding 16S rRNA genes (Fig. 1) indi-

cate that these taxa were less successful in adapting to the supply of different DOM sources than the other three key taxa. We therefore interpreted enhanced abundance fluctuations in *N. caesariensis* and *Alcanivorax* sp. as specialist behaviour. On the other hand, the other taxa apparently dealt well with the different DOM sources and featured more generalist behaviour. Both *N. caesariensis* and *Alcanivorax* sp. were only slightly involved in carbohydrate metabolism pathways (Fig. 3). Both reference strains have been shown to grow only on a restricted range of carbon sources, including organic acids for *N. caesariensis* (Arahal *et al*., 2007) and aliphatic hydrocarbons and fatty acids for *Alcanivorax* sp. (Yakimov *et al*., 1998; Green *et al*., 2004). Limited ability to metabolize the carbohydrates that were introduced with the phytoplankton exudates (Landa *et al*., 2013a) may have caused other species to out-compete *N. caesariensis*, particularly in the diatom treatment of Exp_SwDi. Furthermore, the ability of *N. caesariensis* to incorporate cobalt and synthesize vitamin B_{12} (Fig. 3A) was likely advantageous for this taxon in the vitamin-depleted control. However, in contrast to the bulk community, this taxon did not decrease its relative expression of B_{12} synthesis genes in the diatom treatment (Fig. 3A). Possibly *N. caesariensis* is adapted to marine environments with B_{12} limitation and is not capable of regulating its biosynthesis on the transcriptional level. However, this trait is energetically expensive (Martens *et al*., 2002), and may have been disadvantageous for *N. caesariensis* when expressed in the vitamin-enriched phytoplankton treatment, potentially contributing to reduced competitiveness. Only some prokaryotes possess genes for vitamin B_{12} synthesis, while most other organisms depend on uptake of B_{12} released by these prokaryotes (Roth *et al*., 1996). Our results imply that vitamin B_{12} levels are not only critical for eukaryotic phytoplankton but also impact the ecology of co-occurring bacterial communities (Koch *et al*., 2012; Sañudo-Wilhelmy *et al*., 2012).

Taxa of the genus *Alcanivorax* are usually a minor component of unpolluted marine waters, but they can increase to high abundances in oil-contaminated systems (Kasai *et al*., 2002). Isolates from this genus have also been shown to grow on aliphatic hydrocarbons and on fatty acids (Yakimov *et al*., 1998; Green *et al*., 2004). We could detect several indicator genes of *Alcanivorax* sp. involved in alkane degradation (K00529, K05297, K00496; Supporting Information Table S2), as well as a trans-enoyl-CoA isomerase (K13239) that catalyses conformational changes in long-chain unsaturated aliphatic hydrocarbons. None of these genes were significantly enriched between the two treatments, possibly due to the fact that aliphatic hydrocarbons, such as those from the fatty acid component of membrane lipids, were supplied by both phytoplankton exudates. *Alcanivorax sp.* DG881, the genome to which most of the *Alcanivorax* sp. transcripts were assigned (Fig. 1), was isolated from a dinoflagellate culture (Green *et al*., 2004). We speculate that this bacterium associates with phytoplankton blooms in nature, and that the lipids and fatty acids detected in the DOM sources used in our experiments (Supporting Information Fig. S1C) may be a key substrate in this niche.

According to indicator gene analysis, *Alcanivorax* sp. contributed specifically to sulfate uptake (Fig. 3B) and assimilation (gene K00958, Supporting Information Table S2). Expression of genes in these transport processes was strongly increased in the cyano treatment according to the large PI absolute values. The uptake of inorganic sulfate from seawater may increase when organic sulfur compounds, such as taurine, are scarce. According to transporter protein expression, organic sulfur compounds may have been abundant in the diatom treatment and could have originated from diatom polysaccharides that contain substantial amounts of sulfate residues (Alldredge, 1999; Urbani *et al*., 2012).

Neptuniibacter caesariensis and *Alcanivorax* sp. have different life history strategies but share some commonalities that may have been responsible for the specialist metabolisms observed here (Fig. 1). First, both taxa had few genes that were significantly regulated in response to the treatments (Fig. 3B), suggesting little versatility in the usage of the different DOM sources. Second, neither taxon was heavily involved in metabolizing carbohydrates, in agreement with previous characterizations of the reference strains as specialists with a restricted range of growth substrates that do not include sugars (Yakimov *et al*., 1998; Arahal *et al*., 2007). Eukaryotic phytoplankton are generally thought to produce a higher fraction of polysaccharides in their exudates than cyanobacteria (Myklestad, 2000). A reduced adaptive capacity to use different substrates and the restricted use of carbohydrates may therefore be reasons why these taxa were ineffective at utilizing diatom-derived DOM.

The use of continuous cultures as opposed to the addition of DOM at a single time point can provide high reproducibility and stability (Hoskisson and Hobbs, 2005), which in the study facilitated a comparison of the transcriptional response to different DOM sources. By excluding responses to the successive degradation of the DOM source, observed functional and taxonomic differences between transcriptomes must be linked to a specific quantity and quality of the DOM.

We conclude from our data that phytoplankton DOM plays key roles in aquatic systems both as variable sources of labile substrates and indirectly by shifting the interplay among prokaryotes present in the indigenous community. We confirm results from earlier studies that some taxa have a specialist behaviour and feature a more

pronounced response in terms of transcript and 16S rRNA abundance changes than others to DOM source shifts. We identified reduced adaptive capacity and restricted use of carbohydrates as possible reasons for such specialist behaviour. Identification of scenarios that induce a preponderance of specialist versus generalist taxa should provide a valuable perspective on discrepancies concerning the degree of community changes induced by different phytoplankton DOM sources.

Experimental procedures

Continuous cultures and sample collection

Two independent continuous culture experiments were performed, referred to as Exp_SwDi and Exp_CyDi. Each of the experiments consisted of two treatments that differed in DOM characteristics of the growth media. In Exp_SwDi, seawater aged for 6 weeks [control, 74 μM dissolved organic carbon (DOC)] was compared with the same aged seawater amended with DOM from a diatom culture (*Phaeodactylum tricornutum*) (seawater + diatom DOM, 87 μM DOC). In Exp_CyDi, seawater aged for 6 weeks (63 μM DOC) was amended with DOM from the *cyanobacterium Synechococcus* sp. WH7803 (seawater + cyanobacterial DOM, 73 μM DOC) or with DOM from the *P. tricornutum* (seawater + diatom DOM, 75 μM DOC). These phytoplankton strains were obtained from a culture collection at the Observatoire Océanologique de Banyuls.

The continuous culture system and the seawater sampling are described in detail in Landa and colleagues (2013a,b). Seawater for the growth media and the prokaryotic community that served as inoculum was collected in the northwest Mediterranean Sea (42°28.3′N, 03°15.5′E) at 3 m depths. For the growth media, roughly 300 l were collected in September 2009 for Exp_SwDi and in January 2010 for Exp_CyDi and each stored in a polycarbonate (PC) carboy in the dark at room temperature for 6 weeks to allow degradation of most of the labile DOM. The phytoplankton cultures were grown in low DOM (15 μM DOC) artificial seawater amended with nutrients according to the Guillard's F/2 medium, and DOM was harvested in early stationary phase by collecting the filtrate after sequential filtration through a combusted GF/F filter (90 mm diameter, Whatman, Kent, UK) and an acid-washed, ultrapure water rinsed 0.2 μm PC filter (90 mm diameter, Nuclepore filter Whatman, Kent, UK). Concentrations of DOC in the diatom exudate were 151 μM and 138 μM for Exp_SwDi and Exp_CyDi respectively, and the DOC concentration in the cyanobacterial exudate was 78 μM. In Exp_SwDi, diatom-derived DOM was mixed with seawater to obtain a final concentration of 13 μM C phytoplankton-derived DOM in the diatom treatment. In Exp_CyDi, each of the DOM sources was mixed with seawater to obtain final concentrations of 15 μM C phytoplankton-derived DOM in the cyano and the diatom treatments. Nitrogen (NO_3^- in Exp_SwDi, and NO_3^- and NH_4^+ in Exp_CyDi) and phosphorus (PO_4^{3-}) were added in excess to the initial cultures as well as to the growth media to assure that organic carbon was the limiting factor for prokaryotic growth (Supporting Information Table S1).

The prokaryotic cultures were prepared by inoculating the growth media with a natural prokaryotic community (< 0.8 μm filtrate) in a 10:1 ratio (final volume of cultures 6 l). The prokaryotic communities were collected on the starting day of the experiment (14 November 2009 for Exp_SwDi and 1 March 2010 for Exp_CyDi) in the NW Mediterranean Sea as described above. Prokaryotic growth was maintained in batch mode until stationary phase was reached, and cultures were then switched to continuous mode for the duration of the incubation. For both experiments, duplicate cultures were established for each treatment. However, in one of the control cultures from Exp_SwDi the abundance of prokaryotes decreased over time, so this replicate was eliminated from further analyses. The dilution rate in Exp_SwDi was 0.27 d^{-1} and in Exp_CyDi was 0.28 d^{-1}, and cultures were maintained over five and six generations respectively.

Sampling for metatranscriptomic analysis

After 15 days, samples for metatranscriptomic analysis were siphoned from the incubation vessels into a combusted glass bottle. Roughly 1 l (in duplicate) was immediately filtered through nitrocellulose membranes (GSWP, Millipore, Billerica, MA, USA, 0.2 μm pore-size, 25 mm diameter) using a peristaltic pump. The filtration time did not exceed 10 min. From each continuous culture, two filters (serving as technical replicates) were collected for RNA extraction (Table 3). These filters were stored in RNAlater (Life Technologies, Carlsbad, CA, USA) at –80°C. Further subsamples for DNA extraction (100 ml) were filtered onto polyethersulfone filters (SUPOR, Pall Cooperation, Port Washington, NY, USA; 0.2 μm pore-size, 13 mm diameter) and stored in lysis buffer (Qiagen, Valencia, CA, USA) at –80°C. Deoxyribonucleic acid was extracted with the Qiagen kit following the manufacturer's instructions. The extracted DNA was later used to produce antisense rRNA to subtract rRNA from the metatranscriptome samples as described below.

RNA processing and sequencing

Ribonucleic acid was extracted as previously described (Gifford *et al*., 2011). Briefly, frozen filters for RNA extraction were vortexed with PowerSoil beads (MOBIO, Carlsbad, CA, USA) in 2 ml RLT buffer (Qiagen, Valencia, CA, USA) after the addition of artificial mRNAs as internal standards as described elsewhere (Satinsky *et al*., 2013), and then were processed following the instructions of the RNAeasy Kit (Qiagen, Valencia, CA, USA). Deoxyribonucleic acid was removed from the extracted RNA using the turboDNAse (Applied Biosystems, Austin, TX, USA). To further remove rRNA, we used a protocol based on hybridization with antisense rRNA amplified from DNA of the same samples (Stewart *et al*., 2010). Analyses of the DNA-free RNA extract (Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA) indicated the presence of eukaryotic rRNA in Exp_CyDi but not in Exp_SwDi; we therefore subtracted eukaryotic rRNA from the samples of Exp_CyDi in addition to bacterial rRNA using the same antisense rRNA method. The remaining RNA was amplified using the Message Amp II-Bacteria kit (Life Technologies, Grand Island, USA) and finally reverse transcribed into complementary DNA (cDNA) using

Table 3. Overview of the experimental setup.

experiment	treatment	DOC input (μM)	DOC medium (μM)	culture	technical replicates
Exp_SwDi	control	74 ± 3 (*n* = 4)	74 ± 2 (*n* = 10)	control1	control 1a
					control 1b*
				control2	control 2a*
					control 2b*
	diatom	87 ± 7 (*n* = 4)	75 ± 2 (*n* = 10)	diatom1	diatom1a
					diatom1b
				diatom2	diatom2a
					diatom2b
Exp_CyDi	cyano	73 ± 3 (*n* = 11)	75 ± 2 (*n* = 12)	cyano1	cyano1a
					cyano1b
				cyano2	cyano2a
					cyano2b*
	diatom	75 ± 2 (*n* = 11)	75 ± 2 (*n* = 12)	diatom1	diatom1a
					diatom1b
				diatom2	diatom2a*
					diatom2b

control = aged seawater control; diatom = addition of DOM derived from *Phaeodactylum tricornutum*; cyano = addition of DOM derived from *Synechococcus* sp. WH 7803; 1,2 = suffixes for the cultures (biological replicates); a,b = suffixes for the technical replicates; technical replicates marked with asterisks indicate those that were lost during the experimental procedure or excluded from data analyses for reasons mentioned in the manuscript. Concentrations of dissolved organic carbon (DOC) were measured by (Landa *et al*., 2013a,b) in the input medium and the culture (mean values ± SD are given, *n* = number of time points for which chemical analyses were carried out).

SuperScript III First-Strand Synthesis System for RT-PCR (Invitrogen, Carlsbad, CA, USA) and NEBNext mRNA Second Strand Synthesis Module (New England Biolabs, Ipswich, MA, USA). Transcripts were sheared to approximately 220 bp, and paired-end sequencing was performed at the Duke Institute for Genome Sciences and Policy (Illumina GAIIx platform). Sequences are available via the CAMERA database (http://camera.calit2.net) under accession number CAM_P_0001134.

Bioinformatics analysis

The SHE-RA software package (Rodrigue *et al*., 2010) was used to join paired-end reads, and low-quality pairings were removed using 0.5 as the cut-off value. Further quality trimming was carried out with the SeqTrim pipeline (Falgueras *et al*., 2010) with the parameters set as follows: minimal read length = 100; maximal frequency of Ns = 0.18; minimal nucleotide quality for a called base = 20; window width = 10. Non-mRNA reads (rRNAs, internal standards) were detected using Usearch [with the UBLAST nucleotide algorithm set to standard parameters (Edgar, 2010)] by searching against a sequence database containing reference rRNA gene sequences from taxa in major phylogenetic lineages (Gifford *et al*., 2013). All reads with a bitscore ≤ 50 were categorized as putative mRNA sequences and kept for further analyses.

The potential mRNA reads were compared in a local BLASTX search (Altschul *et al*., 1997) against the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/) RefSeq database (version 49) and top hits with a bitscore ≥ 40 were kept for information on taxonomic affiliation, with the exception of hits (defined by GI numbers) that were only detected once in the total data set. Transcripts were sorted into genome bins, referred to as 'taxa', based on the closest hit to a bacterial reference genome.

Reads that were affiliated with genomes of prokaryotic origin (Bacteria, Archaea) were functionally assigned to orthologs of the Kyoto Encyclopedia of Genes and Genomes (KEGG, Kanehisa *et al*., 2007). For this purpose, a local BLASTX search against the full KO-database provided by KOBAS 2.0 (http://kobas.cbi.pku.edu.cn/home.do; download: February 2012) was performed using the Usearch UBLAST protein algorithm while adjusting the settings to keep the 10 first hits and reject up to 1000 sequences. The best hit that could be assigned to a KEGG ortholog was kept for further statistical analyses. Hits were selected according to cut-off criteria tested previously (Mou *et al*., 2008): e-value ≤ 0.1, identity ≥ 40%, and alignment coverage ≥ 23 amino acids. In the remainder of the manuscript, KEGG orthologs are referred to as 'genes'.

We found evidence for possible contamination of two technical replicates with genomic DNA (Supporting Information Fig. S2 and S3) and the reads from these two replicates were therefore excluded from further analyses (Table 3).

Characterization of the most active community members

Transcripts assigned to a taxonomic bin that represented ≥ 5% of the total metatranscriptome in a culture were used for more detailed analyses. It was assumed that these reads were derived from the most abundant and active taxa in the experiments, and they are referred to as 'key taxa'.

In order to estimate the cell abundance of these key taxa and verify their presence in the samples, we used 16S rRNA from a previously analysed pyrosequencing data set derived from the same experiments (Landa *et al*., 2013a,b). 16S rRNA gene sequences for the key taxa were identified by a BLASTN search of all available OTU representative sequences against 16S rRNA gene sequences from reference genomes of the key taxa. None of the OTUs in the pyrosequencing library had the 16S rRNA gene of *Alcanivorax borkumensis* as the first hit, even though a number of metatranscriptome reads binned to this genome. Instead, all *Alcanivorax*-like OTU representative sequences affiliated with *Alcanivorax sp.* DG881 as the first hit or an

equally good hit. Consequently, although the transcriptome binning indicated the presence of two *Alcanivorax* taxa (*Alcanivorax borkumensis* and *Alcanivorax sp.* DG881), 16S rRNA genes point to the presence of only one population more closely related to *Alcanivorax* sp. DG881. We therefore pooled the mRNA reads binning to *Alcanivorax borkumensis* and *Alcanivorax sp.* DG881 into a single genome bin (*Alcanivorax sp.*) for all downstream analyses. To check the consistency of binning, we compared the transcriptome composition of the key taxa after normalizing to the same number of reads by subsampling. Transcripts were subsequently clustered using Bray–Curtis dissimilarity and Ward's linkage.

Differential expression analysis

Differential expression analyses were performed using the DESeq package (Anders and Huber, 2010). Reads from technical replicates (Table 3) obtained from one culture were pooled, resulting in two biological replicates (cultures) per treatment for statistical analyses. The biological replicate for the control was lost (Table 3), and this sample was therefore processed using the DESeq protocol for comparisons without full replication. We increased the detection power by removing the least abundant genes (the lowest 40% quantile, about 0.1% of the considered reads) before performing comparisons between the treatments (Anders and Huber, 2010). Comparisons between the experiments were not carried out because the different starting populations would have made taxonomic binning inconsistent across studies. Differential expression analyses between treatments were performed for: (i) the total metatranscriptome, which characterized changes in bulk function of the community and (ii) key taxa, in order to learn more about their individual functional responses to different DOM sources.

Characterizing changes in pathway expression using KEGG and the path index

To account for the annotation uncertainties arising when a gene falls into multiple KEGG pathways, we developed a path-index parameter (PI) that is based on the results of the differential expression analyses of the complete data set and information in the KEGG database. First, the probability that a gene was actively involved in a certain pathway (*pko*) was estimated by the reciprocal value for the number of pathways this gene is assigned to (*np*) according to the KEGG database (information downloaded: 09/27/2012) as:

$$pko = \frac{1}{np}$$

We excluded all pathways involved in the classes 'Human Diseases' and 'Organismal Systems' for the calculation of *pko* because the prokaryotic organisms in our samples are not expected to express pathways in these two classes. Next, we identified modules within pathways that specifically differed between treatments by marking genes that were enriched or depleted. In the case of transporters, genes were grouped into modules according to their substrate specificity. For simplicity we refer in the text to both, pathways or subordinate modules as pathways.

The PI for pathway *j* was then estimated as:

$$PI_j = \sum_{i=1}^{n} \frac{pko_{ij} \times \log_2(fc_{ij})}{n_j}$$

where *n* is the number of genes detected in pathway *j* of the compared treatments, and fc_{ij} is the fold change of gene *i* in pathway *j* between the treatments as given in the output of the differential expression analysis. The *fc* for genes with no hit in one treatment was set to a minimal estimation based on depth of sampling of the metatranscriptome, if the normalized abundance (given by the base mean parameter in the DESeq program) for this gene in the other treatment was > 1. Otherwise, we set $fc = 1$. The log transformation of the *fc* provided a more balanced spread in the distribution of the resulting PIs by avoiding extreme values and giving less weight to low abundance genes with high *fc*. The PI therefore provides a quantitative estimate of the relative transcriptional increase or decrease in a group of genes organized in pathways or functionally similar groups. In the subsequent presentation of PIs we concentrate on pathways involved in the classes metabolism and transporter proteins [ABC transporters (path:ko02010) and phosphotransferase system (PTS) (path:ko02060)], since genes involved these functions are likely most relevant for understanding transcriptional responses to DOM.

Indicator genes

Indicator genes describe those genes that are predominantly expressed by an individual taxon (> 50% of the transcripts coding for this gene). For the calculation, we considered all genes assigned to the key taxa in order to focus on expression differences among them. We determined indicator genes for each key taxon in the experiment they were abundant in, as described previously (Gifford *et al.*, 2013), with the modification that expression outliers were not preselected. We instead multiplied the indicator value with the frequency of transcripts from gene *j* in taxon *i* within culture *c*, as suggested in the original indicator value method (Dufrene and Legendre, 1997). Because of considerably different read numbers per taxon in different cultures, taxon/culture-specific weighting factors were incorporated. Thus, the indicator value of gene *j* in the transcriptome of taxon *i* would be:

$$IV_{ji} = \left(\sum_{c=1}^{n} \left(\frac{a_{ci}}{a_i} \times gp_{cj} \right) \right) \times \frac{x_{ij}}{\sum_{i=1}^{s} x_{ji}} \times 100$$

where a_i is the total number of reads in taxon *i*, a_{ci} is the number of reads of taxon *i* in culture *c*, gp_{cj} is the presence (i) or absence (0) of gene *j* in culture *c*, *n* is the total number of replicates considered. x_{ij} is the expression frequency of gene *j* in the transcriptome of taxon *i*, and *s* is the number of selected taxa in culture *c*. We designated gene *j* as an indicator gene for taxon *i* if $IV_{ij} > 50$ and ≥ 10 hits to this gene were detected.

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Supporting information

Additional supporting information is available in the online version of the article at the publisher's web-site.

Fig. S1. Chemical characteristics of the input media and the two DOM sources published previously by (Landa *et al*., 2013b). Composition (mol%) of total hydrolysable neutral sugars (A) and amino acids (B) in the diatom DOM medium and the cyanobacterial DOM medium. van Krevelen diagram based on analysis by FT-ICR MS showing ratios of H/C and O/C for compounds specific of diatom DOM and cyanobacterial DOM in the PPL-extracted fraction (C). For the two phytoplankton DOM sources, the PPL recovery efficiency was 8% of total DOC. Areas tentatively assigned as carboxylic-rich alicyclic molecules (CRAM), proteins, lipids, condensed hydrocarbons and amino sugars are also shown.

Fig. S2. Dendrograms (Bray–Curtis dissimilarity, Ward's linkage) based on a normalized count data set after taxonomic (A) and functional (B) binning of the sequence data. Filled circles indicate technical replicates from Exp_SwDi and open circles indicate technical replicates from Exp_CyDi.

Fig. S3. In order to display differences in expression patterns between the outliers cyano2b/diatom1a (Exp_CyDi, Supporting Information Fig. S1) and the remaining technical replicates, this heatmap highlights genes that were significantly differently expressed between outliers and the remaining technical replicates. The heatmap illustrates that differences between the outliers, and the remaining samples were mostly due to genes that were highly enriched in the outlier samples, but low in abundance or undetected in the remaining samples. This pattern might be caused by contamination with genomic DNA. DNA contamination would also explain why samples cyano2b/diatom1a (Exp_CyDi) clustered as outliers in the functional but not in the taxonomic cluster analysis (Supporting Information Fig. S1). Significance was tested following the differential expression analysis in the DESeq package (Anders and Huber, 2010) and a significance level of $P < 0.1$ with the *P*-value adjusted for multiple testing using the Benjamini–Hochberg procedure. The displayed subset of genes was not scaled for the heatmap but originates from the total data set, which was normalized by sample according to the DESeq package. SwDi or CyDi before the technical replicate designates its origin from Exp_SwDi and Exp_CyDi.

Table S1. Concentrations of dissolved organic carbon (DOC), nitrate + nitrite (NO_3^-+NO_2^-) and phosphate (PO_4^{3-}) in the input media and the biological replicates during the experimental period. In Exp_CyDi, nitrogen was also added in the form of NH_4^+ to the input medium (20 μM final concentration). Mean values ± SD are given. For each time point, the average value of the two replicate + DOM cultures was used. *n* = number of time points for which chemical analyses were carried out.

Table S2. List of all genes with KEGG number (ko) that were analysed for differential expression in pathways in the metabolism class, and in transporter protein categories [ABC transporters (path:ko02010), phosphotransferase system (PTS) (path:ko02060)]. Kegg_colour gives the colour codes used in the colour-mapping tool provided by KEGG (http://www.genome.jp/kegg/tool/map_pathway2.html) in order to

map significantly (intense colour) or non-significantly (light colour) enriched (green) and decreased (red) genes (with respect to the diatom treatment in both experiments). Genes with pko = 1 are indicated with a blue border, with the pko value representing the probability of a gene contributing to a certain pathway. The colour coding was used for grouping of genes after visual inspection in order to use these groups of genes as units for PI calculation. The units of genes used for PI calculation are designated in the column pathway/module. The fold change indicates an enrichment or decrease of a gene in the diatom treatment of both experiments, if values are >0 or <0 respectively. The terms class, category, pathway and description indicate the KEGG BRITE nomenclature. *P*-values adjusted with the Benjamini–Hochberg procedure were obtained for each gene from differential expression analysis for all genes or transcriptomes of the key taxa. Genes are highlighted in red if significantly decreased and green if significantly increased in the diatom treatment in Exp_SwDi and Exp_CyDi (significance level: padj <0.1). Indicator genes (IV >50, more than 10 reads) are displayed in yellow (*gammaproteobacterium* IMCC1989), blue (*N. caesariensis*), violet (*Alcanivorax sp.*), pink (*C. psychrerythraea*) or turquois (*Glaciecola sp*. HTCC2999).