Linking the exometabolome of selected organisms of the *Roseobacter* group to marine dissolved organic matter - a microbiological perspective

Dissertation

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Gerrit Mathias Wienhausen

geboren am 25. September 1986 in Braunschweig

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Erstgutachter:	**Prof. Dr. Meinhard Simon**
Zweitgutachter:	**Prof. Dr. Hans-Peter Grossart**
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Summary

Marine dissolved organic matter (DOM), one of the largest global carbon reservoirs, comprises an enigmatic blend of organic molecules. Heterotrophic bacteria play a key role in DOM ramification, releasing a diverse bouquet of exometabolites. Thus on the one hand they facilitate the global DOM complexity and likewise drive myriads of unidentified interactions in the marine microbial metabolic network. Vitamins in particular, known as indispensable cofactors for the vitality of all living organisms, are an essential element in the metabolic network that control the prosperity of marine microbial communities.

By reason that phytoplankton organisms constitute the foundation for the majority of marine DOM, exudates of *Thalassiosira pseudonana* and its uptake and transformation by selected *Roseobacter* group members were assessed, applying the untargeted ultrahigh resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). The diverse blend of hundreds of complex molecules released by the diatom revealed unique utilization and diversification patterns when provided to individual roseobacters. Our results suggest that individual bacteria evolved specialized traits for different organic compounds and thus engaged different ecological roles in the global carbon turnover.

In order to understand causations for the vast non-assignable blend of detected bacterial exometabolites, such were correlated to intracellular processes, determined via transcriptome. One third of all exometabolites were positively correlated to cellular mechanisms, exhibiting similarities in their molecular characteristics dependent on the intracellular function correlated to. Thus our findings indicate that the bacterial exometabolome is in parts a consequence of the intracellular metabolome and provides an insight into the genesis of bacterial derived DOM in nature.

The detection of vast numbers of exometabolites released by *Phaeobacter inhibens* and *Dinoroseobacter shibae* by FT-ICR-MS raised the question of their extracellular function. Thus molecular formulas were screened against a strain specific metabolite database, predicted by its genome. Those identified were further fragmented by MS/MS if applicable. Combined, both strains revealed 43 known metabolites and metabolite precursors, encompassing variable vitamins, amino acids and secondary metabolites. The wealth of extracellularly identified metabolites indicates the relevance of bacterial exometabolome in the marine microbial

metabolite network, stimulating growth and allocating lacking genetic capabilities in the microbial world.

Vitamins and vitamin precursors represent a noticeable large part of identified metabolites in both exometabolomes. Thus, promising vitamin metabolites (vitamins B12 and B1, α-ribazole, 4-amino-5-hydroxymethyl-2-methylpyrimidine, 4-methyl-5-(β-hydroxyethyl)thiazole) were amended to natural microbial communities sampled from different Pacific Ocean provinces. The allocation of vitamin metabolites, except vitamin B1, induced a significant increase in the bacterial community activity, determined by leucine incorporation. This result highlights the role of vitamin traffic in nature and indicates an unanticipated relevance of vitamin precursor for the remodelling or allocation of lacking genetic traits in the microbial world.

In conclusion, my dissertation demonstrates the relevance and uniqueness of how individual *Roseobacter* species diversify algae derived organic matter in nature. Exometabolomes of *Roseobacter* group members appear as consequence of intracellular metabolism and possess a marketplace of metabolites, supposedly driving the marine microbial metabolic network. Furthermore, this study provides novel insights on the marine microbial vitamin traffic and the relevance of its availability, especially of vitamin precursor, for the microbial community.

Zusammenfassung

Marines gelöstes organisches Material, einer der größten globalen Kohlenstoffspeicher, verbirgt eine rätselhafte Mischung an komplexen organischen Molekülen. Vervielfältigungsprozesse des organischen Materials werden maßgeblich durch heterotrophe Bakterien umgesetzt. Das facettenreiche Gemisch an bakteriellen Exometaboliten ist erstaunlich, nicht nur weil es im großen Maßstab zur Diversifizierung des globalen gelösten organischen Materials beiträgt, sondern auch weil es mutmaßlich zahlreiche Interaktionen im marinen mikrobiellen Metabolitnetzwerk forciert. Insbesondere Vitamine, bekannt als unverzichtbare Kofaktoren für die Vitalität aller lebenden Organsimen, sind ein essentieller Bestandteil des Metabolitnetzwerks und kontrollieren den Erfolg mariner mikrobiologischer Gemeinschaften.

Da Phytoplanktonorganismen die ursprüngliche Grundlage für den Großteil des gelösten organischen Materials im Meer darstellen, wurde das Exsudat von *Thalassiosira pseudonana* und dessen Aufnahme sowie Umwandlung durch einzelne Vertreter der *Roseobacter* Gruppe mittels eines ultra-hochauflösenden Fourier-transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) bestimmt. Die facettenreiche Mischung hunderter komplexer Moleküle, ausgeschieden von Diatomeen, wurde von einzelnen *Roseobacter* Vertretern unterschiedlich genutzt und diversifiziert. Unsere Ergebnisse verdeutlichen, dass Bakterien spezialisierte Eigenschaften entwickelt haben, um unterschiedliches organisches Material abbauen und somit verschiedene ökologische Funktionen im globalen Kohlenstoff Kreislauf einnehmen zu können.

Um die Ursachen der hohen Anzahl an nicht zuordenbaren bakteriellen Exometaboliten besser verstehen zu können, wurden diese mit intrazellulären Prozessen korreliert, welche als Transcriptom ermittelt wurden. Ein Drittel aller Exometabolite zeigten positive Korrelationen und erwiesen Ähnlichkeiten in ihren molekularen Strukturen und Besonderheiten, wenn sie mit der gleichen intrazellulären Funktion korrelierten. Deshalb deuten unsere Forschungsergebnisse daraufhin, dass das bakterielle Exometabolome eine Konsequenz des intrazellulären Metaboloms ist und somit kann ein besseres Verständnis für die Entstehung von bakteriell umgewandeltem gelöstem organischem Material in der Natur entstehen.

Die unzähligen Exometabolite, ausgeschieden durch *Phaeobacter inhibens* bzw. *Dinoroseobacter shibae*, weisen ungeklärte extrazelluläre Funktionen auf. Um diese

Besonderheit zu verstehen, wurden die detektierten molekularen Formeln gegen eine genombasierte, stammspezifische Metabolomdatenbank abgeglichen. Identifizierte Metabolite wurden, wenn möglich, mittels MS/MS weitergehend fragmentiert. Insgesamt wurden 43 bekannte Metabolite und Metabolitvorläufer identifiziert, die verschiedensten Vitamine, Aminosäuren und Sekundärmetabolite umfassen. Die Fülle an extrazellulär identifizierten Metaboliten kennzeichnet die hohe Relevanz von bakteriellem Exometabolom im Bezug zum marinen mikrobiologischem Metabolitnetzwerk. Zum einen kann es Wachstums stimulierend wirken und zum anderen werden Metabolite bei fehlender genetischer Fähigkeit in der mikrobiellen Welt zur Verfügung gestellt.

Vitamine und deren Metabolitvorläufer repräsentieren eine auffallend große Fraktion des identifizierten Anteils beider Exometabolome. Deshalb wurden besonders vielversprechende Vitaminmetabolite (Vitamin-B12 and -B1, α-ribazole, 4-amino-5-hydroxymethyl-2-methylpyrimidine, 4-methyl-5-(β-hydroxyethyl)thiazole) zu natürlichen mikrobiellen Gemeinschaften, die aus unterschiedlichen pazifischen Ozeanprovinzen beprobt wurden, hinzugefügt. Die Bereitstellung der Vitaminmetabolite, mit Ausnahme von Vitamin-B1, induzierten einen signifikanten Anstieg in der Aktivität der bakteriellen Gemeinschaft, die mittels Leucinaufnahme bestimmt wurde. Dieses Ergebnis unterstreicht die Wichtigkeit des Vitaminaustauschs in der Natur und zeigt eine unerwartete Relevanz von Vitaminvorläufern für die Umgestaltung von Vitaminen und deren Bereitstellung aufgrund von fehlenden genetischen Eigenschaften in der mikrobiellen Welt.

Insgesamt zeigt meine Dissertation die Relevanz und Einzigartigkeit von individuellen *Roseobacter* Gruppen Vertreter in Bezug auf die Vervielfältigung von Algenexsudaten in der Natur. Die *Roseobacter*-Exometabolome treten als Konsequenz vom intrazellulären Metabolismus auf und bieten einen Marktplatz an Metaboliten, die wahrscheinlich einen Teil des marinen mikrobiellen Metabolitnetzwerks darstellen. Weiterhin gibt diese Studie einen Einblick in natürliche Prozesse des marinen mikrobiellen Vitaminaustauschs und unterstreicht die Bedeutung der Verfügbarkeit, insbesondere der Vitaminvorläufer, für die mikrobielle Gemeinschaft.

List of publications

My dissertation contains 4 manuscripts of which one is published and three are in submission. In addition, one short chapter on genetic relevance of vitamin B1 and B12 genes in the *Rhodobacteracea* family is included. My contribution of each manuscript and chapter are denoted in the following.

Publications / manuscripts:

Wienhausen, G., Noriega-Ortega, B.E., Niggemann, J., Dittmar, T., and Simon, M. (2017). The Exometabolome of Two Model Strains of the Roseobacter Group: A Marketplace of Microbial Metabolites. Front. Microbiol. 8. (1985)

> *Experimental design, sampling and parameter measurement was carried out in collaboration (**G.W.** and BN. N-O.), data was analysed by (**G.W.**) and the manuscript was written by (**G.W.** and M.S.)*.

Wienhausen, G., Noriega-Ortega, B. E., Voget, S., Niggemann, J., Dittmar, T., and Simon, M. Exometabolome diversity patterns of a *Roseobacter* model strain are linked to its transcriptome patterns.

> *Experimental design, sampling and parameter measurement was carried out in collaboration (**G.W.** and BN. N-O.), data was analysed by (**G.W.** & BN. N-O.) and the manuscript was written by (**G.W.** and M.S.)*.

Wienhausen, G., Heyerhoff, B., and Simon, M. Vitamin B1, B7 and B12 synthesis capabilities of major marine bacterial groups and circumvention of lacking genetic capabilities by metabolite allocation.

*Experimental design (**G.W.**), sampling and parameter measurement (**G.W.** and B. H.), genome screening and analysis (**G.W.**) and the manuscript was written by (**G.W.** and M.S.)*.

Wienhausen, G., Jarling, R., Niggemann, J., Dittmar, T., and Simon, M. Bacterioplankton growth in oceanic systems is controlled by vitamins B1 and B12 and biosynthetic precursors.

*Experimental design and set up aboard as well as sampling (**G.W.**), parameter measurement (**G.W** and M.S.), α-ribazole was manufactured in collaboration (**G.W.** and R.J.), data was analysed by (**G.W.**) and the manuscript was written by (**G.W.** and M.S.)*.

Noriega-Ortega, B. E., **Wienhausen, G.**, Marco Mühlenbruch, Simon, M., Dittmar, T., and Niggemann, J. Differential diversification of algal organic matter by members of the *Roseobacter* group.

*Experimental design and pre-experiments (**G.W.** and BN. N-O.), sampling (M.M.), parameter measurement (**G.W**., BN. N-O. and M.M.), data was analysed by (**G.W.**, BN. N-O. and M.M.) and the manuscript was written by (**G.W.**, BN. N-O., J.N. and M.S.)*.

Contribution to scientific conferences

Wienhausen, G., B. E. Noriega-Ortega, S. Voget, J. Niggemann, T. Dittmar and M. Simon. The exometabolome of *Phaeobacter inhibens* DSM 17395 depends on substrate source and growth stage. VAAM , March. 2015, Marburg, Germany. (poster)

Noriega-Ortega, B. E., **G. Wienhausen**, M. Simon, D. Dittmar, and J. Niggemann. Comparison of the exometabolome composition of two members of the *Roseobacter* clade, VAAM , March. 2015, Marburg, Germany. (oral presentation)

Wienhausen, G., B. E. Noriega-Ortega, S. Voget, J. Niggemann, T. Dittmar and M. Simon. Linking the exometabolome of a model organism of the *Roseobacter* clade to its transcriptome, SAME 14, Aug. 2015, Uppsala, Sweden. (oral presentation)

Noriega-Ortega, B. E., **G. Wienhausen**, M. Simon, D. Dittmar, and J. Niggemann: Exometabolome composition of two members of the *Roseobacter* clade, SAME 14, Aug. 2015, Uppsala, Sweden. (oral presentation)

Noriega-Ortega, B. E., **G. Wienhausen**, M. Simon, D. Dittmar, and J. Niggemann: Is the molecular diversity of marine dissolved organic matter already imprinted in the exometabolome of single strains? OSM2016, Feb. 2016, New Orleans, USA. (oral presentation)

Wienhausen, G., B. E. Noriega-Ortega, J. Niggemann, T. Dittmar and M. Simon. The Exometabolome of Two Model Strains of the *Roseobacter* group: A Marketplace of Microbial Metabolites. ISME-16, Aug. 2016, Montreal, Canada. (poster)

Noriega-Ortega, B. E., **G. Wienhausen**, M. Simon, D. Dittmar, and J. Niggemann *: Bacterial exometabolomes contribute to the chemodiversity of marine dissolved organic matter. ASM2017, Feb. 2017, Honolulu, USA. (oral presentation, *presenting author)

Wienhausen, G., Noriega-Ortega, B. E., Niggemann, J., T. Dittmar and M. Simon (2017). The Exometabolome of Two Model Strains of the *Roseobacter* group: A Marketplace of Microbial Metabolites. SAME14, Aug. 2017, Zagreb, Croatia. (poster)

List of abbreviation

AHL	N-Acyl-Homoserine Lactone
AdoCbl	Adenosylcobalamin
ASW	Artificial Seawater Medium
BP	Bacterial production
BQH	Black Queen Hypothesis
CCorA	Canonical-Correlation Analysis
COG	Clusters of Orthologous Groups
CRAM	Carboxyl-Rich Alicyclic Molecules
CTD	Conductivity Temperature Depth
DCAA	Dissolved Combined Amino Acids
DCNCHO	Dissolved Combined Neutral monosaccharides
DFAA	Dissolved Free Amino Acids
DFNCHO	Dissolved Free Neutral monosaccharides
DHPS	2,3-dihydroxypropane-1-sulfonate
DMB	5,6-dimethylbenzimidazole
DMSP	Dimethylsulfoniopropionate
DOC	Dissolved Organic Carbon
DOM	Dissolved Organic Matter
DON	Dissolved Organic Nitrogen
DSM	Deutsche Sammlung für Mikroorganismen
DSR	Deep Atlantic Seawater Reference
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray Ionization
EqUp	Equatorial Upwelling
FDR	False Discovery Rate
FT-ICR-MS	Fourier-Transform Ion Cyclotron Resonance Mass Spectrometry

GC-MS	Gas Chromatography-Mass Spectrometry
HET	4-methyl-5-(β-hydroxyethyl)thiazole
HMP	4-amino-5-hydroxymethyl-2-methylpyrimidine
HPLC	High Performance Liquid Chromatography
IAA	Indol 3-Acetic Acid
MB	Marine Broth
MDL	Method Detection Limit
MF	Molecular Formula
mRNA	Messenger Ribonucleic Acid
NEqPIW	North Equatorial Pacific Intermediate Water
NMR	Nuclear Magnetic Resonance
NPKM	Nucleotide activity Per Kilobase of exon model per million Mapped reads
NZCP	New Zealand Costal Province
NPF	North Pacific polar Frontal region
OD	Optical Density
PCA	Principal Component Analysis
PCoA	Principal Coordinate Analysis
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RPKM	Reads Per Kilo base per Million
SynASW	Synchronized Artificial Seawater Medium
SLDOM	Semi-Labile Dissolved Organic Matter
SPE	Solid Phase Extraction
SPSG	South Pacific Subtropical Gyre
SRDOM	Semi-Refractory Dissolved Organic Matter
TDA	Tropodithietic Acid
TIC	Total Ion Current
WT	Wild Type

1 Introduction

1.1 Dissolved organic matter – Diversified by and basis for bacterial metabolism

Marine dissolved organic matter (DOM), also referred to as marine geometabolome, consists of myriads of complex organic molecules of which the majority is yet undefined. Oceanic DOM derives primarily from marine primary producers, only a minor fraction of 0.7 – 2.4% is introduced by terrestrial riverine input (Hansell *et al*., 2009; Koch *et al*., 2005). Generally DOM concentrations are fairly low (34 -~80 µmol C kg^{-1}), but the enormous dimension of our world's oceans makes marine DOM an essential carbon storage (Hansell *et al*., 2009; Hansell and Carlson, 1998; Hopkinson *et al*., 1997; Kähler *et al*., 1997; Ogawa *et al*., 1999). In its entity, marine DOM comprises 662 Pg Carbon which is almost equivalent to atmospheric carbon storage (~750 Pg C) and contains 200 times more carbon than the entire oceanic particulate organic matter (POM, Hansell *et al*., 2009). In global marine processes, DOM is a critical factor in the microbial loop and the global dissolved organic carbon (DOC) cycle (Azam *et al*., 1983; Hedges *et al*., 1992). The paradox of marine DOM, acting as essential global carbon storage and simultaneously being the basis for heterotrophic bacterial life is well-founded in its reactivity spectrum. Marine DOM can be fractioned into labile and semi-labile (SL) DOM as well as semi-refractory (SR) and refractory DOM (Figure 1).

The pool of organic carbon with highest turnover rates of hours to days, the labile DOM, comprises compounds such as mono and polysaccharides, amino acids, polypeptides, lipids or fatty acids and a large variety of other organic compounds that are easily accessible and utilizable by marine heterotrophic bacteria. The great majority of labile DOM compounds are formed by marine primary producers that control the atmospheric carbon flux into the ocean via CO^2 fixation into organic molecules. The turnover rate of the SLDOM is highly variable, ranging from month to years, believed to be dependent by microbial community composition, macronutrient availability and other environmental factors (Carlson *et al*., 2004). Generally, the incorporation of labile and SLDOM into bacterial biomass and the resulting return of dissolved organic carbon to higher trophic levels, is described as the "microbial loop" (Azam *et al*., 1983). The fraction of SRDOM behaves similarly to that of SLDOM. Both are detected above the pycnocline, whereas SLDOM is detected above the seasonal pycnocline and SRDOM requires a permeant pycnocline. However, their contribution to the long term carbon sequestration is important (Hansell, 2013).

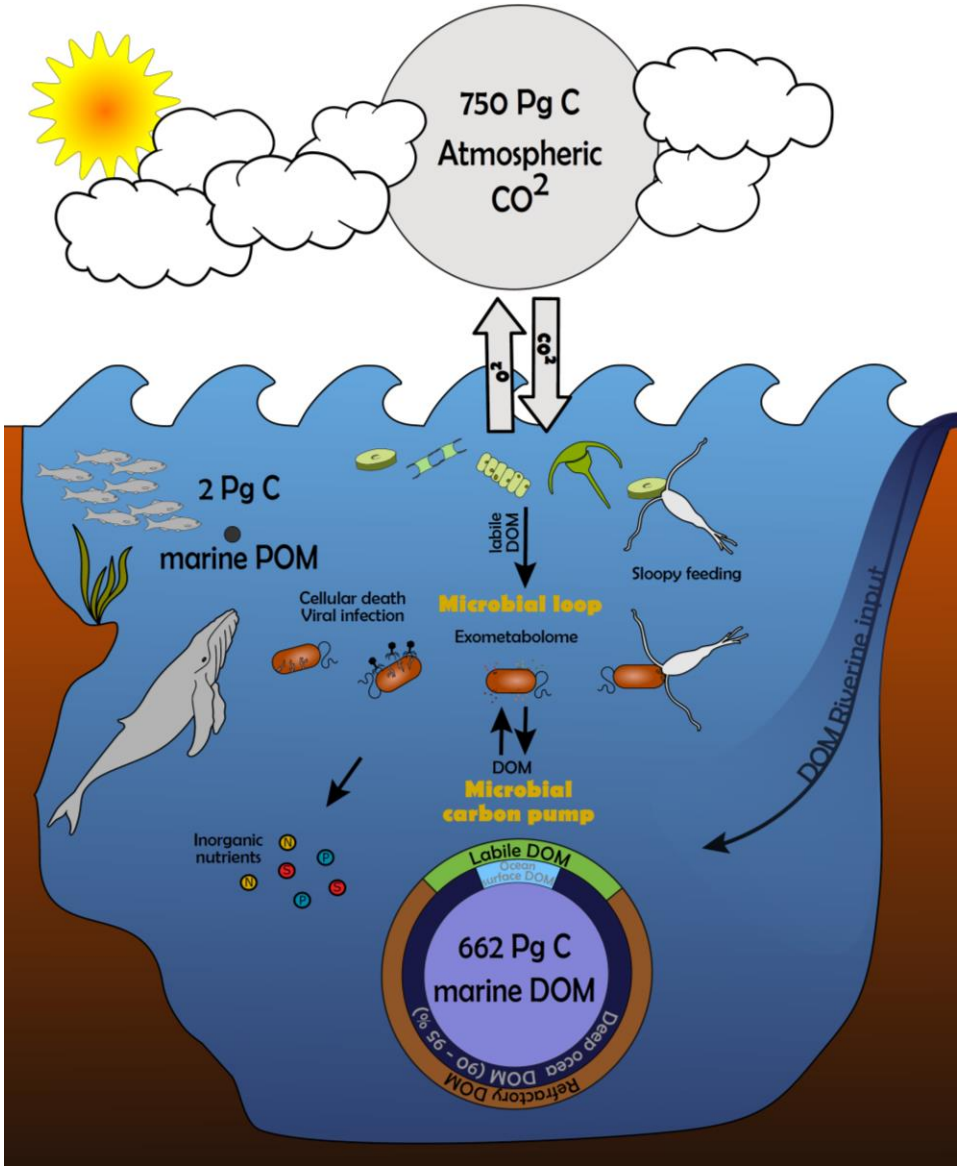

Figure 1 | Global marine dissolved organic matter (DOM) transformation processes and carbon storage bodies. This conceptual scheme illustrates the marine carbon cycle and storage and was adapted from Buchan *et al.* (2014). Atmospheric carbon (CO2) is incorporated by marine phototrophs and released by respiration. Heterotrophic bacteria diversify and transform labile DOM into biomass. Bacterial DOM is released by cell lyses (viral infection, sloppy feeding or cellular death) or exometabolome formation (excretion or diffusion). The carbon storage of atmospheric CO2, POM and DOM is given in circles. Likewise shares of labile and refractory DOM as well as surface (0-200 m) and deep (200 m-bottom) ocean DOM are presented.

Refractory organic compounds are present everywhere in the ocean and comprise the largest fraction of marine DOM (90 – 95%), largely contributing to the carbon sequestration. Of freshly produced microbial derived DOM, only a minor fraction (0.4 – 5%) escapes biotic and abiotic decomposition as refractory DOM for several thousand years after their transformation

(Osterholz *et al*., 2015). Residual refractory DOM accumulates and thereby sustains a stable equilibrium state over a geological time span (Hansell *et al*., 2009).

Currently, three hypotheses are debated to explain the causes for the global DOM persistence from microbial degradation. The environmental hypothesis discusses a scenario in which DOM persistence is caused by the limitation of essential resources in nature, such as metabolites or elements for microbial metabolism that prevent further microbial DOM degradation. The alternate intrinsic stability hypothesis assumes that complex molecules accumulate that are not further degradable by abiotic transformation and biotic metabolism. Another debated scenario, the molecular diversity hypothesis, supposes that the low concentration of individual compounds limits the uptake for microbial metabolism (Hansell and Carlson, 2014). In fact, Arrieta *et al*., (2015) already demonstrated that low DOM concentrations in the deep ocean are cause for slow microbial growth.

New technological approaches, in particular the ultra-high resolution Fourier-transform ion cyclotron resonance mass spectrometry (FT–ICR–MS) coupled to electrospray ionization (ESI), has enabled the detection of thousands of precise molecular masses in one sample of seawater. This technique has widened our understanding of the enormous chemodiversity of marine DOM and the special role heterotrophic microbes play in this context. At present we know that bacterial communities and the complex pool of DOM are intimately linked in a mutual relationship. The vast DOM diversity arises from bacterial DOM decomposition and processing, also introduced as the concept of the microbial carbon pump (Hedges, 1992; Jiao *et al*., 2010; Landa *et al*., 2016; Osterholz *et al*., 2015; Yang *et al*., 2016). In fact, recent studies demonstrated that thousands of complex molecules are excreted by single bacterial strains when grown on one labile substrate, highlighting the relevance of bacterially mediated DOM processing (Romano *et al*., 2014). In marine ecosystems, complexity and size of molecular compounds as well as its availability are decisive for microbial degradation (Kamjunke *et al*., 2016; Logue *et al*., 2016; Walker *et al*., 2016). However, even low DOM concentrations, as identified in ultra-oligotrophic environments, are sufficient for microbial growth and consequently also DOM diversification (Schwedt *et al*., 2015). On the other hand, DOM availability and composition can effect bacterial community dynamics and composition (Judd *et al*., 2006; Tada *et al*., 2017). Geometabolome and metagenome correlation studies revealed a link between molecular compound classes and even specific molecules to microbial groups (Osterholz *et al*., 2016; Sharma *et al*., 2014).

In numerous studies many scientists have analyzed the mutual linkage of the global geometabolome and marine microbes which allowed us to depict global processes (Azam *et*

al., 1983; Hedges *et al*., 1992). Yet, the linkage of the complex DOM pool and heterotrophic bacterial communities features myriads of paradoxes and uncharacterized traits. To better understand these processes we aim to identify the extent of active bacterial DOM formation and unveil cellular mechanisms leading to the formation. Further, the identification of individual molecules will give us an insight into the complexity of microbial metabolite dependencies in nature.

1.2 The *Roseobacter* group – Globally distributed and metabolically active

The *Roseobacter* group, long presumed to be monophyletic and thus formally termed "*Roseobacter* clade", comprises members that belong to the *Rhodobacteraceae* family which falls into the *Alphaproteobacteria* subclass of the class *Proteobacteria* (Buchan *et al*., 2005; Newton *et al*., 2010; Simon *et al*., 2017). Since the first two "*Roseobacter* clade" eponymous strains (*Roseobacter denitrificans* and *Roseobacter litoralis*) were described by Shiba (1991), 70 genera and 170 strains were identifed in 2014 (Pujalte *et al*., 2014) and by now presumably far more.

Members of the *Roseobacter* group dwell in various marine habitats, but are especially prominent as metabolically active and abundant bacterial group in phytoplankton blooms. In such high DOM substrate environments, roseobacters follow periodical population succession patterns (Buchan *et al*., 2014; Giebel *et al*., 2009; Gifford *et al*., 2014; Pinhassi *et al*., 2004; Riemann *et al*., 2000; Teeling *et al*., 2012). Also the prominent *Roseobacter* model organisms *Phaeobacter inhibens* (DSM 17395) and *Dinoroseobacter shibae* (DFL-12) were isolated form or identified on macroalgae or in phytoplankton blooms (Biebl *et al*., 2005; Gram *et al*., 2015, 2015; Segev *et al*. 2017; Thole *et al*., 2012; Wagner-Döbler *et al*., 2009)

It is believed that in succession of continuous nutrient supply by phytoplankton associations at evolutionary times of diatom occurrence in the Mesozoic, members of the *Roseobacter* group evolved specific metabolic and physiologic evolutionary adaptational strategies (Luo *et al*., 2014). The production of secondary metabolites, such as the antibiotic tropodithietic acid (TDA; Brinkhoff *et al*., 2004), algicides (roseobacticdes A and B; Seyedsayamdost *et al*., 2011) and several N-acyl-homoserine lactones (AHLs; Cude and Buchan, 2013; Lerat and Moran, 2004; Williams *et al*., 2007) indicate the roseobacter-algae associated lifestyle. A specialized metabolism facilitates the recycling and transformation of phytoplankton derived organic

compounds, such as the algal osmolyte dimethylsulfoniopropionate (DMSP), urea, polyamines, taurine, glycine betaine, methylated amines, phosphoesters and phosphonates as well as various other aromatic compounds (Chen, 2012; Dickschat, 2010; González *et al*., 1999; Moran *et al*., 2004, 2007; Newton *et al*., 2010). The complexity of identified metabolic traits are a key function for global marine cycles, such as the microbial loop (Azam *et al*., 1983) and the microbial carbon pump (Hedges *et al*., 1992).

Recent findings suggested that the success of the *Roseobacter* group adapting to variable ecological niches and high abundance in phytoplankton blooms, arose from various genetic causes. Most *Roseobacter* group isolates carry uncommonly large genomes with higher gene contents, interestingly including specialized transporters, that allow a fast response to enhanced organic compound availability (Giovannoni *et al*., 2005; Moran *et al*., 2007; Newton *et al*., 2010). In addition, large and essential genetic contents are encoded on extra-chromosomal elements, which can easily be transferred and allow a fast adaptation to varying environmental factors (Luo *et al*., 2014).

As a consequence of its global distribution, high abundance, fast genetic adaptation, and diverse metabolic traits, the *Roseobacter* group plays a key role in global biochemical cycles and therefore perfectly suites our study aim to unravel processes of bacterial DOM formation. The fact that several roseobacter strains, including *P. inhibens* and *D. shibae*, were extensively studied will help us to investigate the connection between intracellular processes and released organic molecules as well as the itemization of bacterially-mediated DOM compounds.

1.3 Bacterial exometabolome – Link between intra- and extracellular space

All living organisms sustain their vital cellular processes by metabolic reactions, the chemical transformation of molecules. The foundation for all intracellular mechanisms, including the production of metabolites, is the transcription of functional genes into messenger ribonucleic acid (mRNA) molecules, and its entity is defined as the transcriptome. Available mRNA are translated into active proteins that then chemically transform metabolites. Hence, metabolites are the downstream products of gene transcription and enzyme activity and reflect cellular processes (Fiehn, 2002; Tweeddale *et al*., 1998). Reversely, metabolites also possess the ability to control enzyme activity by allosteric regulation (Monod *et al*., 1965) and gene transcription by riboswitches binding (Barrick *et al*., 2004).

Equally important for the survival of most microbes are the complex and variable interactions with other cells and their environment. Exometabolomics, originated as sub-field of metabolomics, studies the extracellular metabolites (exometabolites) to gain a deeper understanding on exogenous metabolite exchanges and their biochemical transformations. In fact, the entity of exometabolites derived by single organisms is also referred to as exometabolome and in marine environments expands into the global pool of DOM (see Figure 2).

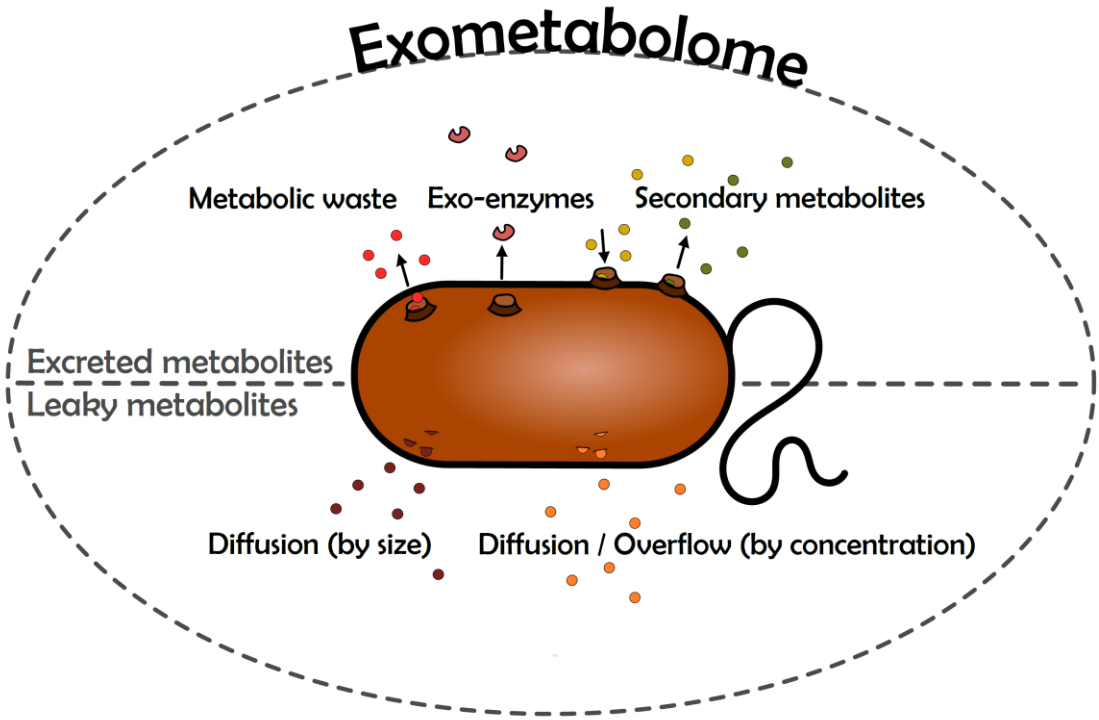

Figure 2 | Bacterial exometabolome formation scenarios. Bacterial exometabolites are released by excretion or as consequence of the cellular leakiness. Excreted compounds may derive as metabolic waste, exo-enzymes, or secondary metabolites. Diffusion or overflow exometabolome formation is dependent on the size and concentration of a given "leaky" metabolite.

Recent studies that aimed for the detection of single exometabolites with relevance of particular exogenous processes, usually apply MS-MS or nuclear magnetic resonance (NMR; Amin *et al*., 2015; Fiore *et al*., 2015; Johnson *et al*., 2016; Kujawinski *et al*., 2009). In contrast, applying the ultra-high resolution ESI FT-ICR-MS allows the simultaneous detection of several thousand accurate chemical formulas and enables the reflection of the major share of the exometabolome (Romano *et al*., 2014). Even molecular building blocks behind given molecular formulas within a complex DOM can be identified by applying FT-ICR-MS based fragmentation (Osterholz *et al*., 2015; Pohlabeln and Dittmar, 2015).

Several studies have applied FT-ICR-MS to measure exometabolomes, comprising hundreds and thousands of exometabolites, released by single bacterial or eukaryotic plankton strains (Longnecker *et al*., 2015; Romano *et al*., 2014). Apportioning this previously unexpected blend of detected molecular formula to known metabolites or the identification of the molecular structure remains one of the largest challenges in the field of exometabolomics. In experimentally generated exometabolomes, only ~3% of all exometabolites were assignable to known strain specific metabolites, but the molecular structure and function of the residual exometabolites remains undefined (Romano *et al*., 2014).

Generally, exometabolome describes all metabolites present outside of a living cell and does not differentiate how they are formed. For instance, bacterial metabolites derive as a result of diffusion or excretion (see Figure 1; Azam and Malfatti, 2007; Jiao and Zheng, 2011) and possibly cell division. Hence, the blend of exometabolites changes depending on its formation scenario. In nature, the release of microbially derived metabolites by cell lyses (zooplankton sloppy feeding, viral infection, cellular death) contribute to the global pool of geometabolome, however it does not reflect the actual cellular metabolism and thus are not considered as part of the exometabolome.

At present, the composition and function of the majority of exometabolites cannot be satisfactorily answered. Particularly puzzling is the division of exometabolites that are leaked or excreted (see Figure 2). The superior term “leaky metabolites” includes both and has only recently been denoted as such in the theoretical conceptual work of metabolic exchange (Morris *et al*., 2012).

Membrane diffusion is size and concentration dependent by means of a given metabolite (Nikaido, 2003; Nikaido and Rosenberg, 1981). Yet, the content of membrane diffusion that contributes to the exometabolome remains largely unresolved. In contrast, targeted studies on excreted metabolites were intensively studied in the last decades. Such (exo)-metabolites can fulfill various functions in the extracellular space and classified as secondary metabolites, exoenzymes and metabolic waste. Secondary metabolites are non-essential for cellular growth or reproduction, but function as defense, quorum sensing (cellular communication) or growth factors and are usually specific to individual species (Brinkhoff *et al*., 2004; Cude and Buchan, 2013; Lerat and Moran, 2004; Williams *et al*., 2007). The excretion of exoenzymes drives variable exogenous biological processes and enables environmental interactions. Exoenzymes function as virulence factors, carry out macromolecule cleavage or digest nutrients (Arnosti, 2011). Disposal of xenobionts, such as reactive oxygen species (ROS) or common intracellular toxins are essential for cellular survival and likewise contributes to the overall exometabolome

(Smirnova *et al*., 2015; Smirnova and Oktyabrsky, 2005). Glutathione-S-transferases (GST) and multidrug and toxic compound extrusion (MATE)-type transporter are cellular mechanisms that facilitate the binding, transformation, detoxification and excreting of these intracellular waste products (Moriyama *et al*., 2008; Vuilleumier, 1997).

At present, large fractions of the exometabolome entity still escape our analytical window. The high resolution FT-ICR-MS allows the simultaneous detection of precise molecular formula of thousands of organic compounds. This probable allows us to apportion shares of the exometabolome, identify individual exometabolites and to draw conclusions on their intracellular genesis. Thus we would shed more light on micro scale events that actually lead to global processes, such as the microbial loop and carbon sequestration.

1.4 Vitamins – Essential cofactors for life in the ocean

Vitamins are biologically active compounds with pivotal impact on marine ecosystem dynamics and several were identified as share of bacterial exometabolomes. In nature, the class of water soluble vitamins, also known as B-vitamins, is present in varying concentrations as part of the global marine DOM pool. By reason of the commonly required B-vitamin function as cofactors for enzymatic reactions (coenzymes) involved in many essential metabolic pathways, their availability is crucial for the survival of all living organisms (Dowling *et al*., 2012; Jurgenson *et al*., 2009). Cultivation experiments and genome survey results show a lack of *de novo* vitamin synthesis by a broad range of phytoplankton as well as bacterial strain, including the order *Rhodobacterales*. Several studies demonstrated strong correlations of bacterial and phytoplankton community dynamics and the availability of different B-vitamins (Carlucci and Silbernagel, 1969; Droop, 1957; Haines and Guillard, 1974; Heal *et al*., 2017; Provasoli and Carlucci, 1974).

In the ocean, B-vitamin availability undergoes strong fluctuations, depending on the oceanic province, water depth, community structure and seasons (Bonnet *et al*., 2010; Menzel and Spaeth, 1962; Sañudo-Wilhelmy *et al*., 2006, 2012; Suffridge *et al*., 2017, see Figure 3). Suitable methods for the enrichment and detection of ambient oceanic vitamin concentrations have only been established for a small fraction of B-vitamins, including the important vitamins B_1, B_7 and B_{12}. However, considering the phycosphere, a microbial habitat that governs cell to cell metabolite exchange, concentrations can widely vary from the ambient large scale environment (Seymour *et al*., 2017).

The most studied vitamins with respect to their synthesis pathways, cofactor functions, environmental relevance and oceanic concentrations are vitamins B_1 and B_{12}. Cobalamin (generally known as vitamin B_{12}), a bioactive corrinoid cofactor has been identified as a highly relevant link between bacteria and phytoplankton (Croft *et al*., 2006). In fact, *de novo* vitamin B_{12} synthesis is exclusively performed by bacteria and archaea and consequently, its production and supply is a significant interplay factor on the domain level (Croft *et al*., 2005; Cruz-López and Maske, 2016; Grant *et al*., 2014; Provasoli, 1963; Sañudo-Wilhelmy *et al*., 2006). *De novo* B_{12} synthesis underlies vast variations within phylogenetic phyla and order. A large majority of *Rhodobacterales* and *Cyanobacteria* genomes possess genes for *de novo* B_{12} synthesis, whereas globally abundant SAR11 or *Bacteroidetes* strains disclose an obligate exogenous B_{12} requirement (Sañudo-Wilhelmy *et al*., 2014). Current scientific knowledge estimates one third of all marine bacteria can de novo synthesize B_{12} and therefore being potential providers (Dirks 2014). Interestingly, all prokaryotes feature cobalamin-dependent enzymes, relevant in a large variety of intracellular processes of several metabolic pathways and nucleotide biosynthesis, whereas half of all phytoplankton organisms are cobalamin non-facultative, evolving B_{12}-independent methionine synthesis enzyme (JR Roth *et al*., 1996; Sañudo-Wilhelmy *et al*., 2014).

Vitamin B_1, also referred to as thiamin, is required by all living organisms. Still, *de novo* thiamin synthesis remains a privilege to some organisms, and hence its public availability is essential for all auxotrophs. For instance, several members of the *Roseobacter* group, *Flavobacteriia*, or cosmopolitan members of the SAR11 clade are seemingly thiamin auxotrophs (Carini *et al*., 2014; Giovannoni *et al*., 2005; Sañudo-Wilhelmy *et al*., 2014).

However, recent finding give rise to the assumption that the vitamin network conceals a far more complex level of connectedness than just the exchange of final vitamin metabolites. Gene streamlining analysis of vitamin pathways revealed a loss of single genes, rather than lacking the whole pathway (Carini *et al*., 2014; Croft *et al*., 2006; Sañudo-Wilhelmy *et al*., 2014). The final cofactor of thiamin synthesis is formed by the conjunction of the two independently synthesized moieties, 4-amino-5-hydroxymethylpyrimidine diphosphate (HMP-PP) and 4-methyl-5-(2-phosphoethyl)-thiazole (HET-P). Many bacterial and eukaryotic phytoplankton genomes encode the kinase for the final synthesis step (Begley *et al*., 1999; Rodionov *et al*., 2002; Sañudo-Wilhelmy *et al*., 2014), but partially lack the capability of synthesizing one of the moieties. For instance, cosmopolitan members of the SAR11 clade lack the HMP synthesis (Carini *et al*., 2014) and several green algae, including cryptophytes and

dinoflagellates, grow on exogenously provided HET enabling their vitamin B_1 auxotrophy (Droop, 1957; Lwoff, 1947; Turner, 1979).

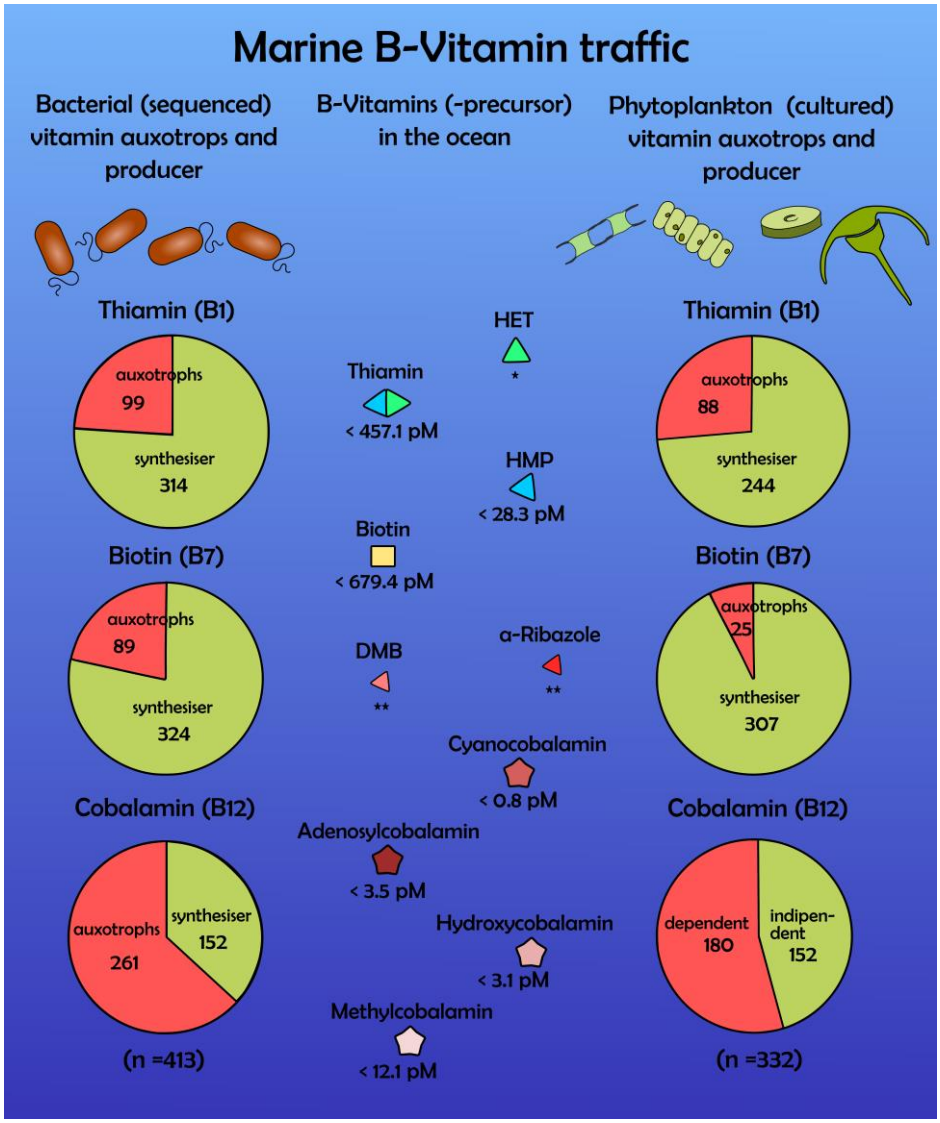

Figure 3 | B-vitamin traffic in the ocean. Shown are bacteria (sequenced) and phytoplankton organisms (cultured) that require (auxotroph) or synthesis vitamins B_1, B_7 or B_{12} (synthesizer; tested by Sañudo-Wilhelmy *et al.* 2014). Furthermore, highest oceanic concentrations detected (*=below detection limit, ** = no suitable method for marine samples) of vitamins B_1 (thiamin), B_7 (biotin) and variable B_{12} – analogs (Cyanocobalamin, Adenosylcobalamin, Hydroxycobalamin, Methylcobalamin) and vitamin precursors (HET = 4-methyl-5-(β-hydroxyethyl)thiazole, HMP = 4-amino-5-hydroxymethyl-2-methylpyrimidine, α-ribazole, DMB = 5,6-dimethylbenzimidazole) are illustrated to give an overview of the vitamin B traffic in the marine ecosystem (detected by Sañudo-Wilhelmy *et al.* 2014 and Johnson *et al.* 2016).

Vitamin B_{12} synthesis and remodeling is far more complex than previously assumed. Generally corrinoid cofactors are simplified as B_{12}, however several chemical variants exist distinguished by the lower ligand attached. The structure of the corrinoid cofactor affects its binding, function and catalysis (Lengyel *et al*., 1960; Renz, 1971; Stupperich *et al*., 1987; Yi *et al*., 2012).

Microbes are capable of importing specific lower ligands or corrinoids from the environment and attach preferred lower ligands or even remodel corrinoids (Crofts *et al*., 2013; Dirks *et al*., 2014; Gray and Escalante-Semerena, 2010). The availability of lower ligands can even change the corrinoid synthesis (Keller *et al*., 2014; Men *et al*., 2014). These findings indicate specific adaptation mechanisms to low bioactive corrinoid cofactor concentrations in nature and suggest a relevant function of lower ligands in the environment. Helliwell *et al*., (2016) demonstrated that representatives of major phytoplankton groups can remodel pseudocobalamin, commonly produced by cyanobacteria, by the aid of 5,6-dimethylbenzimidazole (DMB). DMB is a building block of the fundamental lower ligand α-ribazole. When attached to a corrinoid ring it forms the coenzyme B_{12} complex which is known to be the most distributed and relevant bioactive B12-analog representative. Recent studies detected α-ribazole within the exometabolome of several marine bacteria and in the open ocean, indicating its importance in the marine environment (Johnson *et al*., 2016; Wienhausen *et al*., 2017).

Overall, B-vitamins are essential biochemical cofactors for life. However, the ubiquitous outsourcing of the metabolic capability synthesizing vitamins among various marine microbes makes vitamin traffic indispensable for the survival and dynamic of microbial community. In fact, natural vitamin and vitamin precursor availability is an important factor explaining the network connectedness of microbial communities (Fuhrman and Steele, 2008, see section 1.5). Thus, in order to better understand marine vitamin traffic, the release of vitamin metabolites, including metabolic precursor and the impact of their allocation on microbial communities should be investigated.

1.5 Microbial networks and dependencies – When many become one

Microbial life in the marine world is built as a dynamic network that fuels a mosaic web of metabolic interactions. In recent years, precise and cost-efficient sequencing has elucidated microbial community structures, microbial correlations and periodic ecological interactions (Gilbert *et al*., 2012; Riemann *et al*., 2000; Teeling *et al*., 2012). Yet, a major challenge in marine microbial ecology remains to understand the network genesis and levels of dependencies on an organism or metabolite exchange level.

Microbial connectedness usually derives from metabolic dependencies. In marine habitats, the availability of microbial relevant metabolites, so called public goods, is crucial for a large fraction of microbes. However, the availability lowers the selection pressure to maintain crucial genes. Consequently, gene deletion is favored over a gene insertion and results in an overall genome reduction. This tendency of outsourcing metabolic functions in order to reduce cellular costs is known as genome streamlining (Giovannoni *et al*., 2005; Gross *et al*., 2009; Mira *et al*., 2001; Figure 4.A).

The evolutionary genesis of genome streamlining is explained by the black queen hypothesis

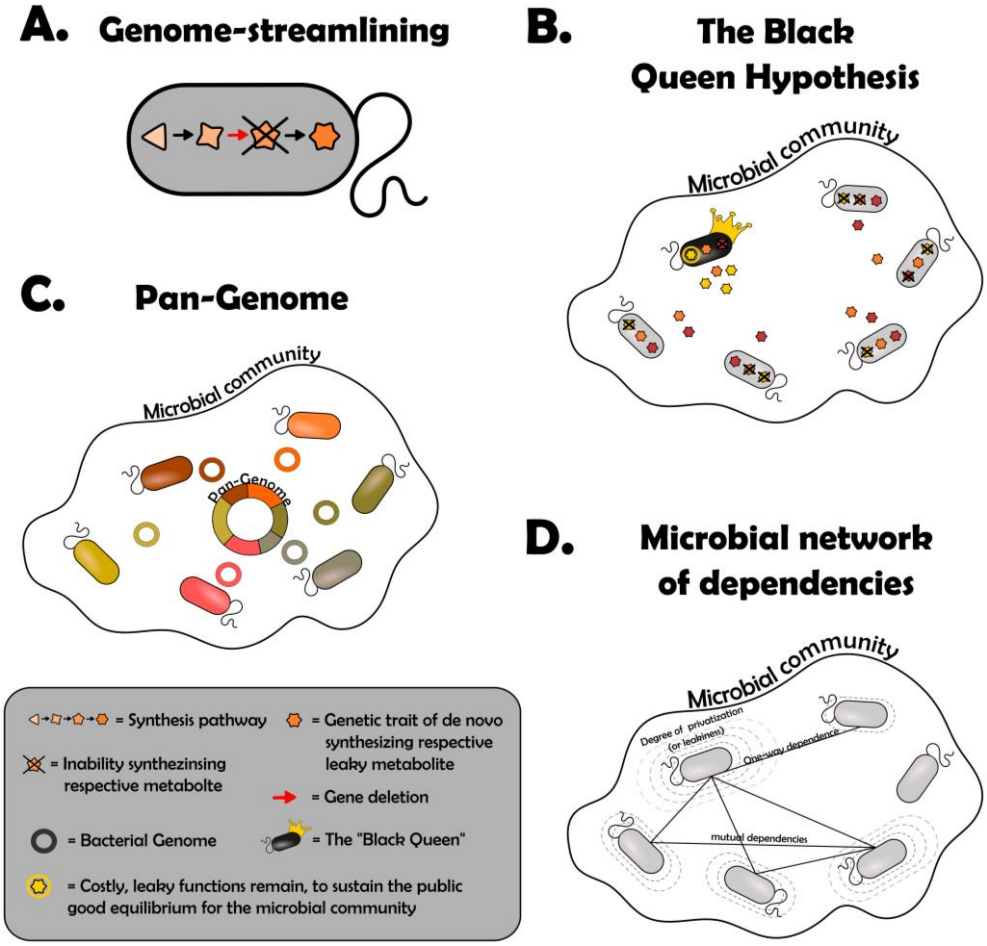

Figure 4 | Theoretical concepts and hypothesis explaining microbial community structures. These schemes illustrate concepts and hypothesis that elucidate our present understanding of microbial community networks. (A) presents the concept of genome streamlining, illustrating the deletion of one gene in a metabolic pathway. (B) shows the black queen hypothesis, elucidating the maintenance of a single gene by the black queen bacterium, to sustain the equilibrium of the leaky function for the community. (C) illustrates the concept of the pan-genome that considers the entity of bacterial genomes in a given bacterial community as the pan-genome. (D) elucidates microbial community network and dependency formations due to the degree of metabolite privatization.

(BQH; Morris *et al*., 2012) in which the black queen refers in a figurative sense to the queen of spades in the card game "Hearts". The aim is to avoid holding the queen of spades last. In the case of gene loss, this means that the last organism within a community left providing an essential public good is forced to keep this genetic trait in order to retain the survival of the dependent microbial community. In other words, gene reduction can only proceed until the production of public goods is just sufficient to support the equilibrium of a metabolite state (Figure 4.B). Despite the suitable attempt explaining evolutionary dependency and community interactions, the BQH lacks to elucidate the total microbial network complexity. For instance, cellular fitness decelerates by the increasing metabolic functions that are lost as a consequence of gene streamlining (D'Souza *et al*., 2015). Another neglected aspect by the BQH is that gene streamlined organisms are enforced to conserve acquisition strategies for the uptake of the lost metabolic function (Cordero *et al*., 2012).

In general, genome streamlining appears to be quite common among marine bacteria and as a consequence genomes are reduced in size (Giovannoni *et al*., 2014). Indeed, the inability to isolate the majority of bacterial strains is believed to derive from lacking essential genetic functions, usually substituted by the community. Numerous isolated bacteria can only be revived and grown, when essential metabolites are substituted. Such compounds are often observed to be vitamins or vitamin related compounds (Carini *et al*., 2014; Gray and Escalante-Semerena, 2010; Paerl *et al*., 2015; Sañudo-Wilhelmy *et al*., 2014). Hence, the evolutionary success of prokaryotes arose from the overall gene reservoir and thereby the metabolic potential provided as public good with in a defined bacterial collective. This phenomenon was termed the pan-genome, reflecting the sum of all genes that are present in a set of bacterial members, summarized as bacterial community (Fullmer *et al*., 2015; Tettelin *et al*., 2005; figure 4.C).

Bacteria live in complex and diverse communities in variable habitats. In marine environments, the exchange of metabolites is indispensable for the collective microbial success. The gain of fitness by outsourcing metabolic functions is accompanied by the disadvantage of becoming dependent on ecological partners. Disentangling the microbial network of dependencies, ranging from independent provider or beneficiary organisms to mutual dependent microbes, remains in its total complexity unsolved (Faust and Raes, 2012). The level of metabolite privatization (or leakiness) is a key factor shaping the outcome of microbial networks. Intermediate metabolite leakiness favors mutual interdependencies, whereas in contrast, one-way dependencies arise when metabolites are easy accessible due to low privatization. Consequently, stronger force on metabolite privatization results in lower interconnections

within a microbial community, whereas in contrast, increasing metabolite leakiness raises public benefits (Estrela *et al*., 2016; Figure 4.D).

In general, the bacterial exometabolome and from a larger perspective the geometabolome were so far mostly neglected when analyzing microbial community networks. To better understand microbial community dynamics it is essential to unveil many of the myriad existing metabolic dependencies. Therefore, the study of bacterial exometabolome and the accentuation of the relevance of individual exometabolites will help to achieve this goal.

1.6 Outline and aims of my thesis

My PhD thesis is focused on the mutually dependent interlinkage of marine microbes and the complex pool of marine DOM. Thereby, special emphasize is placed on microbial derived exometabolome especially that of metabolically active *Roseobacter* group members, which contribute on a large scale to the global marine geometabolome. I further aim to investigate the mosaic nature of the marine microbial metabolite cross feeding, with a focus on vitamins, which are pivotal organic molecules for the marine ecosystem.

To mimic and analyze bacterial diversification mechanisms in natural phytoplankton blooms, I produced and assembled phytoplankton derived exometabolome of an axenic diatom culture. Furthermore, bacteria with differing genetic and metabolic traits were cultivated on the complex blend of diatom derived organic compounds. The degradation and diversification patterns were analysed using the ultra-high resolution ESI FT-ICR-MS (Chapter 2).

In Chapter 3 the exometabolome of two selected *Roseobacter* group members, *Phaeobacter inhibens* and *Dinoroseobacter shibae* were apportioned and itemised by ultra-high resolution FT-ICR¬MS. I scanned the detected MF against genome-predicted metabolites, known to be produced by both organisms, to elucidate the bacterial exometabolome composition and their environmental relevance. To test gene streamlining among marine microbes and the consequent need for metabolite substitution by the detected exometabolites, I amended the most pivotal identified (exo)-metabolites to defined batch cultures to identify natural interactions.

In Chapter 4, I assessed the transcriptome along with the exometabolome of *Phaeobacter inhibens* at variable growth conditions. Correlation patterns gave an overview of exometabolite classes that presumably derive as a consequence of upregulated intracellular processes. Thereby, conclusions on global marine bacterial DOM diversification processes can be gained.

Finally, I present an overview (chapters 5 and 6) of vitamin B_1 and B_{12} gene streamlining of members of the *Roseobacter* group, to elucidate the relevance of vitamin traffic in the marine ecosystem. To understand the natural significance of vitamin traffic, mesocosm experiments were established on two scientific Pacific Ocean cruises. Effects of vitamins B_1, B_{12} and the respective precursors 4-methyl-5-(β-hydroxyethyl)thiazole (HET), 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and α-ribazole such as cellular activity, biomass production and phytoplankton community were analysed over time.

Chapter 2

Diversification of diatom-derived dissolved organic matter by single strains of the *Roseobacter* group

Beatriz E. Noriega-Ortega*, **Gerrit Wienhausen*,** Marco Mühlenbruch , Meinhard Simon, Thorsten Dittmar and Jutta Niggemann

Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Oldenburg, Germany

* These authors contributed equally

Keywords: DOM diversification, FT-ICR-MS, *Roseobacter* group, diatom exudate, *T. pseudonana,* carbon cycling

Chapter 2

Abstract

Phytoplankton exudates provide a diverse source of dissolved organic matter (DOM) for heterotrophic bacteria which rapidly degrade and transform it during phytoplankton blooms in marine pelagic ecosystems. The focus of most studies so far was on how bacteria degrade marine DOM or distinct low and/or high molecular weight fraction. Even though there is recent accumulating evidence that marine DOM is composed on hundreds to thousands of different molecules the role of bacteria in creating this enormous diversity is still largely unexplored. Therefore we studied how three strains of the *Roseobacter* group, *Phaeobacter inhibens*, *Dinoroseobacter shibae* and *Ruegeria pomeroyi*, degrade and diversify freshly exuded DOM of the diatom *Thalassiosira pseudonana*. The composition of the DOM, obtained after gentle sand filtration to avoid any cell damage of the growing diatom, was analyzed by high performance liquid chromatography and Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS). Its solid-phase extracted fraction consisted of around 1300 different mass formulas (MF). The results show that the three strains largely consumed dissolved amino acids and monosaccharides of the exuded DOM but greatly transformed the DOM by removing dozens of and producing around 30% of new MF of the DOM analyzed by FT-ICR-MS. Each bacterial strain had a distinct impact on the transformation of the DOM and *P. inhibens* exhibited the greatest and *R. pomeroyi* the least impact. The findings show that a single bacterial strain can greatly enhance the diversity of freshly exuded diatom-derived DOM and indicate that bacterial processing of DOM is an important process in the diversification of DOM in the oceans.

Introduction

Phytoplankton primary production is a major source of dissolved organic matter (DOM) in the oceans. In particular in nutrient-rich pelagic ecosystems such as upwelling and frontal regions and in coastal seas diatoms greatly dominate phytoplankton communities and fuel pelagic food webs and the microbial loop as energy and carbon source via DOM (Nelson *et al*., 1995; Falkowski *et al*., 1998). Heterotrophic bacteria partly incorporate DOM into biomass, modify and remineralize it. The quality, i.e. molecular composition and the fractions of labile and more refractory DOM compounds affects the bacterial metabolism and controls the bacterial growth efficiency (Del Giorgio and Cole 1998; Weiss and Simon 1999; Baña *et al*., 2014). During bacterial processing of phytoplankton-derived DOM its quality and molecular composition undergoes changes because of selective consumption and modifications but also due to release of DOM components such as carbohydrates by bacteria (Kujawinski *et al.,* 2011; Hahnke *et al*., 2013; Osterholz *et al*., 2015). The major components of characterizable labile and phytoplankton-derived DOM constitute dissolved combined amino acids and carbohydrates (Biersmith and Benner 1999, Weiss and Simon 1999, Benner and Amon 2015). Recent studies applying Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), however, revealed that the composition of marine DOM and also of the exometabolome of heterotrophic marine bacteria and phytoplankton algae is far more complex than assumed before on the basis of classical chemical characterization methods. This approach revealed that DOM is composed of thousands of so far unknown molecular formulas (MF; Dittmar and Stubbins 2014; Longnecker *et al*., 2015; Romano *et al*. 2014; Fiore *et al*., 2015). However only a small fraction (<3%) of the myriads of MF detected by FT-ICR-MS in exometabolomes of marine bacteria and the DOM were identified (Romano *et al*., 2014; Fiore *et al*., 2015; Wienhausen *et al*., 2017). The specific impact bacteria may have on the diversity of phytoplankton- or bacteria-derived fresh DOM while processing it has not been examined by the ultrahigh resolution FT-ICR-MS approach. This insight, however, is important for a more in-depth understanding of the role bacteria play in the diversification and structuring the composition of the refractory DOM persisting in the oceans for decades to millennia.

In order to test the impact of bacteria on the consumption and diversification of phytoplankton-derived DOM we grew three model bacteria of the *Roseobacter* group on DOM freshly exuded by the diatom *Thalassiosira pseudonana* and analyzed how these bacteria consumed the DOM and altered its chemical composition. Members of the *Roseobacter* group were shown to be prominent components of bacterioplankton communities during phytoplankton blooms (West

et al., 2008; Buchan *et al*., 2014; Wemheuer *et al*., 2014; Teeling *et al*., 2016; Segev *et al*., 2017). The DOM was harvested by gentle sand filtration (Barovski *et al*., 2009) and supplied to cultures of *Phaeobacter inhibens* DSM 17395, *Dinoroseobacter shibae* DSM 16493 and *Ruegeria pomeroyi* DSM 15171. *Phaeobacter inhibens* DSM 17395 is a purely heterotrophic bacterium (Thole *et al*., 2012) and strains of this species have been found during phytoplankton blooms (Gifford *et al*., 2014; Segev *et al*., 2017). *Dinoroseobacter shibae* is photoheterotrophic, grows symbiotically with dinoflagellates (Wagner-Döbler *et al*., 2010) and has also been found associated to algae in natural phytoplankton blooms (Gifford *et al*., 2014; Milici *et al*., 2016; Segev *et al*., 2017). *Ruegeria pomeroyi* has also been found during phytoplankton blooms (Gifford *et al*. 2014) and was shown to grow in a symbiotic relationship with Synechococcus (Christie-Oleza *et al*., 2017). We hypothesize that these strains of the *Roseobacter* group utilize different fractions of the algal exudate and that they have different impacts on the diversification on the diatom-derived DOM.

Materials and Methods

The basic approach was to set up batch cultures of *P. inhibens*, *D. shibae* and *R. pomeroyi* supplemented with *T. pseudonana*-derived exudates. During growth subsamples were withdrawn to analyze the concentration of dissolved organic carbon (DOC), dissolved amino acids and carbohydrates by high performance liquid chromatography (HPLC) and the DOM composition by FT-ICR-MS. We performed a series of cleaning steps to prevent organic contaminations that could potentially bias our high sensitivity techniques. All materials were acid-washed (pH 2, ultrapure water). Furthermore, all glassware was combusted at 500°C for 3 hours. All chemicals used were from analytical grade or higher.

Culture set up, incubation, sampling and enumeration of bacteria

To obtain the algal exudate, *T. pseudonana* cells were incubated axenically in synchronized artificial seawater medium (synASW-medium). This novel medium combines the ASW-medium used for cultivation of bacteria (Zech *et al*., 2009) and F/2 medium applied for cultivation of algae (Guillard and Ryther 1962) in order to support the growth of bacteria as well as algae. This medium contained per L 22 g NaCl, 3 g Na2SO4, 0.7 g KCl, 0.098 g KBr, 0.003 g H3BO3, 0.003 g NaF, 0.024 g SrCl2 6H2O, 1 g CaCl2 2H2O, 7 g MgCl2 6H2O, 0.075 g NaNO3, 0.01 g KH2PO4 2H2O, 0.026 g Na2SiO3 5H2O and 0.025 NH4Cl. A solution of 2 g L-1 NaHCO3 was autoclaved separately and added to the salt solution. Trace element solution prepared based on Guillard

and Ryther (1962) and Zech *et al*., (2009) and contained per L 3.15 g FeCl3 6H2O, 5.2 g Na2EDTA 2H2O, 0.01 g CuSO4 5H2O, 0.036 Na2MoO4 2H2O, 0.144 g ZnSO4 H2O, 0.18 g MnCl2 4H2O, 0.19 g CoCl2 6H2O and 0.024 NiCl2 6H2O was added. Per liter, 100 nM thiamin hydrochloride (vitamin B1), 100 nM biotin (vitamin B7) and 100 nM cyanocobalamin (vitamin B12) were added. The diatom was cultured in combusted glass bottles with membrane lid-closed batches of 2 L at 15°C with a light:dark cycle of 16:8 hours (~15-20 µmol photons m2 s-1). At stationary growth phase, the algal exudate was separated from intact cells by filtration over a combusted sand column, consisting of a glass column with a glass fiber bed and acid-washed sea sand (Barofsky *et al*., 2009). Procedural blanks confirmed that filtration through the sand column did not introduce DOM or bacterial cells. The exudate was then filtered through a 0.22 µm polyether sulfone filter to remove particles, algal cells, and bacteria potentially added during the filtration. Exudate was stored at 4°C until further processing.

Bacteria were transferred from a glycerol stock to marine broth (MB) medium and grown to exponential growth phase. To reduce the carry-over metabolism, *P. inhibens* DSM 17395 and *D. shibae* DSM 16493 were cultivated in clean artificial seawater (ASW)-medium amended with 5 mM of glucose, whereas *R. pomeroyi* DSM 15171 was grown on the algal exudate supplemented with 5 mM sucrose and transferred at exponential phase to fresh medium three times. To harvest cells for further transfers to fresh media, cultures of the three strains were centrifuged at 2499 g in acid washed and combusted (3 h, 500°C) glass centrifuge tubes. Cell pellets of *P. inhibens* were washed three times with ASW before inoculation of the final experiment flasks. Cell pellets of *D. shibae* and *R. pomeroyi* were washed only once, in order to minimize cell loss because cell numbers were lower for these two strains. In the final experimental setup, bacteria were cultivated using algal exudate as the carbon source. All experimental flasks, except for negative controls, were inoculated with an initial cell density of 2.5 105 cells ml-1 and incubated at 28°C. Samples for the analysis of DOC, carbohydrates and amino acids and DOM characterization and for enumeration of bacterial cell numbers were taken at the beginning of the incubation (T0), during the exponential (T1) and stationary growth phase (T2). Sampling points were determined previously by establishing growth curves of the selected strains on algal exudates. Samples for DOC were filtered through Whatman GF/F filters, acidified to pH 2 and stored in brown sealed glass vials until analysis, for amino acids and carbohydrates through 0.2 µm polyethersulfone filters and stored frozen at -20 °C and for the analysis of the DOM composition also through 0.2 µm polyethersulfone, acidified to pH 2 and kept in brown sealed glass vials until further processing. Bacteria were counted by

epifluorescence microscopy after staining with SYBRGreen and by using an image analysis system as described in Bakenhus *et al*., (2017).

Amino acid and carbohydrate analysis

Analyses of total dissolved amino acids (TDAA) by HPLC after pre-column derivatization with ortho-phtaldialdehyde and dissolved free neutral (DFNCHO) and combined monosaccharides (DCNCHO) by HPLC and pulsed amperometric detection were done as described by (Wienhausen *et al*. 2017). Detection limits for individual amino acids and monosaccharides were 0.5 and 1.5 nM, respectively.

DOC quantification and DOM characterization

DOC was quantified via high temperature catalytic combustion (Qian and Mopper 1996) using a Shimadzu TOC-VCPH total organic carbon analyzer equipped with an autosampler ASI-V. The method's accuracy was tested during each run using Deep Atlantic Seawater Reference material (DSR, D.A. Hansell, University of Miami, Miami, FL).

Filtered samples for the molecular characterization of DOM were desalted and DOM was concentrated using solid phase extraction (Dittmar *et al*., 2008). 20 ml of filtered and acidified (pH 2, HCl 25%, p.a., Carl Roth, Germany) sample ran through Varian Bond Elut PPL 100mg cartridges (Agilent, USA) by gravity. After extraction, cartridges were rinsed with acidified ultrapure water (pH 2, HCl 25%, p.a., Carl Roth, Germany) to remove remaining salt. The resin was dried with Argon gas and eluted with 1 ml of methanol (HPLC-grade, Sigma-Aldrich, USA). Extraction efficiency with respect to carbon ranged from 8 % to 17 %. Procedural blanks were performed by running acidified ultra-pure water instead of sample.

Ultrahigh-resolution mass spectrometry via FT-ICR-MS was performed on a Bruker Solarix 15 Tesla FT-ICR-MS (Bruker Daltonik GmbH, Bremen, Germany) coupled with electrospray ionization in negative mode. DOC concentration of all samples was adjusted to 10 ppm prior to analysis using a carrier of ultrapure water and methanol (1:1, HPLC-grade, Sigma-Aldrich, USA) in equal parts. 500 scans were accumulated per run in a mass window of 92 to 2000 Da. The spectra were calibrated internally using Bruker Daltonics Data Analysis software package and processed using in house Matlab routines. A prerequisite for direct comparison of the samples is that the total ion current (sum of all intensities detected with the FT-ICR-MS, which is proportional to the amount of injected DOC) is in the same order of magnitude for all samples. Analyte peaks were separated from the noise applying the method detection limit (MDL) described by Riedel and Dittmar (2014). Furthermore, all masses present in procedural blanks

were removed from the data set. Molecular formulae were assigned following the procedure described by Koch *et al*., (2007) with maximum elemental abundances of CnHnOnN4S1. Masses detected in at least two out of three biological replicates were included in further data analysis.

Multivariate statistics and diversity quantification

A list of detected masses with their relative intensity in each sample was obtained from the FT-ICR-MS analyses. Signal intensities were normalized after blank correction by dividing the signal intensity of each individual peak of one sample by the sum of all signal intensities in the respective sample. Additionally, standardization by Z-scores was done prior to principal component analysis (PCA). PCA was performed on the normalized and standardized data with the software R version 3.3.1 (RCoreTeam 2016) using the function "prcomp".

Van Krevelen plots were created using the elemental ratios of molecular formulae detected via FT-ICR-MS that significantly decreased or increased in the incubations as compared to the original exudate. The significance was determined by one-tailed t-tests on the triplicate normalized relative intensities of each molecular formula with a confidence level of 0.05.

The Shannon index (Shannon 1948) was calculated per sample using R package "vegan" function "diversity" with loge. The resulting values of the biological triplicates were averaged. Functional chemical diversity was calculated as described by Mentges *et al*., (2017). This measure compares the functional diversity of mixtures of chemical compounds considering the chemical properties of MF.

Results

Chemical characterization of *T. pseudonana* exudate

The *T. pseudonana*-derived exudate contained 590±10 µM C from which 21 µM C were bound in TDAA, 1.5 µM C in DFNCHO and 46 µM C in DCNCHO, respectively (Fig. 1). Thus, 68.5 µM C, equivalent to 12% of total DOC, were characterized by HPLC analyses. Amino acid composition of the exudate was dominated by valine (32 %), whereas other amino acids (aspartate, glutamate, asparagine, histidine, serine, arginine, glycine, threonine, alanine, tyrosine and methionine) accounted individually for 2-14 % (Fig. 2). Eighty percent of DFNCHO constituted of glucose, followed by 10 % Xylose, 7 % arabinose and 3 % galactose. DCNCHO contained 96 % glucose and 3 % galactose (Fig. 3).

The solid phase extractable fraction of the exudate was analyzed with FT-ICR-MS. The extraction efficiency of the exudates was 8.9 ± 1.3%, corresponding to 42–60 µM C of solid-phase

extractable DOM. Dissolved carbohydrates, THAA and solid phase extractable DOC together accounted for up to 22 % of total DOC of the exudate (Fig. 1 B-E). In total, 2845 masses were

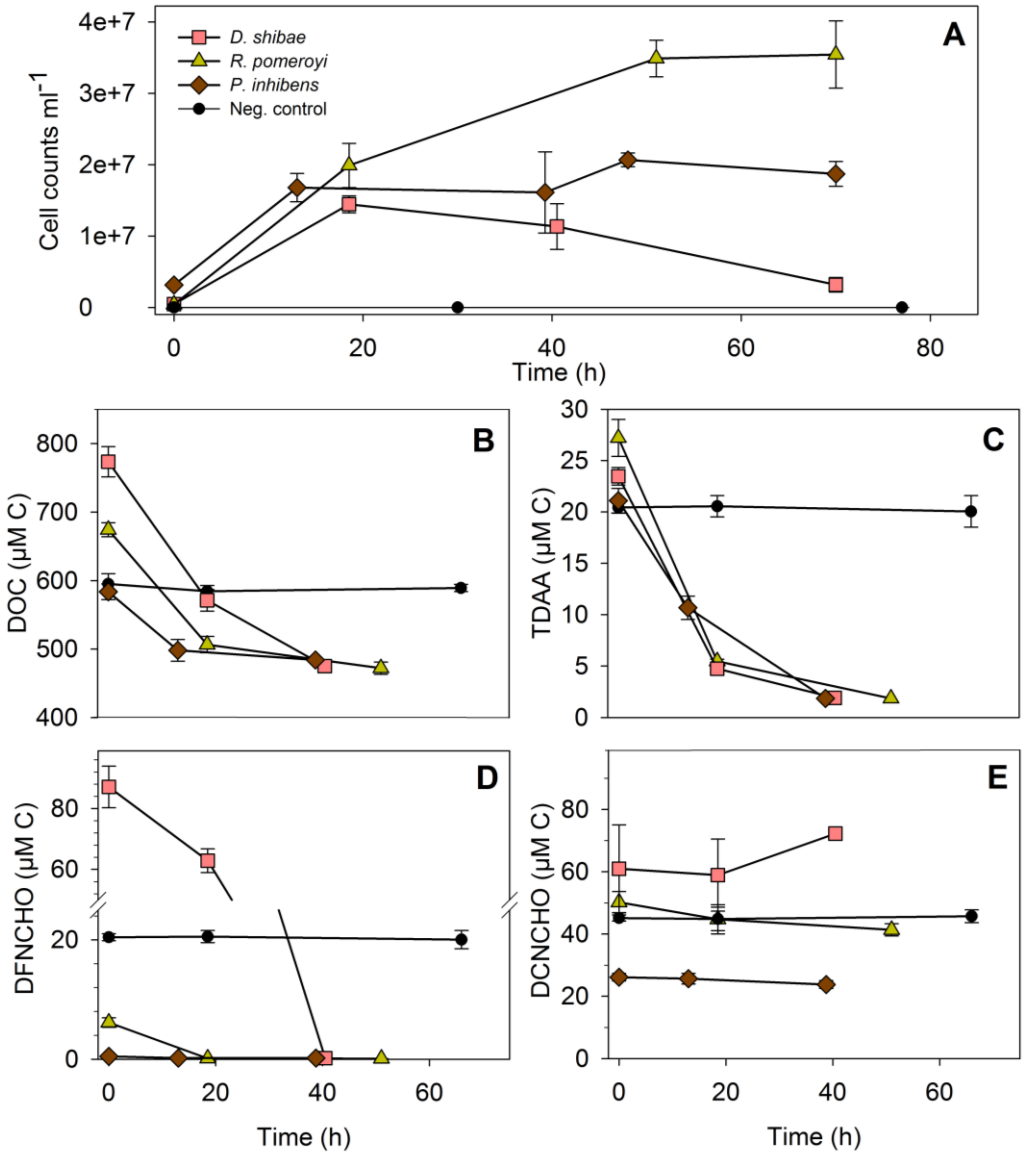

Figure 1 | Bacterial growth on the diatom exudate and mean DOC, TDAA, DFNCHO and DCNCHO consumption. A) Mean cell numbers of triplicate cultures of *P. inhibens*, *D. shibae*, *R. pomeroyi* and a sterile control over time. The time points used for further organic matter analysis were determined based on cell density in order to obtain samples from exponential and stationary growth phases. Mean DOC (B), TDAA (C), DFNCHO (D) and DCNCHO (E) concentrations in the three cultures over time. Error bars represent the standard deviation of the triplicates.

detected, of which 1820 got MF assigned.

Masses were detected in the mass range 94 to 993 Da, with a higher density and intensity of signals at around 300 Da (Fig. S1). Masses with a MF assigned were classified into categories predicted from their MF and elemental ratios. Most molecular formulae detected by FT-ICR-MS

were unsaturated (70 % of all formulae detected), from which 44% were aliphatic compounds. Compounds assigned to the peptide category accounted for 15% of all detected formulae (Fig. S1).

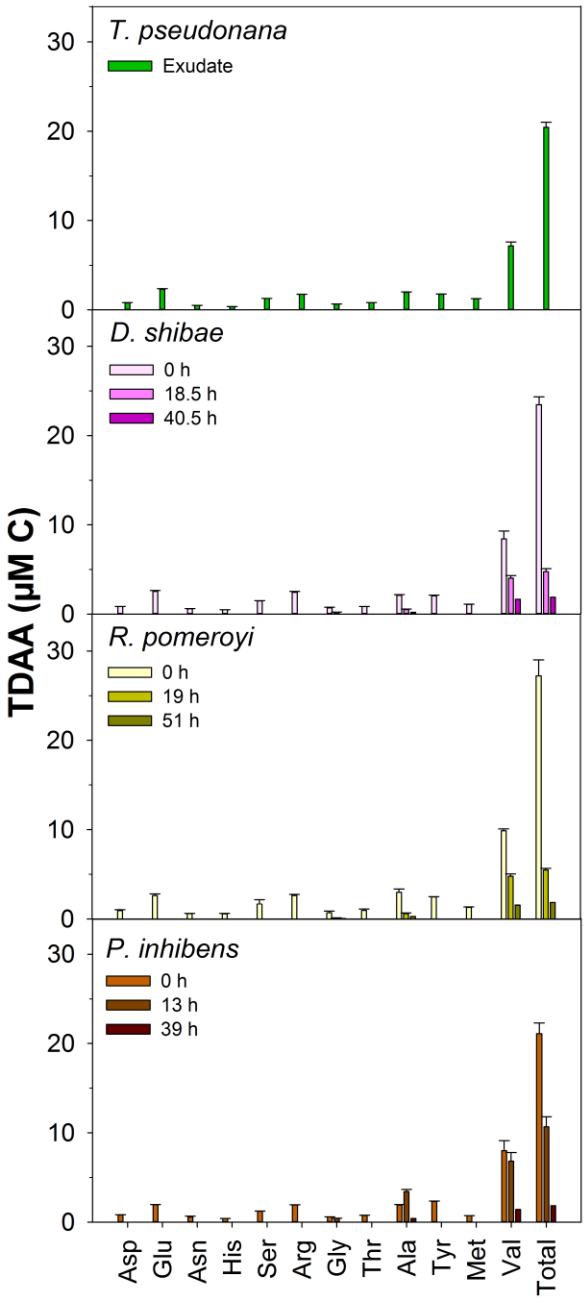

Figure 2 | Concentrations of TDAA of individual dissolved amino acids in the *T. pseudonana* exudate and at time points T0, T1 and T2 in the triplicate cultures of *D. shibae*, *R. pomeroyi* and *P. inhibens*. Error bars represent the standard deviation of the triplicates.

Bacterial growth on algal exudate and DOM utilization

Cell densities increased up to two orders of magnitude from inoculation to the stationary phase. Highest cell numbers were reached by *R. pomeroyi* followed by *P. inhibens* and both strains remained in the stationary phase until the end of the incubation. Highest cell numbers of *D. shibae*, were lower than of the two other strains and reached already after 18 hours but numbers declined thereafter (Fig. 1A).

DOC analysis revealed increased concentrations in the *D. shibae* and *R. pomeroyi* cultures after inoculation, relative to the diatom exudate. We attribute this DOC increase to the bacterial inocula which were not washed as thoroughly as that of *P. inhibens*. DOM not originating from the diatom exudate accounted to 28% and 18% of total DOC in the *D. shibae* and *R. pomeroyi* cultures, respectively. The majority of the excess DOC was in the form of glucose (*D. shibae* 37% free glucose, *R. pomeroyi* 5% free glucose and 39% as part of polysaccharides; Fig. 3).

Additionally, 10% and 21% of the excess DOC in the cultures of *D. shibae* and *R. pomeroyi*, respectively, was in the form of THAA. The bacterial strains consumed 18-20% of the diatom exudate, calculated on the basis of initial DOC concentrations (Fig. 1B).

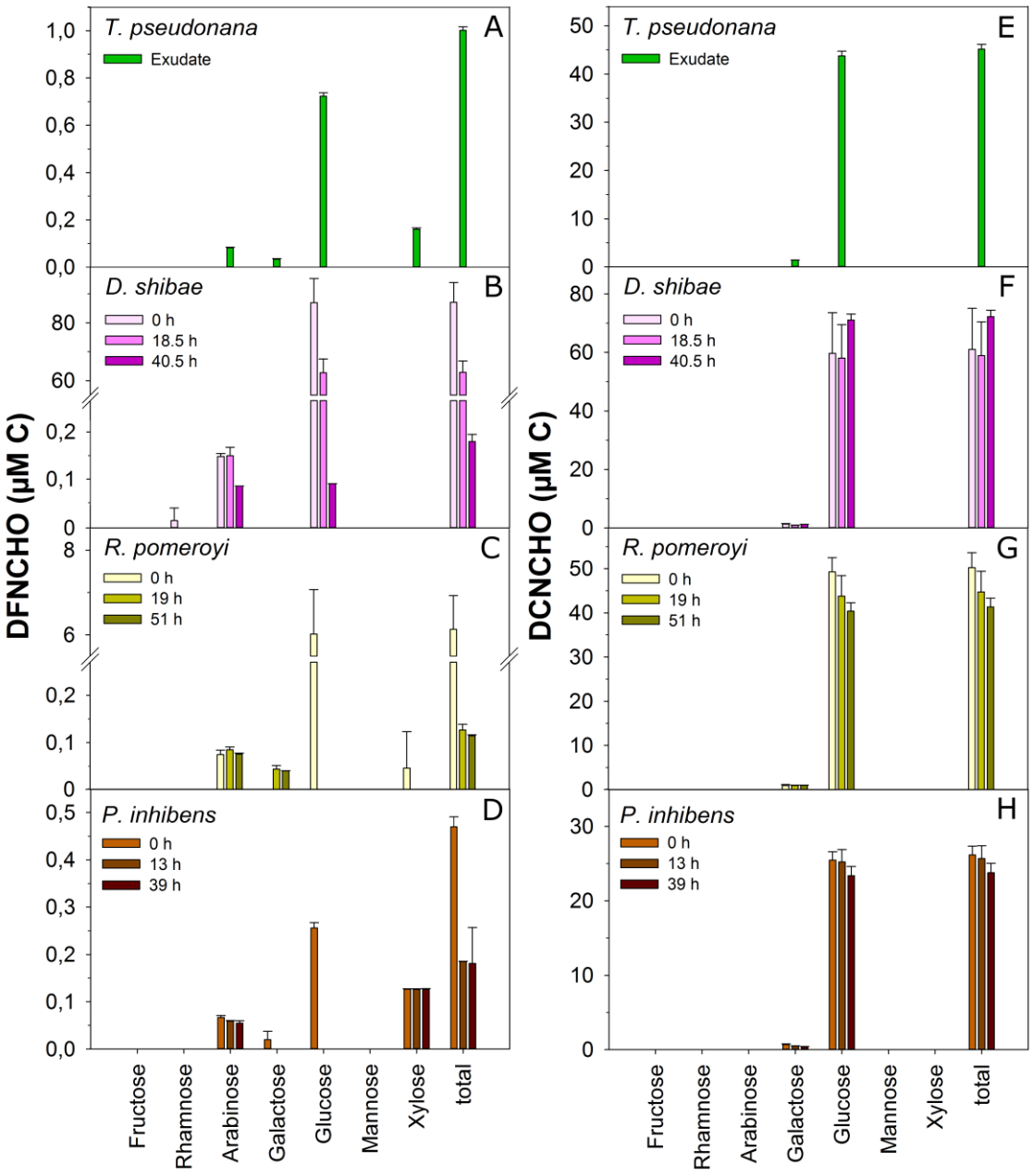

Figure 3 | Concentrations of DFNCHO (A, B, C, D) and DCNCHO (E, F, G, H) of the *T. pseudonana* exudate (A, E) and triplicate cultures of *D. shibae* (B, F), *R. pomeroyi* (C, G) and *P. inhibens* (D, H) sampled during lag, exponential and stationary growth phase. Error bars represent the standard deviation of the triplicates.

Concentrations of THAA rapidly decreased and remained around 5 nM C onwards from T1 in the cultures of all three strains. Most amino acids went below the detection limit except glycine, alanine and valine (Fig. 2).

Concentrations of DFNCHO rapidly decreased in all three cultures and mostly until T1. *D. shibae* and *R. pomeroyi* consumed 89% and 93%, respectively, and *P. inhibens* 61% of the initial concentrations (Fig. 1D). Each strain exhibited different utilization patterns of individual monosaccharides. Dissolved combined neutral carbohydrates were also consumed in all bacterial cultures. In the *R. pomeroyi* and *P. inhibens* cultures 18% and 9% of the initial concentrations, respectively, were removed. In the culture of *D. shibae*, 70% were consumed until T1 but thereafter concentrations increased again and exceeded initial concentrations at T2 when cell numbers had strongly declined.

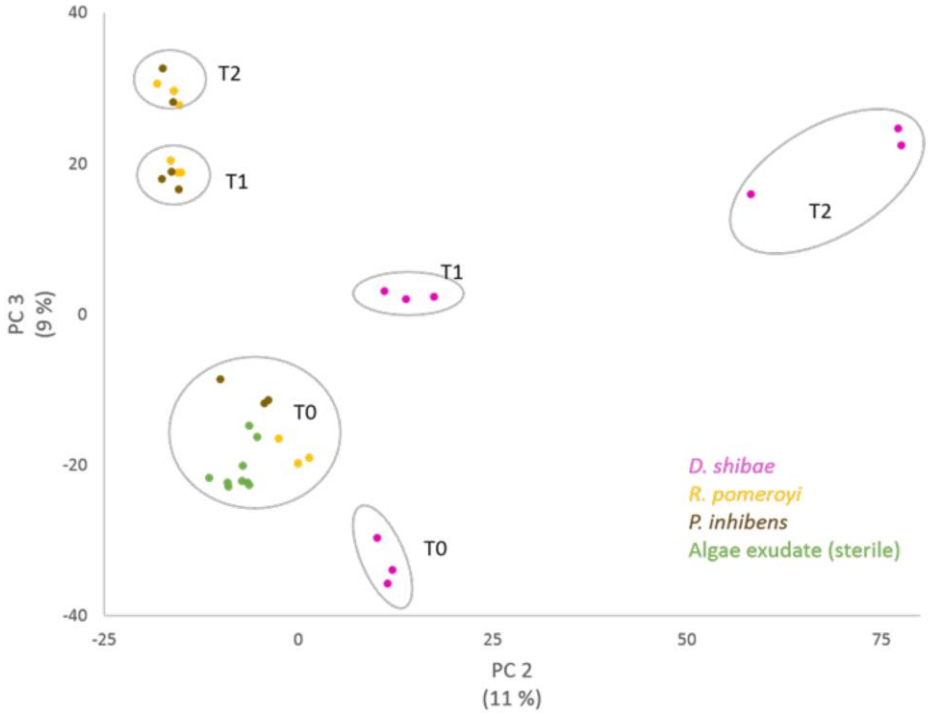

Figure 4 | Principal component analysis of the DOM composition of the diatom exudates and in the triplicate cultures of *D. shibae*, *R. pomeroyi* and *P. inhibens* at T0, T1 and T2. DOM was analyzed by FT-ICR-MS using the normalized relative intensities of masses detected.

Differential utilization of algae exudate

The three bacterial strains growing on the exudate modified the original exudate differently. Principal component analysis revealed that the chemical composition of all initial time points (T0) and sterile controls was similar. Furthermore the samples of T1 and T2 were separated

from the initial points and sterile controls in the PCA space (Fig. 4). This is a reflection of the change in chemical composition over time. Patterns revealed a reproducible trend, shown by the biological replicates of the incubations that grouped together (Fig. 4). Even though the PCA analysis indicates high similarity between incubations of *P. inhibens* and *R. pomeroyi*, van Krevelen plots revealed more detailed consumption patterns (Fig. 6). The three bacterial strains utilized and released different compounds as reflected by the number of MF with a relative decrease or increase masses (Fig. 5). *P. inhibens* exhibited the greatest impact on the diversification of the exudate by utilizing 75 and producing 427 compounds; *D. shibae* utilized 65 and produced 244 new compounds whereas *R. pomeroyi* removed only 45 and newly produced 170 compounds (Fig. 5).

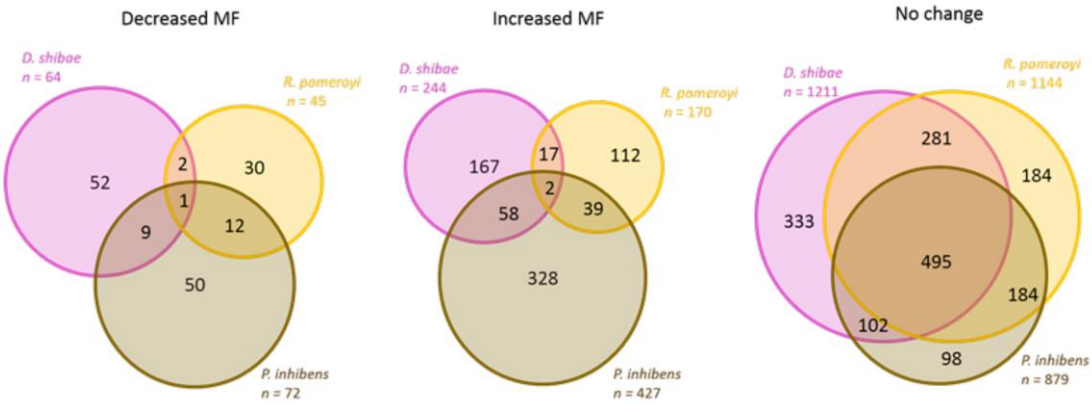

Figure 5 | Venn diagram of the MF detected by FT-ICR-MS in the triplicate cultures of *D. shibae*, *R. pomeroyi* and *P. inhibens* that increased, decreased or did not change in relative intensity over time with a confidence level of 0.05.

Furthermore, the MF that decreased in relative intensity in the *P. inhibens* culture had a higher H/C ratio (1.60) and a lower O/C ratio (0.24) than those in the cultures of the other two strains (*D. shibae* H/C=1.51, O/C=0.29; *R. pomeroyi* H/C=1.55, O/C=0.29). The MF that decreased in the incubations of *R. pomeroyi* and *P. inhibens* had a mean number of nitrogen atoms lower than those that increased or stayed constant in the same incubations (decreased MF: *R. pomeroyi*=0.53, *P. inhibens*=0.56; increased MF: *R. pomeroyi*=0.74, *P. inhibens*=0.70; no change: *R. pomeroyi*=0.85, *P. inhibens*=0.87).

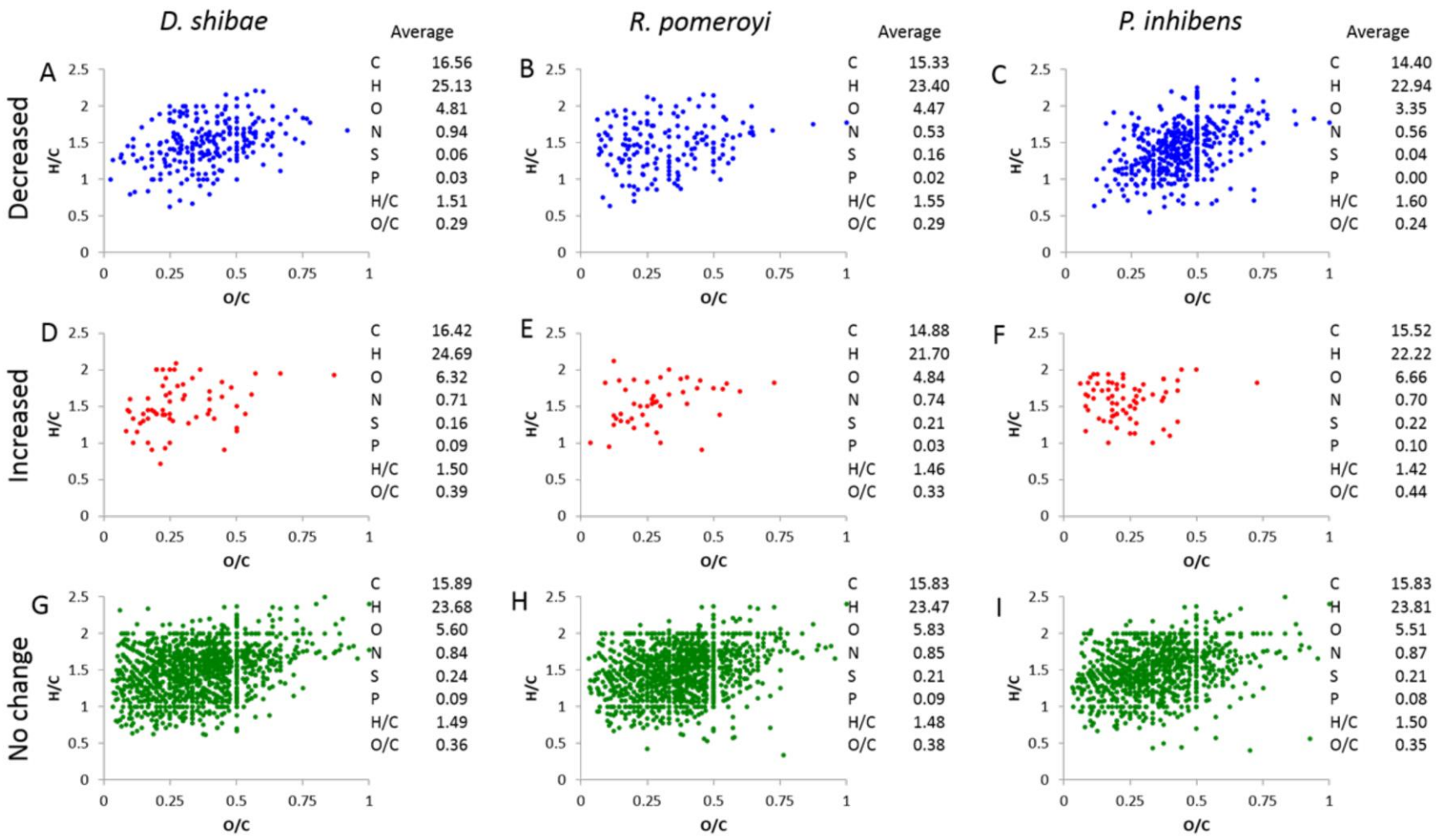

Figure 6 | Van Krevelen plots with the elemental ratios of oxygen/carbon against hydrogen/carbon of all mass formulas (MF) detected by FT-ICR-MS in the triplicate cultures of *D. shibae*, *R. pomeroyi* and *P. inhibens*. A–C: MF that increased in relative intensity from the previous time points; D–F; MF that decreased in relative intensity as compared to previous time points; G–I: MF that did not change in relative intensity over time. The increase or decrease was determined statistically by means of paired t-tests with a confidence level of 0.05. The tables next to the plots are the elemental averages of the different fractions.

The mean number of sulfur atoms in the MF that decreased in relative intensity was lower than in MF that increased or stayed constant in all incubations (decreased MF: *D. shibae*=0.03, *R. pomeroyi*=0.16, *P. inhibens*=0.04; increased MF: *D. shibae*=0.16, *R. pomeroyi*=0.21, *P. inhibens*=0.22; no change: *D. shibae*=0.24, *R. pomeroyi*=0.21, *P. inhibens*=0.21). The fraction of MF that did not change during the incubation with bacteria was mostly shared among the cultures of the different strains and the chemical composition was also similar (Fig. 5 and 6).

Diversification of DOM

In order to quantify the diversity change over time in the incubations, richness, Shannon index and functional diversity as a function of molecular mass were calculated. For this analysis the resulting values were only compared to the initial time point of the respective incubation and not between incubations, because the initial time points of the incubations and the sterile exudates were slightly different.

The richness of detected m/z was highest for the *D. shibae* culture at T0, respectively (Fig. S2). This presumably reflects the carryover of DOM from the preculture which was not washed as thoroughly as the other cultures (Fig. 1 B-E). In all three cultures richness decreased from T0 to T1 and thus during the growth phase. For *P. inhibens* and *D. shibae* richness increased from T1 to T2 and thus during the stationary and/or declining phase, resulting in an overall increase of richness. The *R. pomeroyi* culture on the other hand kept a lower richness at T1 and T2 than the initial time point but was the only culture growing until this time point (Fig. S2). Shannon diversity index takes into account richness and evenness. This diversity index increased in all three cultures at T2 relative to the previous time points (Fig. S2). Functional diversity as a function of molecular mass generally increased in the incubations compared to the initial exudates. This is a consistent trend, however not statistically significant (Fig. S2).

Discussion

***T. pseudonana* exudate provides a diverse blend of organic compounds**

In marine epipelagic ecosystems, phytoplankton exudates are a major source of DOM and fuel microbial food webs. They are a blend of a multitude of organic compounds released as photosynthetic products as well as of senescent and lysing cells. In our experiments we intended to focus on release products of actively growing phytoplankton and used a diatom as a model organism. Therefore, we filtered an axenic *T. pseudonana* culture very gently, using a sand column device to ensure keeping the integrity of the cells (Barofsky *et al*., 2009). To shed more light on the composition of the diatom exudate we assessed amino acids, carbohydrates

and a large fraction of so far unknown organic compounds, applying the untargeted ultra-high resolution FT-ICR-MS. Our results revealed that TDAA, DFNCHO and DCNCHO constitute 11.5% of the exuded DOC. Carbohydrates constituted 7.8% which appears to be a low proportion as compared to reports that this class of biopolymers can constitute variable but even more than 50% of algal-derived and labile DOM (Biersmith and Benner 1999; Weiss and Simon 1999). However, it must be kept in mind that we focused on a very specific component, the exudate of a growing diatom and applied an approach which only detects neutral hexoses and pentoses (Mopper *et al*., 1992) whereas the approaches mentioned either applied a bulk method for carbohydrate analysis (Biersmith and Benner 1999) or HCl for hydrolysis of combined carbohydrates (Weiss and Simon 1999) instead of the more rigorous sulfuric acid hydrolysis we applied. A direct comparison of the bulk method and the HPLC-method showed that the former yields at least 2-3 fold higher values (Mopper *et al*., 1995), presumably because other carbohydrates are included as well, such as tetroses, acidic pentoses and hexoses. Saad *et al*., (2016) analysed the DOM produced by *T. pseudonana* in the late exponential phase by 13C-NMR after SPE and reported that it was largely dominated by amino acids and carbohydrates constituted only around 30%. Because of the sampling time and the completely different methods applied we assume that these results cannot be compared to our results. Further, it is known that *T. pseudonana* produces and releases a variety of organic sulfur compounds including dimethylsulfoniumpropionate and dihydroxypropanesulfonate (Durham *et al*., 2015). From transcriptomic expression patterns of *R. pomeroyi* growing in co-culture with *T. pseudonana*, it can be inferred that exudates of this diatom include dihydroxypropanesulfonate, glycolate, ectoin and carboxylic acids (Landa *et al*., 2014). Concentrations and their relative proportions of total exudates are unknown and we did not detect them with our approaches. Hence, there is good evidence that the DOM of *T. pseudonana* encompasses for more compounds than assessed by our approaches.

The low SPE efficiency we obtained (8.9 ± 1.3%) indicates a high fraction of highly-labile polar organic compounds or colloidal material in the diatom exudate that were presumably not retained by our extraction method. Longnecker *et al*., (2015) also assessed the composition of the exometabolome of *T. pseudonana* by FT-ICR-MS and SPE with an extraction efficiency of 14 – 41% but obtained their samples by filtration thus including a more diverse blend of organic compounds. We assume that our very specific approach to only obtain compounds exuded by intact cells led to this low extraction efficiency. An indication for this high specificity is the fact that Longnecker *et al*., (2015) detected 20% more MF than us even though they used an FT-ICR-MS instrument with only half the sensitivity of ours. Hence, even missing a large fraction of the

exuded DOM our results emphasize that single bacterial strains consuming the exudates of *T. pseudonana* have a great and strains-specific impact on diversifying its composition. Our results thus provide conservative numbers for this diversification process.

Distinct bacterial diversification patterns of diatom exudates

In the ocean, the biogeochemical fate of phytoplankton exudates is largely controlled by the ambient heterotrophic bacterioplankton community which consumes it to a great extent but also transforms it partially into more refractory compounds (Ogawa *et al*., 2001; Lechtenfeld *et al*., 2015; Osterholz *et al*., 2015). The role of single bacteria to consume and transform individual organic compounds exuded by vital phytoplankton algae, however, is rather unknown. Our findings demonstrate that, in addition to the well-known ability to consume TDAA, DFNCHO (Hahnke *et al*., 2013a; Drüppel *et al*., 2014; Wiegmann *et al*., 2014) and to a lesser extent also DCNCHO strains of the *Roseobacter* group diversified the exuded DOM with s strain-specific signature. The rather low degradation of DCNCHO by the three strains reflects the fact that members of the *Roseobacter* group including the three model strains are deficient in hydrolysing polysaccharides (González *et al*., 2003; Biebl *et al*., 2005; Hahnke *et al*., 2013b). Presumably the three strains just took up oligosaccharides which did not need any hydrolysis prior to uptake into the cell.

Assessing the processing of the SPE-DOM fraction of the *T. pseudonana*-derived exudate, we observed unique transformation patterns resulting from degradation and production of distinct MF by each strain. To the best of our knowledge, this decisive process of individual bacteria with large scale implications on structuring the DOM in the oceans has never been examined in such accuracy, tracing changes in the diverse pool of hundreds of individual DOM molecules. Most previous studies focused on the removal and consumption of bulk DOM and some of its constituents (Pedler *et al.*, 2014; Sarmento & Gasol 2012; Polimene *et al.,* 2017; Horňák *et al.,* 2017) and only few studies focused on the production of new compounds with a focus on diversifying the DOM pool (Jiao *et al*., 2011; Romano *et al*., 2014; Johnson *et al*., 2016; Wienhausen *et al*. 2017). In our experiments we examined specifically how strains of the *Roseobacter* group modified the exudate of the growing *T. pseudonana.* In total 16 to 36% of the approximately 1300 MF we detected underwent modifications by the three strains, mainly by producing new compounds. In fact, all three strains produced more MF than they consumed but *P. inhibens* exhibited the greatest impact, in particular by producing 1.8 and 1.5 times more new exometabolites than *D. shibae* and *R. pomeroyi*, respectively. The three strains exhibited further differences in their impact on the H/C and O/C ratios thus demonstrating a strain-

specific imprint on the diatom-derived exudate. However, the three strains appeared to focus their impact on a rather similar fraction of MF because the MF which did not change were rather similar among them and accounted for more than 30% of diatom exudate. This may reflect that all of them affiliate to the *Roseobacter* group and members of other phylogenetic groups presumably target other fractions of such an exudate. Interactions with those bacteria may be necessary to consume and decompose these compounds. On the other hand it cannot be ruled out that they may include compounds which are more refractory and therefore not taken up and decomposed by the strains under the given conditions. Interestingly, the greatest impact of *P. inhibens* on diversifying the exudate is not reflected by its genomic features as compared to the other two strains. *P. inhibens* has a smaller genome and lower number of genes than the other strains and *R. pomeroyi* exhibits the largest genome, the largest number of transport proteins and the largest number of genes in the COG (cluster of orthologous groups or proteins) categories amino acids transport and metabolism and secondary metabolites biosynthesis, transport and catabolism (Table S1).

Our data suggest a profound specialization of fairly closely related bacterial strains on the degradation and turnover of the rather complex exudates produced by actively growing phytoplankton algae in the ocean. The impact on the total DOM including also other fractions, such as of senescent and lysing cells, in diversifying it presumably is even more critical. Our findings suggest a very diverse and fine-tuned alignment of single bacteria to variable C-sources allocated in marine pelagic systems and illustrate that distinct bacterial populations occupy specific niches with respect to their substrate spectra and impact on DOM cycling. This is consistent with the activity and compositional bacterioplankton community changes in response to phytoplankton-derived DOM availability (Teeling *et al*., 2012; 2016; Grossart *et al*., 2005; Wear *et al*., 2015; Dinasquet *et al.,* 2013) and provides an explanation on a molecular chemical level.

As compared to the diversity of exometabolomes of bacterial strains released by growing on single carbon sources the diversity of the diatom exudate was rather low. This may have been the result of the low efficiency of SPE (see above) but may be also a characteristic of the specific nature of the exuded diatom-derived DOM. In various studies, it has been shown that bacterial strains can produce more than 2500 and up to 5000 different MF when growing on a defined mixed or even single carbon source (Romano *et al*., 2014; Johnson *et al*., 2016; Wienhausen *et al*., 2017, Noriega-Ortega *et al*., in submission) as compared to only approximately 1300 MF of the diatom exudate and processed by the bacterial strains in this study. Hence the potential to diversify one or a few carbon sources by a marine bacterium is even greater than indicated by

the results of this study and further emphasizes the significance of this process for structuring the DOM in marine pelagic systems.

Conclusion

We have provided quantitative evidence that individual marine bacterial strains diversify diatom-derived freshly released labile DOM and exhibit distinct transformation patterns while decomposing it, mainly by producing hundreds of new exometabolites. Hence, even individual marine bacteria have a strong impact on the diversification of DOM in marine pelagic ecosystems. The interplay of bacteria with different metabolic traits presumably further transform and diversify the DOM which, together with other non-biological processes such as photochemistry, eventually results in the typical composition and patterns of semi-labile, semi-refractory and refractory DOM (Hansell, 2013) persisting for millennia in the oceans.

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Supplementary information:

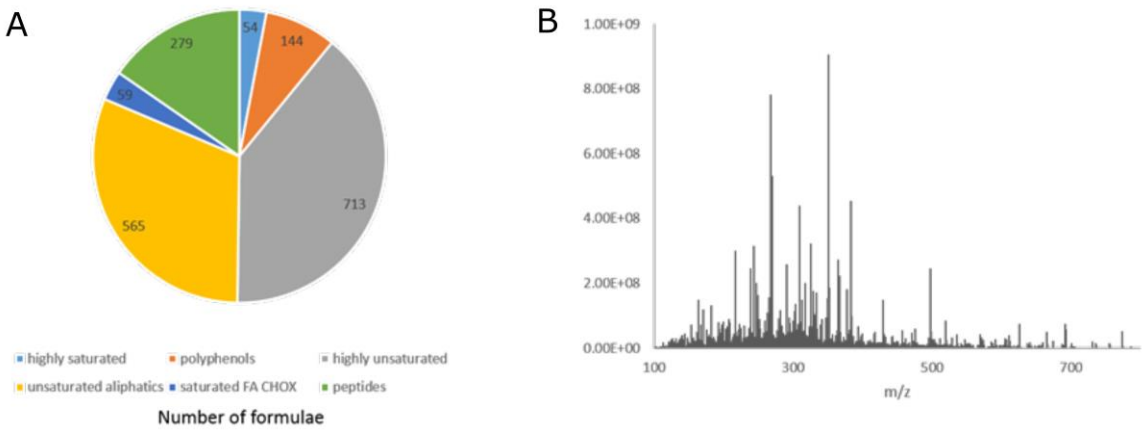
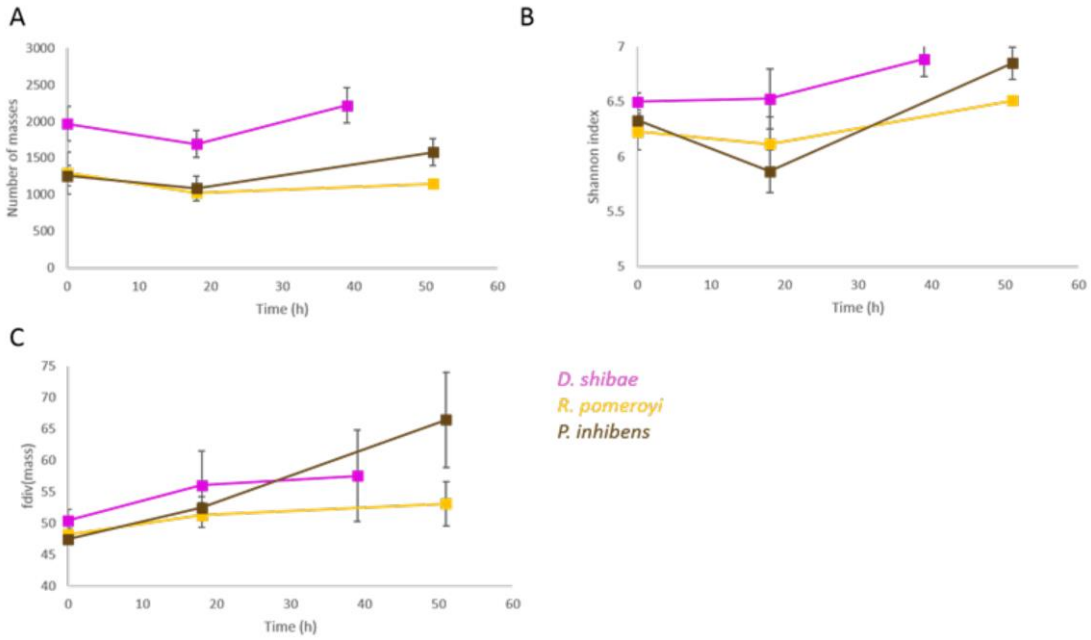
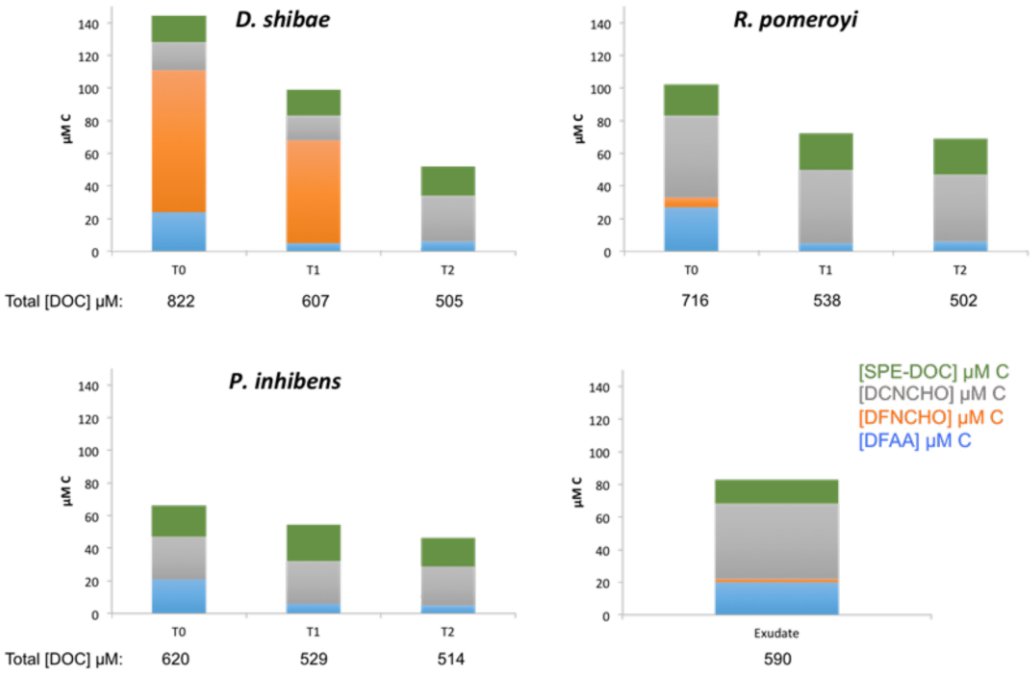


Figure Supplementary figure S1 | *T. pseudonana* exudate composition: A) Molecular chemical classification given as molecular formulae detected by FT-ICR-MS. B) *T. pseudonana* exudate spectrum depicting the averaged intensities of all exudate samples after contamination and noise removal.



Supplementary figure S2 | Diversity measures calculated from the normalized intensities of the masses detected by FT-ICR-MS in the triplicate cultures of *D. shibae*, *R. pomeroyi* and *P. inhibens.* Values are shown as the mean of triplicates and the error bars represent the standard variation. A: richness given by the number of molecular masses; B: Shannon index; C: functional chemical diversity index as a function of molecular mass.



Supplementary figure S3 | Stacked bars of fractions of total DOC detected by the applied methods in the cultures of *D. shibae*, *R. pomeroyi* and *P. inhibens* at time points T0, T1 and T2 (for exact hours of incubation see Figure 1B). SPE-DOC (green): solid phase extractable DOC; DCNCHO (grey): dissolved combined neutral carbohydrates; DFNCHO (orange): dissolved free neutral carbohydrates; TDAA (blue): total dissolved amino acids.

Supplementary table S1 | Genome size, number of total and transport protein coding genes and genes associated to COG categories according to the database IMG.

Genetic characteristics		***D. shibae***	***R. pomeroyi***	***P. inhibens***
Genome sizes (Mbp)		4.42	4.60	4.23
Genes		4271	4355	3960
Transport protein coding genes		322	535	401
COG category	Amino acid transport and metabolism	252	203	203
	Carbohydrate transport and metabolism	102	148	104
	Secondary metabolites biosynthesis, transport and catabolism	407	447	369

Chapter 3

Exometabolome of Two Model Strains of the *Roseobacter* Group: A Marketplace of Microbial Metabolites

Gerrit Wienhausen[1], Beatriz E. Noriega-Ortega[1], Jutta Niggemann[1], Thorsten Dittmar[1] and Meinhard Simon[1]

[1] Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Oldenburg, Germany

Keywords: *Roseobacter* group, DOM, exometabolome, black queen hypothesis

Subject category: Integrated genomics and post-genomics approaches in microbial ^ ecology

Correspondence: Meinhard Simon, Institute for Chemistry and Biology of the Marine Environment, Carl-von-Ossietzky Straße 9-11, Oldenburg, Germany. E-mail: m.simon@icbm.de

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The Exometabolome of Two Model Strains of the *Roseobacter* Group: A Marketplace of Microbial Metabolites

*Gerrit Wienhausen, Beatriz E. Noriega-Ortega, Jutta Niggemann, Thorsten Dittmar and Meinhard Simon**

Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Oldenburg, Germany

Recent studies applying Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) showed that the exometabolome of marine bacteria is composed of a surprisingly high molecular diversity. To shed more light on how this diversity is generated we examined the exometabolome of two model strains of the *Roseobacter* group, *Phaeobacter inhibens* and *Dinoroseobacter shibae*, grown on glutamate, glucose, acetate or succinate by FT-ICR-MS. We detected 2,767 and 3,354 molecular formulas in the exometabolome of each strain and 67 and 84 matched genome-predicted metabolites of *P. inhibens* and *D. shibae,* respectively. The annotated compounds include late precursors of biosynthetic pathways of vitamins B_1, B_2, B_5, B_6, B_7, B_{12}, amino acids, quorum sensing-related compounds, indole acetic acid and methyl-(indole-3-yl) acetic acid. Several formulas were also found in phytoplankton blooms. To shed more light on the effects of some of the precursors we supplemented two B_1 prototrophic diatoms with the detected precursor of vitamin B_1 HET (4-methyl-5-(β-hydroxyethyl)thiazole) and HMP (4-amino-5-hydroxymethyl-2-methylpyrimidine) and found that their growth was stimulated. Our findings indicate that both strains and other bacteria excreting a similar wealth of metabolites may function as important helpers to auxotrophic and prototrophic marine microbes by supplying growth factors and biosynthetic precursors.

Keywords: roseobacter, DOM, exometabolome, black queen hypothesis

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Torsten Thomas,
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****Correspondence:***
Meinhard Simon
m.simon@icbm.de

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INTRODUCTION

The biochemical processes in a living and active prokaryotic cell yield a highly complex blend of metabolites reflecting the catabolic, metabolic and anabolic properties of the organism. On the basis of available genomic information on the metabolic potential, metabolite patterns within the cell, endometabolomics, have been investigated during the recent past in various prokaryotes and as a function of substrate source and growth conditions (Rosselló-Mora et al., 2008; Zech et al., 2009; Frimmersdorf et al., 2010; Paczia et al., 2012; Drüppel et al., 2014). The exometabolome, i.e. the pool of metabolites released into the cell's environment, has also been investigated, showing species- or even ecotype-specific fingerprints as a function of growth stage and conditions (Kell et al., 2005; Villas-Bôas et al., 2006; Paul et al., 2009; Paczia et al., 2012; Romano et al., 2014; Fiore et al., 2015; Johnson et al., 2016).

Most of these studies applied targeted approaches mainly gas chromatography–mass spectrometry (GC–MS), searching for metabolites to be expected from predicted substrate use and metabolic pathways (Villas-Bôas et al., 2006; Zech et al., 2009; Drüppel et al., 2014). Several studies

have used Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS) applying non-targeted approaches and some combined both. They found that the diversity of the endo- and in particular the exometabolome is far higher than expected from targeted approaches, yielding several thousand molecular masses (Rosselló-Mora et al., 2008; Brito-Echeverría et al., 2011; Romano et al., 2014; Fiore et al., 2015; Johnson et al., 2016). Besides the expected metabolites, the metabolome obviously includes a surprisingly high proportion of compounds not expected from predicted metabolic pathways. In particular the exometabolome exhibited a wealth of metabolites, many with so far unknown molecular masses and elemental composition (Rosselló-Mora et al., 2008; Kujawinski et al., 2009; Romano et al., 2014; Fiore et al., 2015). There is some evidence that quite a few of these metabolites are released as a result of an overflow metabolism due to growth on an abundant carbon source (Paczia et al., 2012; Romano et al., 2014). However, under less excessive growth conditions, in addition to well-known exometabolites such as signaling compounds (Dickschat, 2010; Hartmann and Schikora, 2012), vitamins (Sañudo-Wilhelmy et al., 2014), siderophores (Mansson et al., 2011), many other unexpected metabolites are released into the environment. The bacterial secretion of several such microbial metabolites, including the plant hormone indole 3-acetic acid (IAA) and vitamin precursors, has been documented (Zhang et al., 2014; Amin et al., 2015; Fiore et al., 2015; Johnson et al., 2016; Paerl et al., 2017). Analyses of dissolved organic matter (DOM) from Sargasso Sea waters detected several compounds with molecular masses known to be released by a strain of the globally abundant SAR11 clade (Kujawinski et al., 2009). The thiamine (vitamin B_1) precursor 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) was also detected. This precursor is essential for thiamine biosynthesis by members of the SAR11 clade because they lack the gene for its complete biosynthesis (Carini et al., 2014). Thiamine requirements by photoautotrophic eukaryotes can also be met when the phosphorylated form, thiamine diphosphate, is dephosphorylated by a partner. In a bioassay approach Paerl et al. (2015) showed that the thiamine-auxotrophic picoeukaryote *Ostreococcus* sp. could grow when supplied with thiamine diphosphate in the presence of an *Alteromonas* strain that exhibit phosphatase activity.

The exchange of metabolites and precursors between microbes appears to be more common because auxotrophy is much wider distributed among microbes than previously assumed (McRose et al., 2014; Garcia et al., 2015; Paerl et al., 2017). Growth of such organisms depends on mutualistic interactions and supply of metabolites by co-occurring microbes. The Black Queen hypothesis applied this phenomenon to explain genome streamlining of prokaryotes by deleting certain metabolic pathways or parts of it as a reaction to leaky metabolic pathways of other microbes, helpers, thus supplying metabolites or precursors as public goods to the auxotrophic prokaryotes (Morris et al., 2012). It has been proposed most recently that gene loss and niche partitioning may be major drivers in the co-evolution of auxotrophs and helpers (Mas et al., 2016). However, considering the metabolites found in the exometabolome of various microbes and the environment in recent studies it appears that exchange of metabolites has larger ramifications than just explaining genome streamlining of selected microbes (Zelezniak et al., 2015; Estrela et al., 2016). We hypothesize that the exometabolome of helpers includes multiple metabolites and precursors, not only vitamins and growth factors, which are beneficial for other microbes. This release may promote growth of auxotrophic organism and even enhance growth of prototrophic microbes because they may not need to allocate energy to synthesize these precursors.

In order to test this hypothesis we assessed the exometabolome of two model bacteria of the marine *Roseobacter* group, *Phaeobacter inhibens* DSM 17395 and *Dinoroseobacter shibae* DSM 16493, growing on either glucose, glutamate and acetate or succinate, by analysis on a 15 Tesla FT-ICR-MS. *Phaeobacter inhibens* DSM 17395 is a purely heterotrophic bacterium growing in biofilms (Thole et al., 2012; Gram et al., 2015). Strains of this species and the genus *Phaeobacter* have been found in various marine habitats, such as natural biofilms on solid surfaces and during a bloom of *Emiliana huxleyi* (Gifford et al., 2014; Gram et al., 2015; Segev et al., 2016; Breider et al., 2017). *Dinoroseobacter shibae* is photoheterotrophic, grows symbiotically with dinoflagellates (Wagner-Döbler et al., 2010), produces various signaling compounds (Neumann et al., 2013) and has also been found associated to algae in natural phytoplankton blooms (Gifford et al., 2014; Milici et al., 2016; Segev et al., 2016). In order to examine whether identified molecular masses, and more precisely formulas, are also relevant under natural conditions, we screened DOM samples analyzed by FT-ICR-MS from a mesocosm experiment (Osterholz et al., 2015) and the North Sea. It is the first application of such a powerful FT-ICR-MS for exometabolomic studies in combination with the search for molecular formulas in environmental samples thus greatly enhancing the sensitivity and resolution of metabolite identification.

MATERIALS AND METHODS

Growth Conditions

Phaeobacter inhibens DSM 17395 and *D. shibae* DSM 16493 were first grown on marine broth (MB; Difco MB 2216) medium and afterwards repeatedly (5x) transferred and cultivated in artificial seawater (ASW) medium with the addition of a single organic carbon source. Before every transfer and after centrifugation of the cultures the cell pellets were washed three times with ASW medium. All plastic and glass ware used were rinsed with acidified ultrapure water (MilliQ, pH 2) and all glassware additionally combusted for 3 h at 500°C.

The ASW-medium for the *P. inhibens* cultures was prepared as described by Zech et al. (2009) but slightly modified by excluding EDTA from the trace element solution. The cultures were supplemented with glucose (5 mM, 30 mM C; ultrapure brand), acetate (30 mM, 60 mM C; ultrapure brand) or glutamate (20 mM, 100 mM C; ultrapure brand). *Dinoroseobacter shibae* was cultivated in ASW-medium (Soora and Cypionka, 2013) with the same concentrations of glucose as for *P. inhibens* and of glutamate at 7.5 mM (37.5 mM C). Instead of acetate, succinate was used at an initial concentration of 10 mM (40 mM C;

ultrapure brand). Concentrations of the substrates were adjusted due to varying metabolic rate efficiencies to obtain rather equal growth yields as determined by optical density (OD_{600}). Bacteria were cultivated in 500 ml of medium in triplicate 2 liter baffled Erlenmeyer glass flasks at pH 8 at 28°C in the dark on a shaker (100 rpm) and growth was monitored by OD. A sterile flask with media and the respective single carbon source was run as control. Subsamples were withdrawn under laminar flow for the separate analysis of the replicates for dissolved organic carbon, exometabolome-DOM, dissolved free (DFAA) and dissolved combined amino acids (DCAA), dissolved free neutral monosaccharides (DFNCHO), and dissolved combined monosaccharides (DCNCHO) and fatty acids at the start, the lag phase, the mid-exponential and early stationary phase in order to recover the majority of exometabolites released at varying growth conditions. The growth patterns were assessed more precisely than by OD measurements by subsampling every 4–8 h depending on the growth phase patterns for bacterial cell enumeration. Cells were fixed with 2% glutaraldehyde and frozen at −20°C until further analysis.

In order to test the effect of the vitamin B_1 precursors HMP (AstaTech inc., Bristol, PA, USA) and HET [4-methyl-5-(β-hydroxyethyl)thiazole; Sigma Aldrich, Munich, Germany] on the growth of the diatoms *Thalassiosira pseudonana* (CCMP 1335) and *Leptocylindrus danicus* (CCMP 470) these diatoms were grown axenically in ASW medium in a 12:12 h light dark cycle and illuminated at 70 µE. Instead of B_1, the precursors were added at 100 nM final concentration together with vitamins B_7 and B_{12} (100 nM each) and growth was monitored as relative fluorescence against a positive control including all three vitamins at final concentrations of 100 nM each and a negative control including only B_7 and B_{12} at the same concentrations. Axenicity of the diatom cultures was checked microscopically.

Cell Abundance

Cells of the *D. shibae* cultures were enumerated by flow cytometry, those of the *P. inhibens* cultures by epifluorescence microscopy due to the formation of microaggregates. Flow cytometric analyses were done according to Osterholz et al. (2015). Cell aggregates of *P. inhibens* were dispersed by ultrasonication (5 × 10 s at 15 mV; Bandelin Sonopuls HD 200, Bandelin, Berlin, Germany), filtered onto a 0.2 µm polycarbonate membrane, stained for 30 min with SYBR® Green I and counted by epifluorescence microscopy as described (Lunau et al., 2005). At least 1,000 cells were enumerated per filter.

Amino Acids, Mono- and Polysaccharides and Fatty Acids

Aliquots of the cultures were centrifuged at 2,499 g in acid washed and combusted (3 h, 500°C) glass centrifuge tubes. The supernatant was filtered through a 0.22 µm polyethersulfone membrane (Minisart, Sartorius, Göttingen, Germany) and the filtrate stored in combusted 20 ml glass vials at −20°C until further analysis. Concentrations of DFAA and DCAA were analyzed by high performance liquid chromatography (HPLC) after precolumn derivatization with orthophtaldialdehyde (Lunau et al., 2006) and concentrations of DFNCHO and DCNCHO by HPLC and pulsed amperometric detection after desalting (Hahnke et al., 2013). Detection limits for DFAA and DFNCHO were 0.5 and 1.5 nM, respectively. Fatty acid concentrations in the treatments with additions of succinate and acetate were determined by HPLC (Sykam, Fürstenfeldbruck, Germany) equipped with an Aminex HPX-87H column (Biorad, München, Germany) (Graue et al., 2012).

DOM Analyses

Dissolved organic carbon in the filtrates of the bacterial cultures and of the solid-phase extracted DOM (see below) was quantified as described previously (Osterholz et al., 2014). For FT-ICR-MS analyses, filtrates were acidified to pH 2 (HCl 25% p.a., Carl Roth, Germany), extracted via PPL solid phase cartridges (100 mg; Agilent, Waldbronn, Germany) adapted to a concentration of 15 ppm carbon and analyzed by FT-ICR-MS according to Osterholz et al. (2015). The extraction efficiency increased from 2 to 31% on carbon basis in the course of the experiment, as result of the contrary running substrate availability. Extracted DOM was ionized by soft electrospray ionization (Bruker Apollo, Daltonics, Bremen, Germany) and analyzed in positive and negative mode with a 15 T Solarix FT-ICR-MS (Bruker, Daltonics, Bremen, Germany). For each spectrum 300 scans were accumulated in the mass window of 92 to 2,000 Da. An internal calibration list was generated using Bruker Daltonic Data Analysis software for the calibration of the spectra. FT-ICR-MS instrument performance was verified using a laboratory-internal deep ocean DOM reference sample. Detected mass to charge ratios were processed applying a customized routine Matlab script. Molecular formulas were assigned as described by Koch and Dittmar (2006) to molecular masses with a minimum signal-to-noise ratio of 5 (Koch et al., 2007). From the mass spectrograms of the single time points of each culture that of the respective sterile control was subtracted.

Exometabolite Fragmentation

To confirm structures of genome-predicted identified exometabolites, we performed fragmentation experiments using FT-ICR-MS. Therefore, both bacterial strains were cultured again at similar conditions as described above for the exometabolome experiments but the exometabolome was harvested at the time point of the peak concentration of the respective exometabolite. A total of 500 mL was extracted via PPL solid phase cartridges as described above. Extracts were redissolved in a 1:1 MilliQ water/methanol solution at a concentration of 29 ppm carbon and analyzed on the FT-ICR-MS as described above. We selected the exometabolites which were identified as precursors or products of biosynthetic pathways for fragmentation, i.e., 43 of the 107 exometabolites identified in total. However, only seven metabolites had sufficiently high signal intensities in the FT-ICR-MS to perform fragmentation experiments. Limitations to fragmentation experiments include the fact that fragments may not be ionizable and different fragments with rather similar masses may yield overlapping peaks. Exometabolites of interest were isolated in a 1 Da window using the quadrupole unit and collision with argon occurred in the hexapole unit of the FT-ICR-MS. Fragmentation parameters

were optimized for each mass with an isolation window ranging from 0.1 to 1 (m/z), collision energy adjusted by applying 10 to 15 mV and 300 to 700 broadband scans were accumulated per run.

Exometabolite Prediction from Genome Databases and Screening against Natural DOM Samples

Metabolites of *P. inhibens* and *D. shibae*, predicted by the genome database BioCyc (Caspi et al., 2012), were listed with their corresponding molecular masses and molecular formulae (MF). All MF calculated from FT-ICR-MS detected masses were scanned against the genome-based metabolite prediction list. Matches were analyzed in more detail, regarding intra and extracellular function identified by previous studies. Putatively identified MF of exometabolites were screened against MF of DOM data sets of naturally derived and North Sea phytoplankton blooms (Osterholz et al., 2015, 2016; Noriega-Ortega et al., in preparation).

RESULTS

Both model strains of the *Roseobacter* group were grown in batch culture on single carbon sources of three major substrate classes to examine the diversification of the exometabolome as a function of these different substrates. Here we focus on the identified exometabolites whereas the overall exometabolome and its diversity is dealt with in a different publication (Noriega-Ortega et al., in preparation)

Growth and Substrate Utilization

Phaeobacter inhibens reached highest growth with maximum ODs of 0.76, 1.04, and 1.96 on acetate, glucose and glutamate respectively, at 68, 56, and 20 h (**Figure 1**). Respective cell numbers at these time points were 1.4×10^9, 0.6×10^9, and 3.4×10^9 cells mL^{-1} (see supporting information Figure S1). Growth of *D. shibae* reached highest ODs of 1.11, 1.03, and 0.90 on succinate, glucose and glutamate respectively, at 23, 65, and 39 h (**Figure 1**) with corresponding cell numbers of 2.1×10^9, 0.8×10^9, and 3.3×10^9 cells mL^{-1} respectively (see supporting information Figure S1). Substrate concentrations decreased inversely to growth of both bacteria and went below detection limit in the stationary phase except for the culture of *D. shibae* growing on glucose (**Figure 1**).

Amino Acids in the Exometabolome

The analysis of dissolved amino acids within the exometabolome of both strains was biased in the glutamate treatments by the interfering large glutamate peak. Hence, concentrations could only be measured in the stationary phase when glutamate was completely utilized. In the *P. inhibens* cultures, concentrations of DFAA remained below the detection limit of the HPLC analysis at all growth phases but tryptophan, tyrosine and histidine were detected by the FT-ICR-MS analysis (see below). Concentrations of DCAA in the treatment with acetate remained below 4 μM during the exponential growth phase but reached 235.4 μM in the stationary phase (**Figure 2F**). In the treatment with glucose, DCAA concentrations increased continuously during the exponential and stationary phase but reached a final concentration of only 15.6 μM (**Figure 2E**). The glutamate treatment of this culture yielded a concentration of 684.9 μM in the stationary phase (**Figure 2D**). The substrate source had only a minor influence on the composition of the amino acid pool in the exometabolome. During the exponential and stationary phases aspartate, glutamate, glycine, and alanine constituted the highest mol% of DCAA and together at least 60 mol% (**Figure 2**, Table S1). The very high DCAA concentrations in the exometabolome may have been a result of cell lysis. To estimate the extent of potential lysis, we made a mass balance of carbon (C) in DCAA and in the bacterial biomass in the stationary phase. DCAA concentrations of 219.8 and 639.0 μM in the treatments with acetate and glutamate in the stationary phase translate into 11.5 and 32.8 mg C L^{-1}, respectively. On the basis of 50 fg C per cell of large bacteria (Simon and Azam, 1989), typical for fast growing cultures, the bacterial numbers of 1.39×10^9 and 3.44×10^9 cells mL^{-1} in the stationary phase in the acetate and glutamate treatments, respectively, equal 69.5 and 160.9 mg C L^{-1}. Hence, the C bound in DCAA comprises 17.1 and 20.6% of the C bound in bacterial biomass. Therefore, we conclude that at these two conditions, but neither at other conditions nor in the glucose treatments, protein released by lysed cells contributed to the high DCAA concentrations in the stationary phase of the *P. inhibens* culture. Consequently, these time points were not considered for further exometabolome analyses.

In the *D. shibae* cultures, DFAA concentrations were below the detection limit of the HPLC analyses at all growth phases, but tryptophan, tyrosine, phenylalanine and histidine were detected by FT-ICR-MS (see below). Concentrations of DCAA in the exometabolome of the *D. shibae* cultures continuously increased during the exponential growth phase and reached 12.0 and 14.7 μM in the glucose and succinate treatments in the stationary phase (**Figure 3**). The glutamate treatment yielded 23.0 μM in the stationary phase (**Figure 3D**). Glutamate, glycine and alanine dominated the DCAA pool at all substrate conditions and constituted >50 mol% (Table S2). A mass balance of carbon bound in DCAA and the bacterial biomass in the stationary phase of the three treatments showed that DCAA constituted always <1% of the C bound in the biomass of the *D. shibae* cultures. Hence, cell lysis was minimal in these cultures.

Mono- and Polysaccharides in the Exometabolome

The analysis of dissolved mono- and polysaccharides interfered with the addition of glucose as single substrate source. DFNCHO were not detected in the exometabolome at any growth stage of both strains. Concentrations of DCNCHO increased continuously during growth of both strains on glutamate and acetate or succinate with highest concentrations in the stationary phase (**Figures 2**, **3**). Released DCNCHO in the *P. inhibens* cultures were greatly dominated by glucose but galactose, rhamnose, and fucose constituted proportions of up to 22

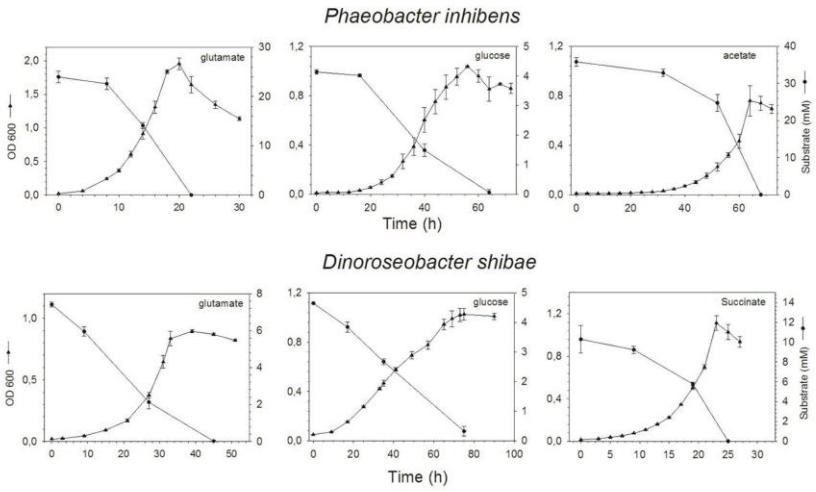

FIGURE 1 | Optical density and concentration of the substrates glutamate, glucose and acetate or succinate over time of *P. inhibens* **(upper)** and *D. shibae* **(lower)**. Note the different scales of the axes.

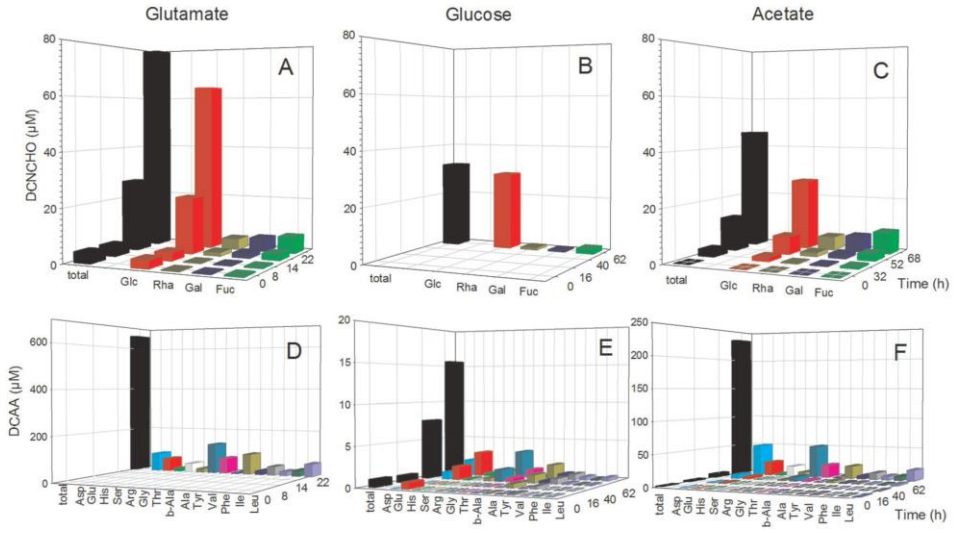

FIGURE 2 | Concentrations of individual neutral monosaccharides and amino acids (AA) bound in total dissolved combined neutral monosaccharides (DCNCHO) and total dissolved combined amino acids (DCAA), respectively, in the *P. inhibens* cultures. DCNCHO **(A–C)** and DCAA **(D–F)** right after inoculation, in the lag, exponential and stationary phase of *P. inhibens* growing on glutamate **(A,D)**, glucose **(B,E)** and acetate **(C,F)**. Note the different scales of the parameters in the different panels. Concentrations of DCNCHO in the treatment with glucose are not available until the stationary phase at 62 h due to interference with the HPLC analysis. Concentrations of AA in the glutamate treatment are only available for 22 h in the stationary phase because high glutamate concentrations until the exponential phase interfered with the HPLC analysis.

mol% in the treatment with acetate (**Figure 2**, Table S2). In the *D. shibae* cultures, DCNCHO concentrations remained lower than in those of *P. inhibens* (**Figure 3**). Only glucose, galactose and rhamnose were detected in the exometabolome. In the succinate treatment, rhamnose and galactose dominated whereas in the glutamate treatment galactose became the dominant

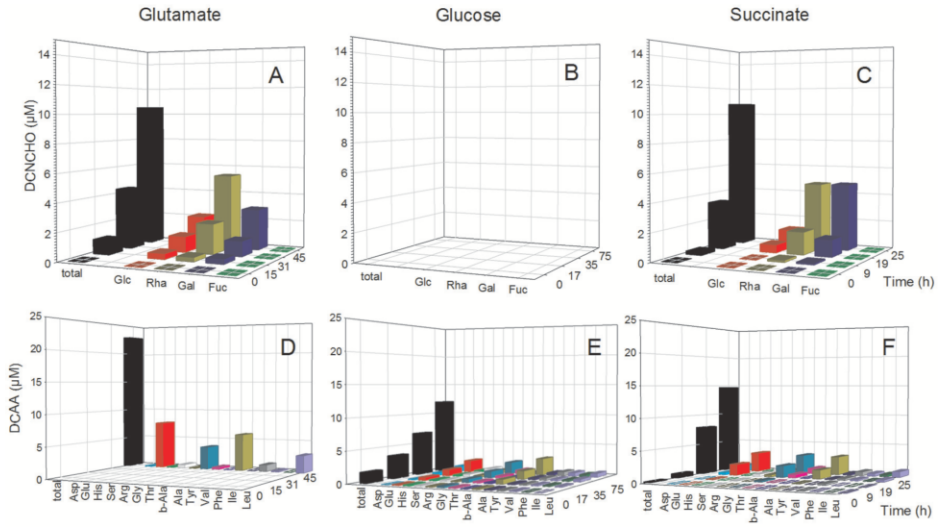

FIGURE 3 | Concentrations of individual neutral monosaccharides and amino acids (AA) bound in total dissolved combined neutral monosaccharides (DCNCHO) and total dissolved combined amino acids (DCAA), respectively, in the *D. shibae* cultures. DCNCHO **(A–C)** and DCAA **(D–F)** right after inoculation, in the lag, exponential and stationary phase of *D. shibae* growing on glutamate **(A,D)**, glucose **(B,E)** and succinate **(C,F)**. Concentrations of CHO in the treatment with glucose are not available due to interference with the HPLC analysis. Concentrations of DCAA in the glutamate treatment are only available for 45 h in the stationary phase because high glutamate concentrations until 31 h interfered with the HPLC analysis.

DCNCHO component in the exponential and stationary phase (Table S2).

Exometabolome Diversity and Exometabolite Identification

Applying the ultrahigh resolution FT-ICR-MS, we detected in total 2767 MF in the exometabolome of *P. inhibens* and 3,354 MF in that of *D. shibae* (**Figure 4**; Tables S3, S4). These numbers include all growth stages and all substrate treatments of both strains, except the stationary phase of *P. inhibens* grown on glutamate and acetate due to suspected cell lysis. The composition of the exometabolome, but not the exometabolites identified (see below) of both strains varied considerably as a function of the substrate utilized and growth stage (Noriega-Ortega et al., in preparation). Scanning all detected exometabolomic MF against the genome-predicted metabolites of *P. inhibens* and *D. shibae* by applying the BioCyc database revealed a match for 67 and 84 exometabolites, respectively (**Figure 4**). In addition, secondary metabolites known to be produced by *P. inhibens* and *D. shibae* were scanned against the exometabolomic MF obtained by FT-ICR-MS, yielding 4 identified compounds in the exometabolome of *P. inhibens* but none in that of *D. shibae* (**Table 1**). Identified exometabolites were further subdivided on the basis of their function and occurrence within the bacterial metabolism (**Figure 4**). For *D. shibae*, 35 metabolites were assigned to biosynthetic pathways (**Table 1**), 16 metabolites to degradation pathways (Table S5) and 33 metabolites originated from spontaneous non-enzymatic chemical-reactions (Table S6). For *P. inhibens*, 36 metabolites were assigned to biosynthetic pathways (**Table 1**), 15 to degradation pathways (Table S5), but only 16 derived from spontaneous non-enzymatic chemical reactions (Table S6). In total 43 different exometabolites of biosynthetic pathways were identified of which 28 were present in the exometabolome of both strains. In the *D. shibae* experiments 19 exametabolites assigned to biosynthetic pathways were detected in >50% of the time points sampled and in the experiments with *P. inhibens* 16 exometabolites. Alpha-ribazol was detected only at three time points in the exometabolome of *D. shibae* but in 88% of the time points of the *P. inhibens* experiments. Pyridoxal-P and histidine were detected only once in the exometabolome of the *D. shibae* experiments but in 43 and 55% of that of the *P. inhibens* experiments, respectively. HET was detected only once in the exometabolome of *P. inhibens* experiments but in 58% of that of the *D. shibae* experiments. All other exometabolites were detected in 10–50% of the samples analyzed in the experiments of both strains.

The detailed analysis showed that 12 (34%) and 10 (28%) of the annotated compounds linked to metabolic pathways of *D. shibae* and *P. inhibens*, respectively, were B vitamins and/or late precursors of biosynthetic pathways of vitamins B_1, B_2, B_5, B_6, B_7, and B_{12} (**Figure 5**, **Table 1**). Five (14%) and 6 (18%) of the annotated compounds were putatively quorum sensing-related metabolites and 10 (29%) and 9 (25%) amino acids and precursors of their biosynthetic pathways (**Figure 5**). Methyl-(indole-3-yl) acetate and tryptophan

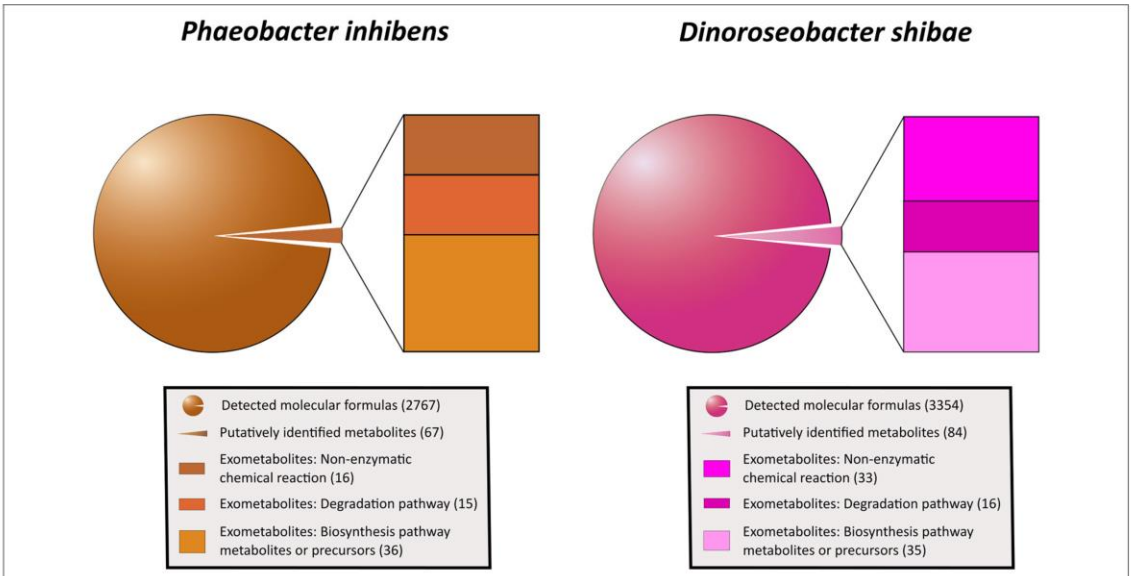

FIGURE 4 | Subdivision of all exometabolites released by *P. inhibens* and *D. shibae* during all growth phases. Given (in parenthesis) are total numbers of detected molecular formulas, putatively identified metabolites and those related to non-enzymatic chemical reactions, degradation and biosynthetic pathways and precursors.

as precursors in the biosynthetic pathways of IAA were annotated as exometabolites of both organisms whereas IAA was annotated only in that of *D. shibae*. Altogether, the complete set of annotated compounds was linked to 22 and 20 biosynthetic pathways in *D. shibae* and in *P. inhibens* respectively (**Table 1**).

To further validate the compounds annotated by the genome-based metabolite prediction approach, the respective molecular masses were isolated and fragmented via positive charge collision in the FT-ICR-MS. We confirmed the presence of 7 metabolites in the exometabolomes of *D. shibae* and *P. inhibens* respectively, including vitamins, vitamin precursors, acylated homoserine lactones (autoinducers) for quorum sensing and TDA (**Table 1**). Support for the correct annotation of the compounds was also provided by their detection in other studies applying similar experimental approaches (**Table 1**). Several identical metabolites were detected by Fiore et al. (2015), Johnson et al. (2016), annotated by Romano et al. (2014), or predicted to be needed exogenously because of lacking biosynthetic pathways in annotated bacterial genomes (Garcia et al., 2015).

Growth of Diatoms on Vitamin B1 and Precursors

In order to test the second part of our hypothesis whether identified precursors indeed enhance growth of prototrophic algae we selected two diatoms, *Thalassiosira pseudonana* and *Leptocylindrus danicus* and grew them with the supplementation of HET, HMP, HET, and HMP or B_1 and other vitamins they require (B_7, B_{12}). Supplementation of HET and HMP resulted in statistically significantly higher growth of both diatoms ($p < 0.01$; Student's *t*-test; Table S7). The growth rate and yield of *L. danicus* was enhanced by 17 and 35%, respectively, relative to a control, whereas the growth rate of *T. pseudonana* was enhanced by 22% but growth yield remained unaffected (**Figure 6**, Table S7). In both diatom cultures the growth stimulation differed among the various growth phases. The addition of vitamin B_1, surprisingly, resulted in a lower growth stimulation of both diatoms than that of the precursors and only for *T. pseudonana* the growth rate was significantly higher than that of the control (**Figure 6**, Table S7).

Identified Exometabolites in Marine DOM

To examine whether exometabolites produced by the two model strains of the *Roseobacter* group also occur in natural or naturally-derived DOM, we screened samples of a mesocosm experiment and from phytoplankton blooms in the North Sea. In the DOM of the mesocosm experiment in which a phytoplankton bloom was induced and various bacteria of the *Roseobacter* group were present (Osterholz et al., 2015), 19 MF were detected which matched those found also in the cultures of both strains. Sixteen and 15 MF were attributed to the cultures of *P. inhibens* and *D. shibae* respectively, and 14 occurred in both cultures. Eight MF included amino acids or biosynthetic precursors, three late biosynthetic precursors of vitamins and cofactors, three were autoinducers of quorum sensing and two the plant auxin IAA and its inactive form methyl-IAA (**Table 1**). In samples from in and outside phytoplankton blooms in the North Sea, 15 MF matched those found in the exometabolomes of both strains. Four of

TABLE 1 | Metabolites, their molecular formula, function, fragmentation results detected in the exometabolome of *D. shibae* and *P. inhibens*, in other studies, a mesocosm (Osterholz et al., 2015) and in a North Sea phytoplankton bloom (Noriega-Ortega et al., in preparation).

Metabolites	Molecular formula	Function	Fragmentation	*D. shibae*	*P. inhibens*	Other studies	Mesocosm	North Sea bloom
HET (4-methyl-5-(β-hydroxyethyl)thiazole)	C6H9NOS	vitamin B1 precursor		+	+			
Thiamine phosphate	C10H15N2O8P	vitamin B1		+	+	G, J		
Dimethyl-D-ribityl-lumazine	C13H18N4O6	vitamin B2 precursor	+	+	+	R		
Riboflavin	C17H20N4O6	vitamin B2	+	+	+	F, R, G, J		
Pantoate	C6H12O4	vitamin B5 precursor		+				
Pantothenate	C9H17NO5	vitamin B5		+	+	G, J		
Pyridoxal	C8H9NO3	vitamin B6 related		+	+		+	+
Pyridoxal phosphate	C8H10NO6P	vitamin B6		+	+			
Dethiobiotin	C10H17N2O3	vitamin B7 precursor			+			
Alpha-ribazole	C14H18N2O4	vitamin B12 precursor	+	+	+	R, J	+	+
Alpha-ribazole-5-phosphate	C14H19N2O7P	vitamin B12 precursor		+	+			+
Pyrroloquinoline quinone	C14H6N2O8	vit. B - cofactor	+	+				
6-(2-amino-2-carboxylatoethyl)−1,2,3,4-tetrahydroquinoline-2,4-dicarboxylate	C14H14N2O6	vit. B - cofactor		+			+	+
Methyl (indole-3-yl)acetate	C11H11NO2	IAA related		+	+		+	
Tryptophan	C11H12N2O2	IAA precursor		+	+	F, R, G	+	
Indole_acetate	C10H9NO2	IAA		+		F		
2,3-dihydroxybenzoate	C7H5O4	siderophore building block		+		F		
3-4-dihydroxybenzoate	C7H6O4	siderophore building block			+			
PAI-1 (N-(3-oxododecanoyl)-L-homoserine lactone)	C16H27NO4	quorum-sensing	+	+	+			
AAI	C12H19NO4	quorum-sensing		+	+		+	
VAI-2	C12H21NO3	quorum-sensing		+	+			
VAI-1	C10H15NO4	quorum-sensing		+	+		+	+
HAI-1	C8H13NO4	quorum-sensing		+	+		+	
N-3-hydroxydecanoyl-L-homoserine lactone	C14H25NO4	quorum-sensing	+		+	J		
Porphobilinogen	C10H14N2O4	AA derivate		+	+		+	+
Tyrosine	C9H11NO3	AA		+	+	R, G	+	+
Arogenate	C10H13NO5	AA precursor		+	+	R	+	+
4-Hydroxy-phenylpyruvate	C9H8O4	AA precursor		+	+		+	+
Phenylalanine	C9H11NO2	AA		+		F, R, G	+	+
L-SDAP	C11H18N2O7	AA precursor		+	+			
2-Isopropylmaleate	C7H10O4	AA precursor		+	+	R	+	
Delta-piperideine-2-6-dicarboxylate	C7H9NO4	AA precursor		+	+		+	+
O-Acetyl-L-homoserine	C6H11NO4	AA derivate		+	+		+	
Histidine	C6H9N3O2	AA		+	+	G, J		
Miraxanthin V	C17H18N2O6	betaxanthine			+		+	+
L-Dihydroxy-phenylalanine	C9H11NO4	betaxanthine			+		+	+
Glutathione	C10H17N3O6S	defense		+	+	R		
Tropodithietic acid	C8H4O3S2	antibiotic	+		+			
Inosine	C10H12N4O5	purin metabolism		+	+	F, R		
Thymidine	C10H14N2O5			+		R	+	+
Deoxycytidine	C9H13N3O4			+	+			
Phenylacetylcarbinol	C9H10O2				+			+
S-Methyl phenylethanethioate	C9H10OS				+	T		

F, Fiore et al. (2015); G, Garcia et al. (2015); J, Johnson et al. (2016); R, Romano et al. (2014); T, Thiel et al. (2010). Garcia et al. inferred the metabolites in cocultures on the basis of genomic information. +, Detected; AA, amino acid; IAA, indole acetic acid.

those were biosynthetic precursors of vitamins and cofactors including those detected in the mesocosm experiment, six were amino acids or biosynthetic precursors, matching those detected in the mesocosm experiment, and one was an autoinducer to quorum sensing found also in the mesocosm experiment (**Table 1**).

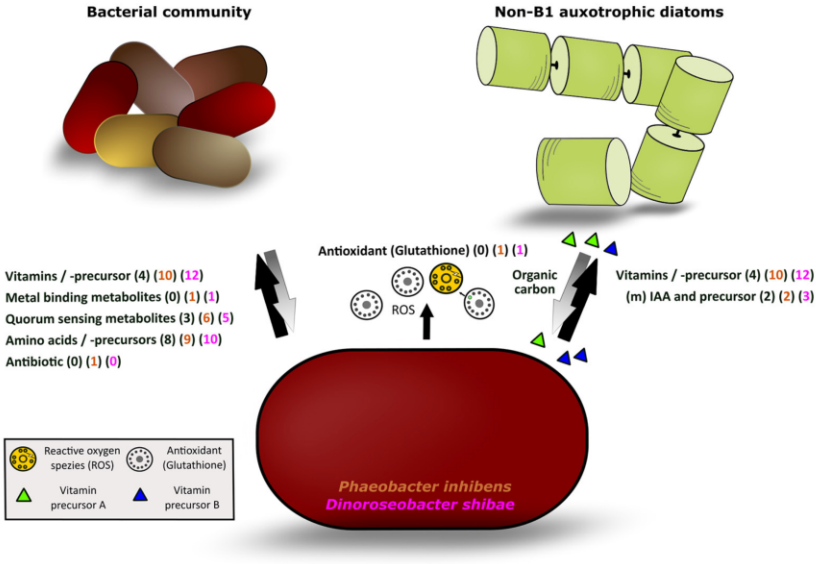

FIGURE 5 | Illustration of the different classes of exometabolites released by *P. inhibens* and *D. shibae* and mutualisms with other bacteria and phytoplankton algae. Black arrow: detected metabolites released by both strains; gray arrow: potential response of the bacterial or phytoplankton communities to the bacteria releasing metabolites; in parentheses, the number of exometabolites of each class detected in the natural phytoplankton bloom (black) and released by *P. inhibens* (brown) and *D. shibae* (pink) are given.

DISCUSSION

Our exometabolome analyses are based predominantly on an untargeted approach using FT-ICR-MS, which enables an ultrahigh resolution of individual molecular masses with a relative but no absolute quantification. The results show that *P. inhibens* and *D. shibae* both exhibit distinct exometabolite patterns consisting of 2,767 and 3,354 distinct MF and variations as a function of the carbon source. We were able to identify 2.3 and 2.6% of the exometabolites of *P. inhibens* and *D. shibae*, respectively, by comparing detected MF and genome-predicted metabolites. The observation that more than 97% of the MF consist of unknown chemical compounds, also reported in previous studies (Romano et al., 2014; Fiore et al., 2015; Johnson et al., 2016), is surprising and illustrates that the majority of MF comprises compounds is not predicted by genome annotated metabolites. There are indications that some of these compounds are metabolic waste (Fiore et al., 2015) but presumably other biological and physico-chemical reactions contribute to the formation of these compounds. The diversity of the exometabolome and implications for a better understanding of the bacterial processing of organic matter to shape the marine DOM pool are discussed elsewhere (Noriega-Ortega et al., in preparation). Here we focus on the identified exometabolites and the implications these findings have for the understanding of mutualistic interactions among bacteria and algae in marine ecosystems. The detection of seven exometabolites was further supported by fragmentation and we did not find a single case of mismatching fragments. We obtained further support of the correct MF by the genome-based exometabolite prediction. Hence our MF assignment is based on three independent methods and thus appears to be a solid base for discussing their significance in the interplay among marine microbes.

Significance of Identified Exometabolites

In both strains we identified MF identical to late biosynthetic precursors of and/or the vitamins B_1, B_2, B_5, B_6, B_7, and B_{12}, IAA and its methylated form, metal-acquisition growth factors, autoinducers for quorum sensing and biosynthetic precursors of several amino acids. Pyridoxal, the dephosphorylated form of vitamin B_6, alpha-ribazole, methyl-IAA and 3 autoinducers were also putatively detected in the DOM of a naturally-derived phytoplankton bloom (Osterholz et al., 2015) and the same vitamin precursors, alpha-ribazole-5 phosphate and one of the autoinducers also in the DOM of a North Sea phytoplankton bloom. Most MF identical to biosynthetic precursors of amino acids were also detected in the phytoplankton bloom samples. On the basis of genomic information of both strains and previous data, the vitamins and autoinducers were expected to be present in the exometabolome (Newton et al., 2010; Wagner-Döbler et al., 2010; Thole et al., 2012). However, the putative detection of late precursors of all B vitamins except one, methyl-IAA and five biosynthetic precursors of amino acids in the exometabolome of

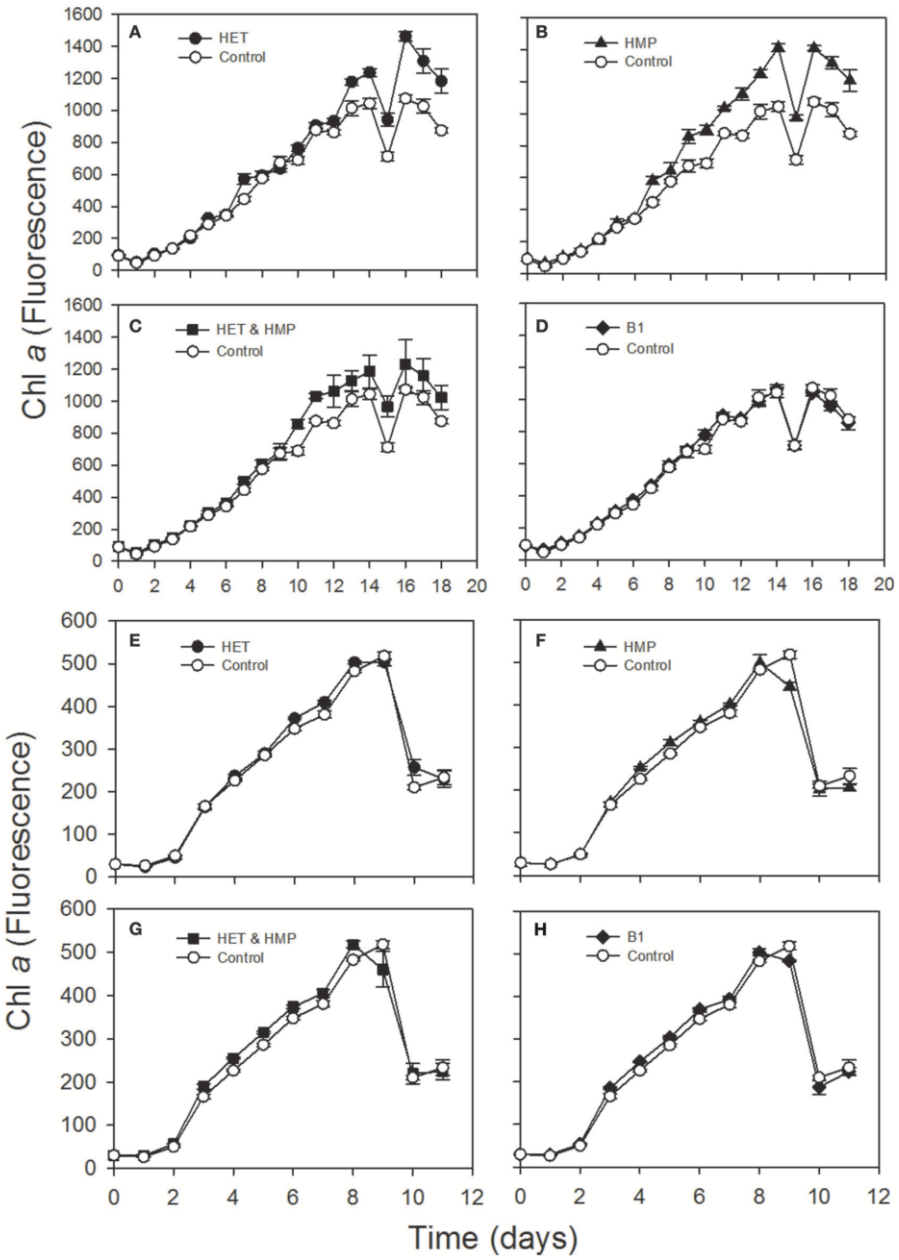

FIGURE 6 | Chlorophyll *a* fluorescence over time of an axenic culture of the diatoms *Leptocylindrus danicus* **(A–D)** and Thalassiosira pseudonana **(E–H)** supplemented with 4-methyl-5-(β-hydroxyethyl)thiazole (HET; **A,E**), 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP; **B,F**), HET & HMP **(C,G)** and vitamin B_1 **(D,H)** and a control without any of these supplements.

each strain and in the DOM of the phytoplankton bloom samples was surprising. It showed that these strains and presumably other microorganisms as well release a much greater variety of exometabolites than expected. Even though previous studies found a few of the precursors we detected, such a rich bouquet of biosynthetic precursors has neither been reported before in any bacterial exometabolome nor in the DOM of phytoplankton blooms.

The B_2 precursor dimethyl-D-ribityl-lumazine and/or the B_{12} precursor alpha-ribazole and two amino acid precursors were detected as exometabolites of a *Pseudovibrio* strain and *Ruegeria pomeroyi* (Romano et al., 2014; Johnson et al., 2016). The final precursor of the thiazole moiety of vitamin B_1, HET, we detected in both strains, is known to be used by several green algae, cryptophytes and dinoflagellates instead of B_1 (Lwoff, 1947; Droop, 1958; Turner, 1979). The pyrimidine moiety of vitamin B_1 which we did not detect, HMP, has been reported to be released by cyanobacteria, a marine betaproteobacterium and the alga *Dunaliella tertiolecta* (Carini et al., 2014). Results of previous research provides evidence that HMP and HET can be used in a salvage pathway for biosynthesis of thiamine by various B_1-auxotrophic eukaryotic algae and by Pelagibacterales (Turner, 1979; Carini et al., 2014; McRose et al., 2014; Paerl et al., 2015). There is most recent evidence, however, that HMP and unknown HET-related precursors can support growth of B_1-auxotrophic microeukaryotic marine algae via various bacteria and that these precursors are present in the open ocean (Paerl et al., 2017). Our results of the growth experiments with *T. pseudonana*, and *L. danicus* indicate that HMP and HET also stimulate growth of these vitamin B_1 prototrophic coastal diatoms. It was unexpected that the effect was even higher than that of the addition of B_1. This result is surprising and implies that *L. danicus* either lacks a vitamin B_1 transporter and/or that in both diatoms these precursors do not only compensate the lacking genetic capabilities of auxotrophic microbes but that HET and HMP have a so far unknown growth-promoting effect on vitamin B_1 prototrophic diatoms. If this stimulatory effect on vitamin B_1 prototrophic organisms is also true for other microorganisms the release of these precursors has even greater implications for controlling growth of these planktonic communities.

Pantoate as a precursor of vitamin B_5 was detected in the exometabolome of *D. shibae*. It is unknown whether this B_5 precursor can be used by marine algae or bacteria. An uptake system for pantoate, however, has been described for *Salmonella enterica* (Ernst and Downs, 2015) and thus is likely to exist in marine bacteria enabling them to use this precursor. Pyridoxal as the dephosphorylated form of vitamin B_6 was detected in the exometabolome of both strains and in the DOM of both phytoplankton blooms. Pyridoxal kinase (EC 2.7.1.35), the enzyme to phosphorylate this inactive form of vitamin B_6, is encoded in the genome of many bacteria as documented by a genomic search (https://img.jgi.doe.gov/cgi-bin/mer/main.cgi). If microbes can take up pyridoxal like *Sacharomyces cerevisiae* (Stolz and Vielreicher, 2003), this inactive form may be a so far neglected source of vitamin B_6.

Dethiobiotin, the last stage in the biosynthesis of vitamin B_7 (biotin), was detected in the exo-metabolome of *P. inhibens*. Extracellularly provided dethiobiotin has been demonstrated to cause diverging effects. Exogenous simultaneous supply of biotin and dethiobiotin caused a growth inhibition in several biotin-requiring fungi and bacteria including *Sacharomyces*, *Sordaria* and *Lactobacilli*, and was termed the anti-biotin effect (Dittmer et al., 1944; Lilly and Leonian, 1944). Contrary observations were reported for other fungi and Lactobacilli and a freshwater cyanobacterium, in which biotin auxotrophy was compensated by dethiobiotin addition (Dittmer et al., 1944; Lilly and Leonian, 1944; Bowman and DeMoll, 1993). Hence the significance of released dethiobiotin in marine microbial communities including the mycoplankton is still open and needs further studies.

Alpha-ribazole, a lower ligand building block of vitamin B_{12}, was released by both strains and detected in the phytoplankton bloom samples. The bioactive corrinoid cofactor vitamin B_{12} has been shown to be a controlling factor of primary production in pelagic ecosystems (Bertrand et al., 2007; Koch et al., 2011). Its cofactor binding, function and catalysis highly depends on the attached lower ligand (Lengyel et al., 1960; Stupperich et al., 1988; Renz, 1999; Yi et al., 2012). It has been shown, however, that the genome of *Listeria innocua*, lacking the genes for alpha-ribazole biosynthesis, encodes a transporter system of alpha-ribazole, cblT, and is able to synthesize B_{12} when this moiety is available as an exogenous source (Gray and Escalante-Semerena, 2010). Remodeling of corrinoids, such as vitamin B_{12}, by complementation of the lower ligand via uptake of exogenous compounds appears to be a common phenomenon within microbial communities (Gray and Escalante-Semerena, 2010; Keller et al., 2014; Men et al., 2014). Available 5,6-dimethylbenzimidazole (DMB), an alpha-ribazole precursor, allows major phytoplankton groups to remodel pseudocobalamin, commonly produced by cyanobacteria, into a usable corrinoid cofactor structure (Helliwell et al., 2016). Our results indicate that corrinoid building blocks are exchanged in marine ecosystems and that roseobacters such as *D. shibae* and *P. inhibens* may be providers of the most common and bioactive corrinoid cofactor lower ligand.

The auxin IAA has recently been identified to be secreted by various freshwater and marine bacteria and to be an important growth factor of various green algae and a diatom (Bagwell et al., 2014; Zhang et al., 2014; Amin et al., 2015, Fiore et al., 2015). It was also found in a phytoplankton bloom in the Pacific, in a eutrophic lake dominated by cyanobacteria and to be produced by *P. inhibens* (Zhang et al., 2014; Amin et al., 2015; Segev et al., 2016). We detected IAA in the exometabolome of *D. shibae* and its precursor tryptophan in the exometabolome of both strains and the naturally derived phytoplankton bloom. Tryptophan has been shown to enhance the production of IAA in *P. inhibens* (Segev et al., 2016) and also in the haptophyte *Emiliania huxleyi* (Labeeuw et al., 2016). It thus appears to be important in controlling the production of IAA in these roseobacters and the haptophyte. In both strains and in the naturally derived phytoplankton bloom, we also detected methyl-IAA, a related compound which was neither detected previously in the exometabolome of marine bacteria nor in marine DOM samples. It has been shown in *Arabidopsis* that methyl-IAA is an inactive form which can be taken up but needs to be demethylated

by an esterase to generate the active IAA (Yang et al., 2008). Because of the more hydrophobic form of methyl-IAA as compared to IAA, these authors suggest that it is more easily transported across the cell membrane, possibly even diffuses, thus enhancing the exploitation of the exogenous supply with the subsequent need to demethylate it intracellularly. Another aspect of hydrophobic compounds released into the water is that enhanced hydrophobicity results in a faster supply through this hydrophilic medium thus reducing the time of action and enhancing the accumulation at the target site, e.g. the cell surface (Maier et al., 1994). Hence it is conceivable that the methylated form of IAA in pelagic ecosystems leads to a more efficient use of this important auxin by phytoplankton.

The metal-binding 2,3-dihydroxybenzoate was detected in the exometabolome of *D. shibae*. This building block of the siderophore enterobactin is known to be secreted by heterotrophic and cyanobacteria under iron-limiting conditions (Young et al., 1967; Byers and Lankford, 1968; Fiore et al., 2015) and to enhance the expression of the 2,3-dihydroxybenzoate-AMP ligase, catalyzing an essential synthesis step toward the formation of enterobactin (Khalil and Pawelek, 2011). Other strains of the *Roseobacter* group are known to secrete enterobactin but not 2,3-dihydrobenzoate (Hogle et al., 2016). Hence, *D. shibae* appears to have the potential to provide 2,3-dihydroxybenzoate to marine bacterioplankton communities, thus inducing enterobactin synthesis and favoring iron uptake by itself and other bacteria. A building block of the siderophore petrobactin, 3,4-dihydroxybenzoate, was detected in the exometabolome of *P. inhibens* and not found before as a bacterial exometabolite. It has been shown that *P. inhibens* is able to produce enterobactin (Thole et al., 2012). Thus, 3,4-dihydroxybenzoate may have a similar role in metal acquisition as 2,3-dihydroxybenzoate (see above).

Glutathione was detected in the exometabolome of both strains and previously found also in that of a *Pseudovibrio* strain (Romano et al., 2014). Besides essential intracellular functions, glutathione is a fundamental extracellular protectant for bacteria, in particular as an antioxidant when reactive oxygen species (ROS) are present (Smirnova and Oktyabrsky, 2005; Montoya, 2013; Smirnova et al., 2015). One reason for the presence of glutathione in the exometabolome may be stress caused by the growth conditions and high cell densities and the possible protection against ROS. On the other hand, glutathione has been found in nanomolar concentrations in the oligotrophic north Pacific (Dupont et al., 2006) even though it is rapidly photooxidized (Moingt et al., 2010). Therefore, it is conceivable that bacteria such as our model strains actively excrete glutathione for protection against ROS produced by photochemical DOM oxidation.

Biosynthetic precursors of amino acids were previously reported in bacterial exometabolomes but interpreted as a result of an overflow metabolism (Paczia et al., 2012; Romano et al., 2014). As we found these precursors also in the DOM of phytoplankton blooms, we suggest that they are either actively secreted or released by dividing and growing cells or due to mortality by grazing or viral lysis of bacterial communities and are not a result of an overflow metabolism. They can potentially be used for amino acid biosynthesis by other bacteria but this pathway has yet to be shown. In support of this suggestion, it has recently been shown that bacterial mutants, missing biosynthetic genes in amino acid pathways, have a growth benefit over the wild type when supplied with the respective precursors or amino acids (D'Souza et al., 2014; Waschina et al., 2016). These authors consider amino acid cross-feeding as a specialized evolutionary mechanism of how bacterial subpopulations can receive mutual benefits by saving biosynthetic costs. Auxotrophy of essential amino acids can potentially be conquered by public amino acid goods and even facilitate metabolic interdependency or symbiosis (McCutcheon and Moran, 2007; Garcia et al., 2015). Our findings of several essential and non-essential amino acids and respective biosynthetic precursors in the exometabolome of both strains and in the DOM of the phytoplankton bloom samples suggests that beneficial amino acid cross-feeding also occurs in marine microbial communities.

The Exometabolome: A Market Place of Microbial Metabolites

The release of a wealth of exometabolites by the two model strains indicates that both of them may function as important suppliers of growth factors as well as of biosynthetic precursors, so-called public goods (Morris et al., 2012), to other pro- and eukaryotes in marine ecosystems. *Phaeobacter inhibens* dwells in biofilms (Gram et al., 2015) and both model strains live in association with microalgae and pelagic environments (Wagner-Döbler et al., 2010; Gifford et al., 2014; Segev et al., 2016) thus suggesting that these exometabolites are released as public goods in biofilm-associated as well as in pelagic marine communities. Biofilms with dense colonization of diverse bacterial communities may include surface-associated habitats but also marine aggregates which often form during phytoplankton blooms (Simon et al., 2002). Bacteria releasing public goods were recently termed Black Queen in the context of the Black Queen hypothesis (BQH; Morris et al., 2012; Morris, 2015), a scenario in which other bacteria benefit from losing genetic traits to synthesize certain growth factors such as vitamins or parts of their biosynthetic pathways when exogenous supply of these compounds is consistently available. Aggregate-associated bacteria acting as Black Queens may also supply free-living pelagic bacteria and phytoplankton algae in the surrounding water with public goods. Genome streamlining features were reported from various pelagic bacteria including *Pelagibacterales*, the SAR86 clade and *Prochlorococcus* (Dupont et al., 2012; Carini et al., 2014; Giovannoni et al., 2014) but also from free-living and symbiotic bacteria dwelling in nutrient-rich or constant environments (Van de Guchte et al., 2006; McCutcheon and Moran, 2007; D'Souza et al., 2014). Supply of released compounds as public goods to other microbes is part of a complex network of microbial interactions and there are quite a few public goods in this context, but also private metabolic goods not shared, which act beyond the concept of the BQH (Morris, 2015; Estrela et al., 2016). Our results in fact indicate that the two vitamin B_1 prototrophic diatoms benefit from supply by the B_1 precursors HET and HMP, a scenario not considered by the BQH. It

must also be kept in mind that in a microbial community mutual interactions exist between the primary producers secreting substrates to the (photo)heterotrophic microbes and that different microbes are distinct in their capabilities, e.g., in hydrolyzing polymers. For instance *Flavobacteria* exhibit a wealth of polysaccharide hydrolyzing enzymes (Teeling et al., 2012) whereas roseobacters are very limited in these polymer-degrading traits (Hahnke et al., 2013). Further, it has been shown that different bacteria, each missing distinct genomic metabolic traits and exhibiting streamlined genomic features, coexist by complementing each other with metabolites for which they are auxotrophic (Garcia et al., 2015). A microbial community with mutual interdependencies between two or among a group of microbes and no unidirectional flows of public and private goods among various bacteria and other microbes appears to be a more suitable model to describe this complex network, a marketplace of microbial metabolites (**Figure 5**; Zelezniak et al., 2015). Such scenarios presumably characterize the dynamic ecosystems in which both model strains dwell: In tighter or looser association with algae and other bacteria on biofilms or during phytoplankton blooms. Both scenarios exhibit many mutual interactions among a multitude of organisms with non-streamlined as well as streamlined genomes.

AUTHOR CONTRIBUTIONS

All authors designed the experiments. GW and BN carried out the experiments, GW identified the exometabolites and wrote the first draft of the manuscript. MS and GW wrote the final version of the manuscript. All authors critically reviewed and added aspects to the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2017.01985/full#supplementary-material

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Chapter 4

Exometabolome diversity patterns of a *Roseobacter* model strain are linked to its transcriptome patterns

Gerrit Wienhausen*[1], Beatriz E. Noriega-Ortega*[1], Sonja Voget[2], Jutta Niggemann[1], Thorsten Dittmar[1] and Meinhard Simon[1]

[1] Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Oldenburg, Germany

[2] Institute for Microbiology and Genetics, University of Göttingen, Göttingen, Germany

* These authors contributed equally

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Chapter 4

Abstract:

The exometabolome of marine bacteria features an unforeseen diverse blend of chemically complex molecules but we know very little about their genesis in the context of intracellular metabolic processes. Therefore, we conducted experiments with a model bacterium of the *Roseobacter* group, *Phaeobacter inhibens*, which we grew on the single carbon sources glutamate and glucose and assessed the exometabolome by Fourier transform ion cyclotron resonance mass spectrometry and the intracellular metabolic activities by the transcriptome. To link exometabolite to transcriptomic patterns we applied a novel multivariate statistical approach which revealed a significant correlation of 41% of all detected exometabolite mass formulas (MF) to 15 distinct clusters of orthologous groups (COG) of proteins. This suggests that a large fraction of bacterial exometabolites is not formed randomly but as the result of distinct intracellular metabolic processes. In some cases the chemical classes of the correlating MF were linked to respective COG categories such as proteins and translation whereas in other cases the classes of correlating MF were distinctly different from the respective COG categories. Eleven percent of the correlating MF were similar to those of refractory dissolved organic matter (DOM) of a sample from one of the oldest water masses of the Pacific suggesting that a small fraction of refractory DOM may originate from actively growing bacteria. The findings of our novel approach suggest that specific metabolic processes of marine bacteria contribute to or may even control the composition of the exometabolome in marine pelagic systems and thus eventually the global DOM diversification.

Introduction

Marine dissolved organic matter (DOM) is a major global carbon reservoir (Hansell, 2013). Recent studies, applying ultrahigh resolution Fourier transform ion cyclotron resonance mass spectrometry (FT-ICR-MS), revealed that DOM encompasses a surprisingly high chemodiversity with thousands of different molecules of which only the mass formula (MF) and the elemental composition are known (Hansell and Carlson, 2014, Dittmar and Stubbins 2014). Applying FT-ICR-MS to assess the exometabolome of monocultures of bacteria, it became evident that individual bacteria growing on single carbon sources release thousands of distinct organic molecules, so called exometabolites, yielding specific chemodiversity patterns (Romano *et al*. 2014, Fiore *et al*. 2015, Johnson *et al*. 2016, Noriega-Ortega *et al*. in prep). Carbon source, growth stage and limiting elements modulate the specific diversity patterns of the exometabolome. Microbial processing of freshly produced DOM, released e.g. by phytoplankton algae, leads to further diversification of the DOM and results in more and more similar chemodiversity patterns of the processed DOM and that persisting in the oceans (Lechtenfeld *et al*., 2015; Osterholz *et al*., 2015). Surprisingly, only very few of the molecules detected in the marine DOM and the exometabolome of individual bacteria, less than 3% and often far less than 1%, match known metabolites, i.e. known from endometabolomic analyses or genome-based predicted metabolic pathways (Fiore *et al*., 2015; Romano *et al*., 2014; Wienhausen *et al*., 2017). This finding indicates that the bacterial metabolism is far more complicated than assumed, and based on targeted approaches to assess the endo- and exometabolome, obviously misses a large unknown amount of metabolites. It is still an open question how the exometabolites are released, either by cell lysis, leakage during cell division, diffusion via the cell membrane or secreted during regular cellular metabolism. There is some evidence that metabolites are released as a result of cell lysis but this does not appear to be the major process as indicated by mass balance calculations and the fact that they are released during exponential growth (Fiore *et al*., 2015; Johnson *et al*., 2016; Wienhausen *et al*., 2017, Noriega-Ortega *et al*. in prep.).

The fact that the composition of the exometabolome is strikingly different and more diverse than that of the endometabolome is also an argument against cell lysis (Brito-Echeverría *et al*., 2011; Johnson *et al*., 2016). The rate of passive metabolite diffusion through the cell membrane is strongly correlated to the size (Nikaido and Rosenberg 1981) and polarity of molecules (Benz *et al*. 1979), the membrane permeability, concentration gradient and existence of porin channels (Nikaido, 2003). These properties may contribute to leakage of

metabolites into the cell's environment. Multifaceted energy dependent exudation mechanisms facilitate the active secretion of various metabolites and compound classes. If the exometabolites are released as intermediate metabolic products of biosynthetic and/or catabolic pathways the occurrence of the exometabolites should be linked to bacterial gene expression patterns, i.e. transcriptomic profiles, as shown for the endometabolome of various prokaryotes applying a targeted approach (Laass *et al*., 2014; Trauger *et al*., 2008). However, it is completely unknown whether and if so, how the many unknown MF detected by FT-ICR-MS in the exometabolome of marine bacteria or their release are linked to or even controlled by intracellular metabolic processes. We hypothesize that transcriptomic patterns reflect molecular features of exometabolites and thus the chemodiversity patterns of the exometabolome of individual bacterial strains.

Therefore, the aim of this study was to test how transcriptomic patterns of *Phaeobacter inhibens* DSM 17395, growing on the single carbon sources glutamate and glucose, are linked to the exometabolome and properties of the exometabolites assessed by FT-ICR-MS. *P. inhibens* is a purely heterotrophic model strain of the *Roseobacter* group exhibiting a versatile secondary metabolism (Beyersmann *et al*., 2017; Thole *et al*., 2012; Wang *et al*., 2016). Strains of this species have been found in various marine habitats, such as natural biofilms on solid surfaces and during a bloom of *Emiliana huxleyi* (Breider *et al*., 2017; Gifford *et al*., 2014; Gram *et al*., 2015; Segev *et al*. 2016). The results show that distinct groups of mass formulas (MF) of the exometabolome are significantly correlated to expression patterns of distinct Clusters of Orthologous Groups of proteins (COG; Galperin *et al*., 2015; Tatusov *et al*., 2000), suggesting that the release of exometabolites is not a random process. The MF of several exometabolites matched MF in the dissolved organic matter (DOM) of North Equatorial Pacific Intermediate Water, ranging among the older water masses in the oceans, and thus suggesting that primary bacterial exometabolites can persist for a long time in the oceans.

Material and Methods

We carried out two growth experiments with *P. inhibens*, one with the type strain *P. inhibens* DSM 17395, carrying all three plasmids and one with a mutant which lacked the pPGA1_65 plasmid encoding a gycosyltransferase and type I secretion system exporting polysaccharides (Thole *et al*., 2012). The rationale for using the type strain and mutant was to enlarge the diversity of the exometabolites. *Phaeobacter inhibens* DSM 17395 was first grown on marine broth (MB; Difco MB 2216) medium and afterwards repeatedly (5x) transferred and cultivated

in artificial seawater (ASW) medium with the addition of glucose or glutamate as single carbon source. Before every transfer and after centrifugation of the cultures the cell pellets were washed three times with ASW medium. All plastic and glass ware used were rinsed with acidified ultrapure water (MilliQ, pH 2) and all glassware additionally combusted for 3 h at 500°C. The growth conditions, experimental analytical procedures are outlined in detail in Wienhausen *et al*., (2017) and Noriega-Ortega *et al*., (in prep.).

The ASW-medium for the *P. inhibens* cultures was prepared as described by Zech *et al*. (2009) but slightly modified by excluding EDTA from the trace element solution and supplemented with glucose (5 mM, 30 mM C; ultrapure brand) or glutamate (20 mM, 100 mM C; ultrapure brand). The strain was cultivated in 500 ml of medium in triplicate 2 liter baffled Erlenmeyer glass flasks at pH 8 at 28 °C in the dark on a shaker (100 rpm) and growth was monitored by optical density (OD_{600}) and cell numbers by epifluorescence microscopy. A sterile flask with media and the respective single carbon source was run as control. Subsamples were withdrawn under laminar flow for the separate analysis of the replicates for dissolved organic carbon and exometabolome-DOM at the start, the lag phase, the mid-exponential and early stationary phase in order to recover the majority of exometabolites released at varying growth conditions. Aliquots of the cultures were centrifuged at 2499 g in acid washed and combusted (3 h, 500°C) glass centrifuge tubes. The supernatant was filtered through a 0.22 µm polyethersulfone membrane (Minisart, Sartorius, Göttingen, Germany) and the filtrate stored acidified to pH 2 (HCl 25% p.a., Carl Roth, Germany) in combusted 20 ml glass vials at 4°C until further analysis. Samples for transcriptomic analyses were obtained from the exponential phase of the glucose-amended culture of the type strain and the exponential and stationary phases of the glutamate-amended culture of the type strain and pPGA1_65 mutant.

DOM analyses

Dissolved organic matter of the solid-phase extracted DOM (see below) was analysed by FT-ICR-MS as described previously (Osterholz *et al*. 2015, Wienhausen *et al*. 2017). Briefly, filtrates were extracted via PPL solid phase cartridges (100 mg; Agilent, Waldbronn, Germany) adapted to a concentration of 15 ppm carbon and analyzed by FT-ICR MS. Extracted DOM was ionized by soft electrospray ionization (Bruker Apollo, Daltonics, Bremen, Germany) and analyzed in positive and negative mode with a 15 T Solarix FT-ICR-MS (Bruker, Daltonics, Bremen, Germany). For each spectrum 300 scans were accumulated in the mass window of 92 to 2000 Da. Detected mass to charge ratios were processed applying a customized routine Matlab script. Molecular formulas were assigned as described by Koch and Dittmar (2006) to

molecular masses with a minimum signal-to-noise ratio of 5 (Koch *et al*., 2007). From the mass spectrograms of the single time points of each culture that of the respective sterile control was subtracted. Only MF with a signal to noise ratio of >5 were considered as exometabolites in the further analyses.

RNA-extraction, transcriptome analysis and data deposition

Cell pellets were shock frozen and stored at -80 °C until further analysis. Total RNA extraction using the RNeasy Kit (Qiagen, Hilden, Germany) was employed according to the manufacture's protocol with only minor modifications. Cells were resuspended in 400 µl TE buffer amended with 15 mg ml^{-1} Lysozyme digesting the cell wall peptidoglycan component prior to extraction and RTL buffer was amended with ß-mercaptoethanol for protein denaturing and reduction. Remaining traces of DNA were removed with DNase treatments (ThermoScientific, Waltham, MA, USA) and subsequently purified and concentrated using RNeasy MinElute Cleanup Kit (Qiagen). RNA samples to be DNA free was guaranteed by running a *P. inhibens* specific PCR (reverse: GCA AGA TCA TGA CAG TTC TG; forward: AAG TCG TAA CAA GGT ARC CGT A). The Ribominus transcriptome isolation kit (Invitrogen GmbH, Karlsruhe, Germany) was used to enrich the mRNA as recommended by the manufacturer. Concentration and denaturation of the total RNA and enriched mRNA was analyzed applying NanoDrop ND-1000 UV-vis Spectrometer (Nanodrop Technologies, Wilmington, DE, USA) and the 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). Random primers pd(N)6 (ROCHE, Mannheim, Germany) were used to transcribe reverse-transcribed mRNA into cDNA and then synthesized by the aid of the SuperScript™ double-stranded cDNA synthesis kit (Invitrogen) to double stranded cDNA. Sequencing was carried out by the FLX 454 Illumina pyrosequencer (Illumina, San Diego, CA, USA) using the cDNA libraries established as recommended by the manufacturer. To compare the RNA-Seq results, the read counts were normalized to remove biases like the length of the transcript and the sequencing depth of a sample. We used the Nucleotide activity Per Kilobase of exon model per million Mapped reads (NPKM), a derivate of RPKM (reads per kilo base per million), as a normalized read count value (Wiegand *et al*., 2013).

Statistics evaluation

The five triplicate samples of which we obtained transcriptomic and exometabolite patterns were used for the statistical evaluation. Only mRNA reads with a likelihood of 0.9 and a false discovery rate (FDR) below 0.05 were taken into consideration for further data analysis. Within a set of triplicates, the absolute number of each mRNA read was normalized to the total sum. Respective mRNA-derived genes were grouped by functional COG categories comprising at least 60 statistically significant genes (Galperin *et al*., 2015; Tatusov *et al*., 2000). Exometabolites detected by FT-ICR-MS were only used for further statistical analysis when assigned to MF and when present in 2 out of 3 samples of each triplicate. Samples were standardized by normalizing absolute peak intensities to the total sum. Correlations of exometabolome and transcriptome data were calculated as described by Osterholz *et al*., (2016) with minor modifications. In this approach at first Bray Curtis dissimilarity matrices were established for the exometabolite and transcriptomic patterns of the transcribed genes in the COG categories. These dissimilarity matrices were computed accounting matrices of relative numbers and intensities of all transcribed genes of a given COG category and exometabolites (Bray and Curtis, 1957; Legendre and Legendre, 1998). Of each matrix a principal coordinate analysis (PCoA) was calculated. Only a subset of MF with a correlation of >90% to a given COG category was used for further statistical evaluation. In order to test for cross-covariance we computed a canonical-correlation analysis (CCorA) by linking all transcribed genes of a given COG category to the exometabolome matrix. To test for a random co-correlation of transcriptional COG categories and exometabolome patterns we calculated a CCorA between the latter and concentrations of total ion current (TIC). In addition a randomized set of mRNAs of all COG categories was generated and correlated to the exometabolome patterns with the same approach as for the data set described above. Correlations of each exometabolome MF to one transcriptomic COG category were presented as van Krevelen plot using color-assignment. All multivariate statistics were performed in R (version 3.2.1, R Development Core Team, http:// cran.r-project.org/) using the 'vegan'-package (Okasanen *et al*., 2012).

Exometabolome comparison to deep ocean DOM sample

To examine whether freshly produced exometabolites persist in the marine geometabolome and which associated metabolic functions, i.e. COG categories, may directly contribute to the formation of recalcitrant DOM compounds we screened the exometabolites of *P. inhibens* with respect to their MF against a DOM sample from the North Equatorial Pacific Intermediate

Water (NEqPIW) obtained from 674 m depth near Hawaii in the oxygen minimum zone. The NEqPIW ranges among the older water masses on earth and can thus be considered to carry primarily recalcitrant DOM.

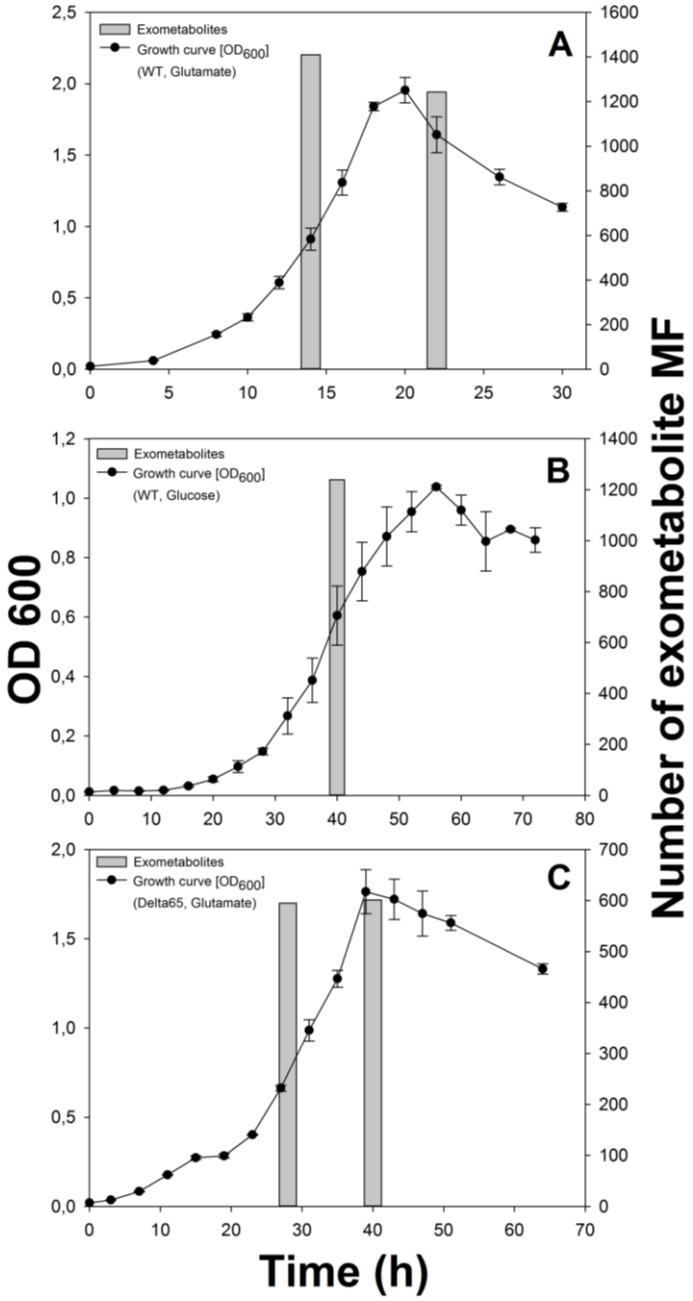

Figure 1 | Growth of *P. inhibens* assessed as optical density (OD 600) over time and number of detected exometabolite molecular formulae (MF) at sampling points of for transcriptomics. A) *P. inhibens* WT grown on glutamate; B) *P. inhibens* WT grown on glucose; C) *P. inhibens* mutant Δ65 grown on glutamate (Glutamate; C) . Note different scales of the axes.

Results

Growing on glucose and glutamate, *P. inhibens* produced a total of 4794 and 3692 distinct MF exometabolites, respectively, and reached the stationary phase after 55 and 20 h, respectively. *P. inhibens* delta65 released 2324 distinct MF exometabolites when grown on glutamate and reached stationary phase after 38 h. A total of 1911 MF was produced when pooling the data of the cultures growing on both substrates. Glucose and glutamate were completely used up when *P. inhibens* entered the stationary phase (Wienhausen *et al*., 2017). Considering only the time points at which transcriptomic analyses and thus further statistical evaluations were carried out, 1239, 1513 and 1239 distinct MF were detected in the

cultures growing on glucose and glutamate WT as well as mutant, respectively, and in total 1911 MF (Fig. 1).

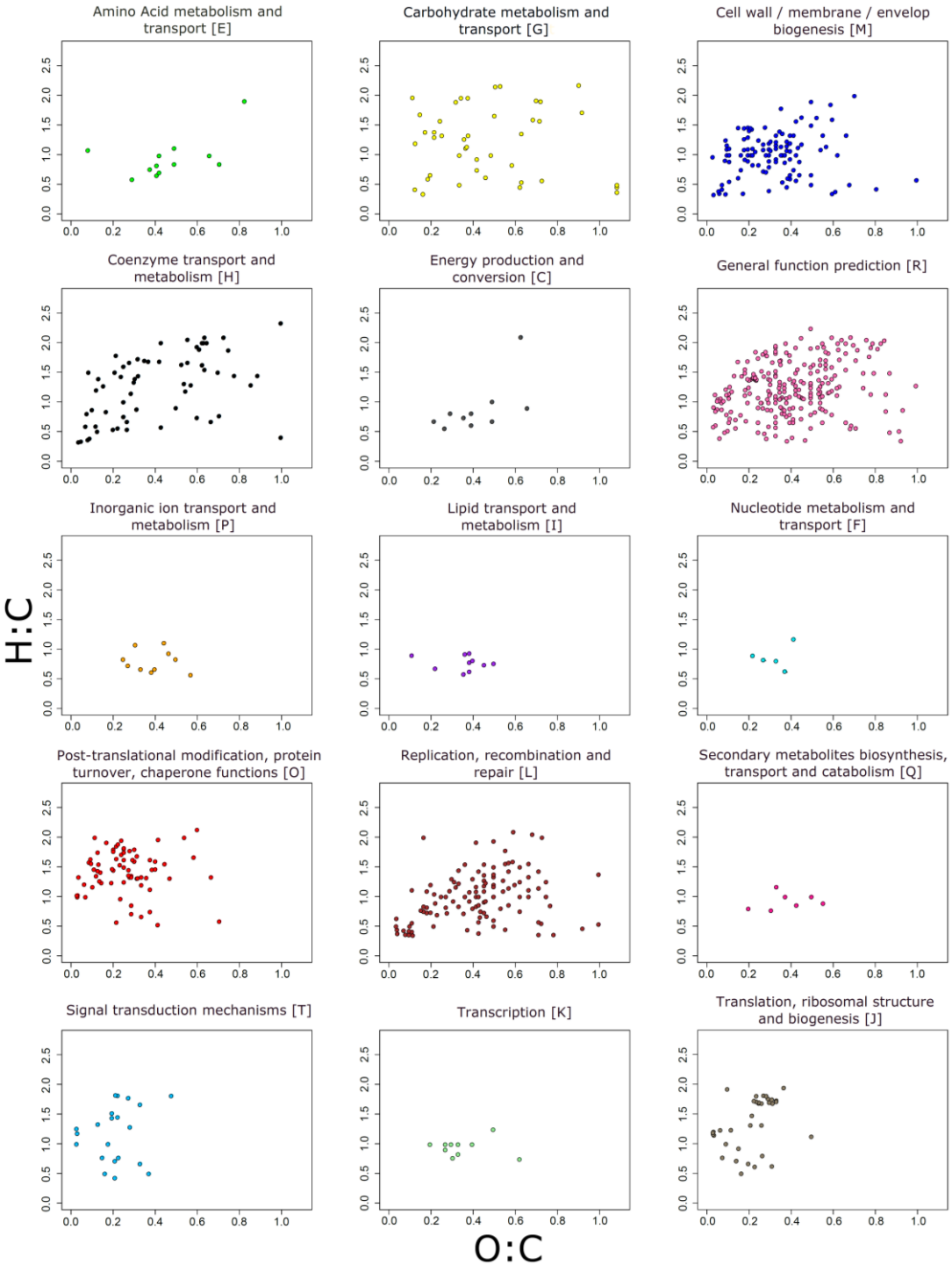

Figure 2 | Van Krevelen diagrams (H:C / O:C) of exometabolite molecular formula correlated with COG-groups.

Genes of all COG categories were highly actively transcribed. The mean NPKM values ranged between 963 for transcribed genes of the COG category cell motility (N) and 3576 for cell wall/membrane/envelope biogenesis (M; Table S1). As the intention of this study was a correlation of the pooled transcripts of the COG categories with the MF detected at the same time points we did not carry out a detailed assessment and comparison of distinct transcriptional patterns of the strain and mutant and the two substrates and different growth stages.

The statistical analysis revealed that 819 exometabolites, i.e. MF (42.9% of all MF detected in the five samples), were significantly correlated to transcribed genes of 15 COG categories encompassing between 60 and 264 genes. A correlation of a randomized exometabolome dataset to these COG categories, applying similar statistical constraints, yielded a positive correlation for only 14.2% of the MF (Supplementary Fig. S1) and a canonical correlation analysis (CCorA) of total ion current with the exometabolome showed no conspicuous feature when displayed as color-coded correlations in van Krevelen diagrams (Supplementary Fig. S2). The number of correlating MF of our experiments with *P. inhibens* ranged between 5 in the COG category nucleotide metabolism and transport (F) to 257 in that general function prediction (R) (Table 1, Fig. 2). For three COG categories (general function prediction (R); replication, recombination and repair (L); cell wall/membrane/envelope biogenesis (M)) more than 100 correlating MF were detected whereas for eight COG categories numbers of correlating MF did not exceed 15 (Table 1). There was no correlation between the number of MF and the number of genes of the correlating COG category. For instance, COG category replication, recombination and repair (L) encompassed only 60 genes but showed a correlation to 125 MF whereas the COG category amino acid transport and metabolism (E), encompassing 264 genes, correlated only with 13 MF (Table 1). Mean masses of the correlating exometabolites ranged between 235.8 for the COG category nucleotide metabolism and transport (F) and 407.8 for that of coenzyme transport and metabolism (H; Table 1). The size range distribution of the correlating MF, however, varied greatly (Fig. 3). Those correlating to COG categories carbohydrate metabolism and transport (G), post-translational modification/protein turnover (O) and signal transduction (T) were broadly distributed with no distinct peak range whereas those correlating to COG categories amino acid transport and metabolism (E), cell wall/membrane/envelope biogenesis (M), general function prediction (R) and replication, recombination and repair (L) exhibited the largest number of MF at the lowest M/Z and a decreasing number with increasing M/Z (Fig. 3).

Further, the elemental composition of the correlating MF varied greatly. The number of carbon atoms per MF ranged from a mean of 9.7 of the MF correlating to COG categories energy production and conversion (H) to 19.7 of those correlating to COG categories post-translational modification, protein turnover and chaperones (O; Table 1). The MF correlating to COG categories lipid metabolism (F), inorganic ion transport and metabolism (I) and secondary metabolites biosynthesis and transport (Q) did not contain any nitrogen. Roughly half of the MF correlating to COG categories carbohydrate metabolism and transport (G), cell wall/membrane/envelope biogenesis (M), coenzyme transport and metabolism (H), general function prediction (R), post-translational modification, protein turnover and chaperones (O), replication, recombination and repair (L) and translation (J) contained between 1 and 3 nitrogen atoms per MF with mean values for the number of nitrogen atom per MF of the respective categories of 0.67 to 2.19 (Table 1, Supplementary Fig. S3). Only two of the MF correlating to COG category amino acid metabolism and transport (E) and only one of those correlating to COG categories energy production and conversion (C) and transcription (K) contained a nitrogen atom (Table 1). Surprisingly, the great majority of the correlating MF contained at least one sulfur atom with mean sulfur atoms per MF ranging from 0.5 to 1.8 (Table 1, Supplementary Fig. S4). Only for the MF correlating to COG categories post-translational modification and repair (O), signal transduction (T) and translation (J) less than 50% of the MF contained a sulfur atom.

The comparison of the correlating MFs with MFs in the DOM sample from the North Equatorial Pacific Intermediate Water showed in 8 of the 16 COG categories at least one match (Table S2). In three categories even 10 to 71 MF matched accounting for 10.3 to 23.3% of the total MF in each category. In total 93 (11.4%) of the correlating MF yielded a match in the DOM sample of the Pacific. The highest proportion of matching MF was found in the range 200 to 300 M/Z (Fig. 4). The mean number of C atoms per matching MF ranged between 11 and 14.2 for the MF correlating to COG categories cell wall/membrane/envelop biogenesis (M), general function prediction (R) and post-translational modification, protein turnover and chaperones (O) with an overall mean of 13.2 (Table 1).

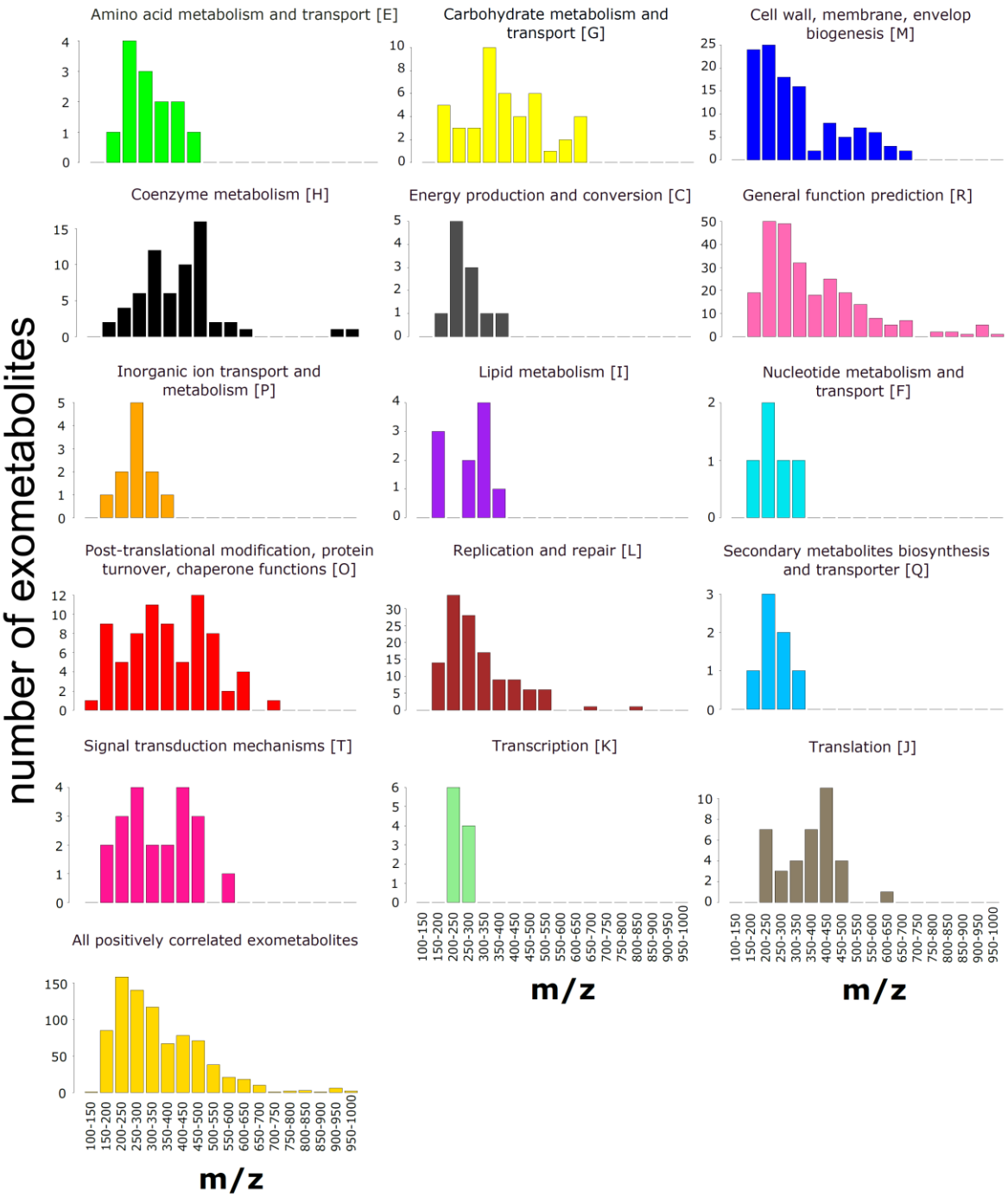

Figure 3 | Molecular size ranges of exometabolites correlating with COG-groups. Size range is subdivided in intervals of 50 m/z. Note the different scales of the axes.

Table 1 | Numbers of exometabolites correlated to functional COG-groups and their respective mean molecular mass (M/Z, mean element ratios (H, O, N, S, P) over C and the mean elements (C, H, O, N, S, P).

Functional COG-group	Number of correlating exometabolites	Mean molecular mass and element ratio						Mean elements				
		m/z	H/C	O/C	N/C	S/C	P/C	C	H	O	N	S
Amino Acid metabolism and transport [E]	13	285.5	0.95	0.49	0.03	0.13	0.01	11.6	11.1	5.2	0.3	1.5
Carbohydrate metabolism and transport [G]	44	378.7	1.23	0.39	0.06	0.06	0.02	17.3	23.1	5.9	1.0	1.0
Cell wall / membrane / envelop biogenesis [M]	116	316.3	1.02	0.33	0.05	0.07	0.01	15.1	15.4	4.6	0.7	1.0
Coenzyme transport and metabolism [H]	63	407.8	1.27	0.41	0.10	0.05	0.01	18.0	22.4	6.4	1.8	0.9
Energy production and conversion [C]	11	254.6	0.86	0.43	0.02	0.19	0.03	9.7	8.5	4.2	0.2	1.8
General functional prediction [R]	257	366.9	1.24	0.41	0.08	0.04	0.01	16.7	21.0	6.2	1.3	0.7
Inorganic ion transport and metabolism [P]	11	277.4	0.79	0.39	0.00	0.16	0.00	11.6	9.3	4.5	0.0	1.8
Lipid transport and metabolism [I]	15	271.8	0.76	0.36	0.00	0.16	0.00	11.6	8.8	4.2	0.0	1.8
Nucleotide metabolism and transport [F]	5	235.8	0.86	0.32	0.04	0.09	0.00	11.0	9.6	3.6	0.4	1
Post-translational modification, protein turnover, chaperone functions [O]	75	379.2	1.42	0.26	0.09	0.02	0.01	19.7	27.7	4.7	1.8	0.4
Replication, recombination and repair [L]	125	304.7	1.00	0.43	0.05	0.10	0.02	13.1	12.5	5.1	0.7	1.3
Secondary metabolites biosynthesis, transport and catabolism [Q]	7	249.9	0.93	0.39	0.00	0.15	0.00	10.6	9.7	4.0	0.0	1.6
Signal transduction mechanisms [T]	21	349.5	1.18	0.22	0.09	0.03	0.01	18.9	23.9	3.8	1.7	0.5
Transcription [K]	10	245.1	0.95	0.35	0.01	0.15	0.00	10.5	9.9	3.6	0.1	1.6
Translation [J]	37	365.0	1.34	0.22	0.11	0.02	0.00	19.4	26.7	3.8	2.2	0.4
Positive correlating exometabolites	819	344.7	1.16	0.37	0.07	0.06	0.01	16.0	19.0	5.3	1.1	0.9
All exometabolites	1911	349.7	1.25	0.31	0.08	0.05	0.01	16.5	20.6	5.2	1.4	0.8

Matching MF were enriched in N with a mean C:N ratio of 10.9 as compared to 27.9 as the overall mean of all MF correlating to COG categories. In contrast, matching MF were deprived in S with a C:S ratio of 220 as compared to 14.5 of the overall mean of all MF correlating to COG categories (Table 1).

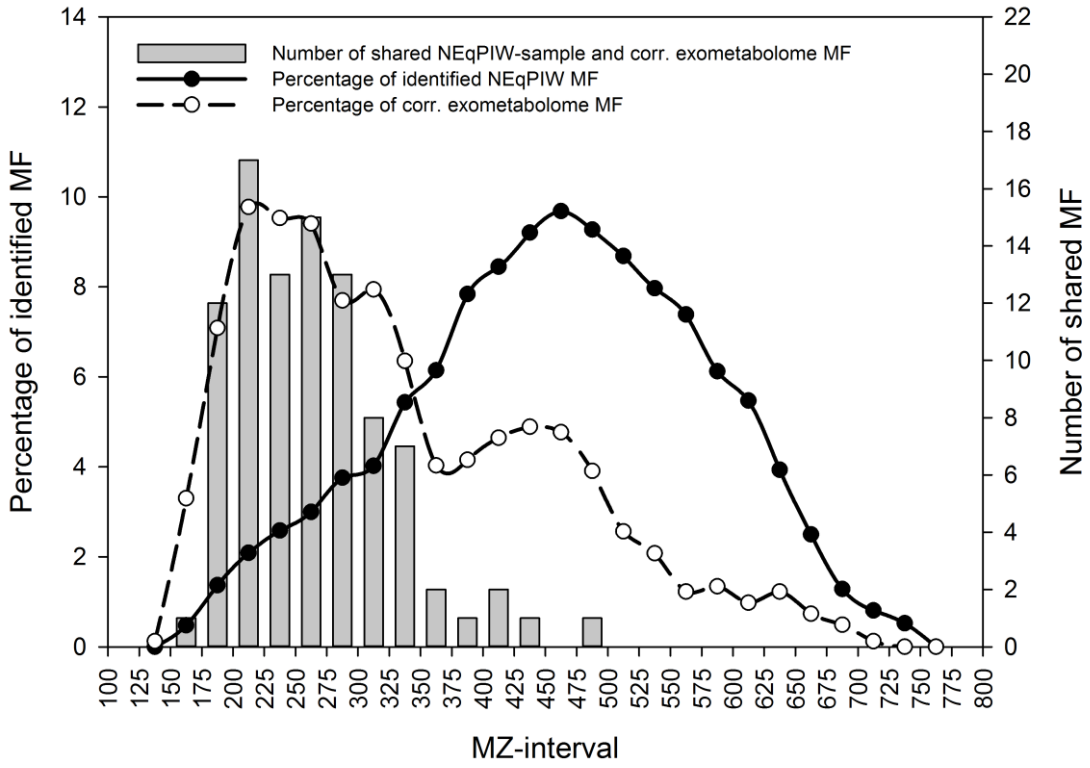

Figure 4 | Percentage per 25 M/Z of detected MF in *P. inhibens* exometabolome (white circle, left Y-axis) and deep sea water NEqPIW-samples (black circle; left Y-axis). Bars present numbers of MF present in both samples (right Y-axis).

Discussion

Recent studies, applying ultrahigh resolution FT-ICR-MS, revealed that marine bacteria release thousands of different exometabolites, yielding strain-specific, substrate-specific and growth stage-specific patterns of the released MF (Fiore *et al*., 2015; Johnson *et al*., 2016; Romano *et al*., 2014, Noriega-Ortega in prep.). It is still an open question, however, how these exometabolites are produced, randomly, e.g. passively released by lysing and/or growing leaky cells and/or as a function of polarity, size or structure, actively secreted and or as specific processes related to metabolic properties of the growing cells. There is some evidence that either of these processes, and possibly others may contribute to producing the greatly diversified exometabolome of marine bacteria. However, whether and if so how the release of

exometabolites is linked to metabolic features of a bacterium has not been specifically addressed so far. Therefore, the aim of this study was to address this point specifically, by linking the MF of the exometabolome of a model bacterium of the *Roseobacter* group, *P. inhibens*, to its metabolic features in the form of the transcriptome, i.e. to transcribed genes of distinct COG-categories (Galperin *et al*., 2015; Tatusov *et al*., 2000). The results show for the first time that patterns of a bacterium's exometabolome assessed by ultrahigh resolution FT-ICR-MS are linked to specific metabolic features. More than 40% of the MF of *P. inhibens* were highly significantly correlated to distinct metabolic features reflected by transcribed genes of different COG categories. The largest proportion of MF (13.4%) correlated to transcripts of COG category general function prediction (R) which includes proteins of general functions but also poorly characterized proteins. This finding indicates that the release of quite a few exometabolites occurs in the context of general metabolic functions of the bacterial cell. It further suggests that the results of this study are not only reflecting specific features of our model bacterium but apply to general metabolic functions of other bacteria as well meaning that just the active metabolism of a growing marine bacterium produces a certain proportion of so far unidentified metabolites released outside the cell and occurring as exometabolites. About 30% of the MF were correlated to COG categories of specific metabolic functions and 20% of them to replication and repair (L), cell wall/membrane/envelope biogenesis (M), post-translational modification, protein turnover and chaperones (O) and coenzyme transport and metabolism (H). It is plausible that enzymatic reactions related to repair, post-translational modification, protein turnover and chaperones produce a certain proportion of metabolic products which are of little use for subsequent intracellular metabolic reactions and therefore released or even secreted into the cell's environment. Cell-surface-related metabolic functions occur in close proximity to the cell's environment which also makes it plausible that a distinct proportion of the respective metabolites are lost outside the cell. Coenzymes are involved in many different metabolic functions and thus their biosynthesis is a crucial process. In the context of these processes quite a few metabolites appear to be released into the cell's environment, possibly as unusable byproducts. On the other hand, the correlation showed that for several COG categories only few MFs exhibited significant correlations such as nucleotide metabolism and transport (F), amino acid metabolism and transport (E), secondary metabolites biosynthesis and transport (Q) and inorganic ion transport and metabolism (P). These metabolic functions obviously were carried out very efficiently despite high numbers of involved genes of each COG category and thus contribute only little to the exometabolome. It appears noteworthy that only 11 MFs of the exometabolome were correlated to COG category amino

acid metabolism and transport (E) whereas 44 to that of carbohydrate metabolism and transport (G) despite the fact that the former involves more than twice as many genes. Hence amino acid metabolism contributes much less to the exometabolome diversity than that of carbohydrates. It is well known that carbohydrates are important components of the cell envelope and released outside the cell by different bacteria (Salton, 1953; Silhavy *et al*., 2010) and thus our findings are in line with these reports of previous studies but provide a refined look into the diversity and elemental composition of the exometabolites produced in the context of the cellular carbohydrate metabolism.

Striking differences occurred with respect to the N- and S-containing MF. Whereas only two of the MF correlating to COG category amino acid metabolism and transport contained an N atom half of the MF correlating to carbohydrate metabolism and transport contained one or more N-atoms. These findings appear counter intuitive to the intracellular metabolism of amino acids and carbohydrates. In the intracellular amino acid metabolism N-containing compounds constitute a major fraction of the metabolites. Therefore it appears surprising that only one exometabolite correlating to this COG category contains an N-atom. In contrast, the great majority of intracellular metabolites of the carbohydrate metabolism does not contain any N-atom. Hence our findings of the correlating MF may indicate that metabolic byproducts, not meeting these features, are secreted outside the cell. It appears plausible and in line with the known metabolism that MF correlating to COGs of lipid metabolism (I) and inorganic ion transport (P) do not contain any N-atom and MF correlating to COG category energy production and conversion (C) include only one with an N-atom. But the fact that MF correlating to COG category transcription (K) only include one MF containing an N-atom and those correlating to secondary metabolite biosynthesis and transport (Q) none with an N-atom is surprising. These findings further indicate that the formation and release of quite a few exometabolites are not random processes even though different from the rather well known endometabolic reactions but also embedded in and presumably controlled by the metabolic network of *P. inhibens*.

It was also surprising that about half of all correlating MF contained at least one S-atom. Sulfur is involved in many metabolic reactions, e.g. via the coenzyme acetyl CoA-SH and in signaling via LuxS (Pereira *et al*., 2013). Bacteria of the *Roseobacter* group exhibit a very elaborate metabolism of organic sulfur compounds including the antibiotics tropodithietic acid and roseobacticides (Durham *et al*., 2015; Newton *et al*., 2010; Thole *et al*., 2012; Wang *et al*., 2016), and 13 volatile organic sulfur compounds have been reported to be released by *P. inhibens* (Thiel *et al*., 2010). Hence the results of our study further emphasize the significance of S-containing metabolites and in particular of exometabolites in the metabolism of *P. inhibens*

and generally in the *Roseobacter* group. However, also other prokaryotes such as cyanobacteria or other *Alphaproteobacteria* than roseobacters release a variety of organosulfur compounds (Fiore *et al*., 2015; Romano *et al*., 2014). The latter authors found a high proportion (41-65%) of S-containing MF in the exometabolome of a *Pseudovibrio* strain assessed by FT-ICR-MS, in particular when grown under phosphorus limitation (Romano *et al*., 2014). As we detected S-containing exometabolites in many different MF correlating to very different COG categories and considering other reports we propose that S-containing exometabolites are more common in the exometabolome also in other marine bacteria not affiliated to the *Roseobacter* group than previously thought. However, they seem to be metabolized rather rapidly, as indicated by a study in the North Pacific (Dupont *et al*., 2006) and the fact that the C:S ratio of the MF matching to MF of North Pacific Intermediate waters was far higher than the overall mean C:S ratio of the exometabolome of *P. inhibens*.

As our approach to link exometabolites to transcriptomic patterns of a bacterium is completely new it is important to constrain the approach and validate it by supporting tests and observations. One important test was that the correlation applying the same approach with a randomized data set yielded a much lower fraction of correlating MF, 14.2% as compared to 42.9%, indicating that our correlation yielded valid results. Another piece of supporting evidence is to examine the correlating MF of a given COG category for their binning into classes of organic compounds identified on the basis of the H:C and O:C ratios, elemental composition and saturation index of the aliphatic and aromatic hydrocarbons such as lipids, carbohydrates, peptides and others (Abdulla *et al*., 2013; Table S3). Large proportions of the MF correlating to COG categories translation (J), post-translational modification, protein turnover and chaperones (O) and coenzyme transport and metabolism (H) binned into the class assigned to proteins (Table S3). These binnings appear consistent with the metabolic functions of the respective COG categories and suggest that a large fraction of these MF in fact are still characterized as proteins and peptides as exometabolites in the COG-specific processes. It appears also consistent that MF correlating to COG category general function prediction (R) are distributed over diverse classes of compound classes with no proportion exceeding 27% (Table S3). Obviously, the very general intracellular metabolic functions also lead to rather diverse exometabolites. However, only 7% of the MF correlating to the COG category carbohydrate metabolism and transport (G) showed characteristics typical of carbohydrates whereas about 60% showed characteristics of condensed hydrocarbons and peptides (Table S3). As mentioned above, these exometabolites may not be typical products of the intracellular carbohydrate metabolism but were released, possibly as waste products. Similar observations and implications seem to be true for MF

correlating to COG category lipid transport and metabolism (I) because no MF binned into the class of lipids whereas 60% binned into the class of carboxyl-rich alicyclic molecules (CRAM; Table S3). This class of molecules has been identified as a prominent class of marine DOM (Hertkorn *et al*., 2006). The core structure of these compounds is similar to sterols and hopanoids, indicating that they are related to lipids and may derive from membranes of marine microbes (Hertkorn *et al*., 2006, 2012). Interestingly, 41% of the MF correlating to COG category cell wall/membrane/envelop biogenesis (M) binned into the class of CRAM which is consistent with the associated metabolism (Table S3). Hence, the binning of the correlating MF into distinct COG categories provides further evidence for specific as well as rather general metabolic processes and production of exometabolites.

An interesting observation was that 11% of the exometabolites matched MF in the DOM sample from the North Equatorial Pacific Intermediate Water which contains predominantly refractory organic molecules persisting for decades to centuries in the ocean. A large proportion of these MF correlated to only several COG categories. It has previously been shown that during a phytoplankton bloom induced in a mesocosm 0.18-0.36% of the DOM produced by bacterial communities matched signatures of the refractory DOM of the North Equatorial Pacific Intermediate Water (Osterholz *et al*., 2015). Further, it has been shown that <2% of the MF of the exometabolome of a *Pseudovibrio* strain matched MF of this Water (Romano *et al*., 2014). Our study extends the observation that even a single bacterium produces exometabolites with MF identical to those of refractory DOM of North Pacific Intermediate waters by showing that they exhibit correlations to intracellular metabolic functions and are presumably not produced randomly. Hence, we provide evidence that a single bacterium produces MF with the signature of refractory marine DOM which may persist for long periods in the marine DOM, i.e. the geometabolome.

Conclusions

We applied a novel approach to correlate MF of the exometabolome of a marine bacterium to transcriptomic patterns. The results show that about 40% of the produced MF correlate to distinct COG categories indicating that the correlating MF are not produced randomly but are the result of specific intracellular metabolic processes. In some cases the class of correlating MF of the exometabolites reflect directly the intracellular metabolic functions, like peptides and translation, whereas in other cases they do not match, like for carbohydrate and lipid metabolism. The latter cases suggest that the correlating MF may be metabolic byproducts

which are released outside the cell. Hence, the exometabolome appears to encompass molecules which are a direct end product of intracellular metabolic processes but others which are byproducts, e.g. metabolic waste, of other intracellular metabolic processes. A small fraction of either exometabolites appears to be similar to refractory compounds of marine DOM, implying that such compounds may be directly produced my marine bacteria and persists in the oceans for long periods. This powerful approach provides novel important insights into the functional link between the exometabolome of single bacteria and the marine geometabolome and by linking the MF of exometabolites to intracellular metabolic processes.

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Conflict of interest

The authors declare no conflict of interest.

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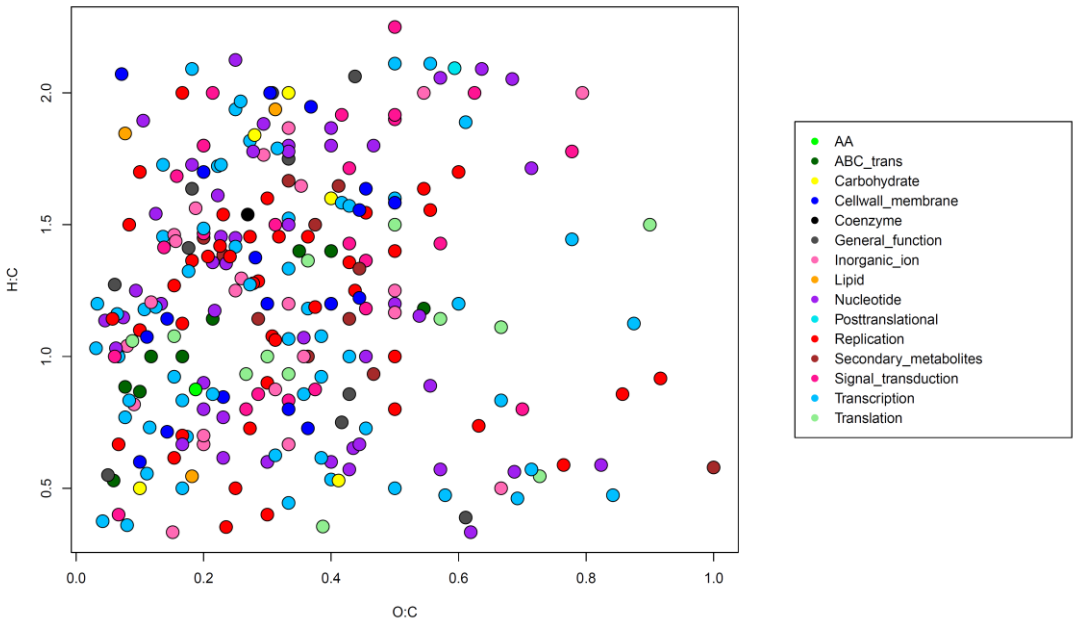
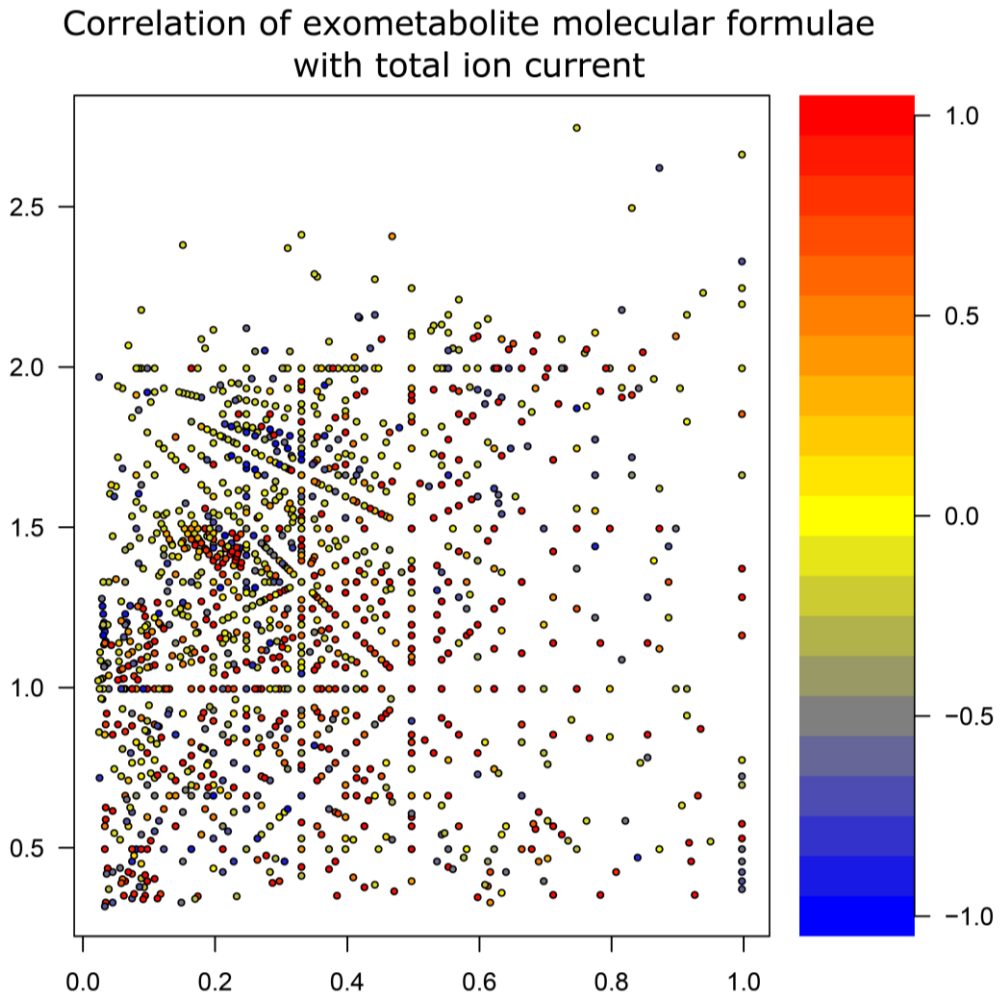
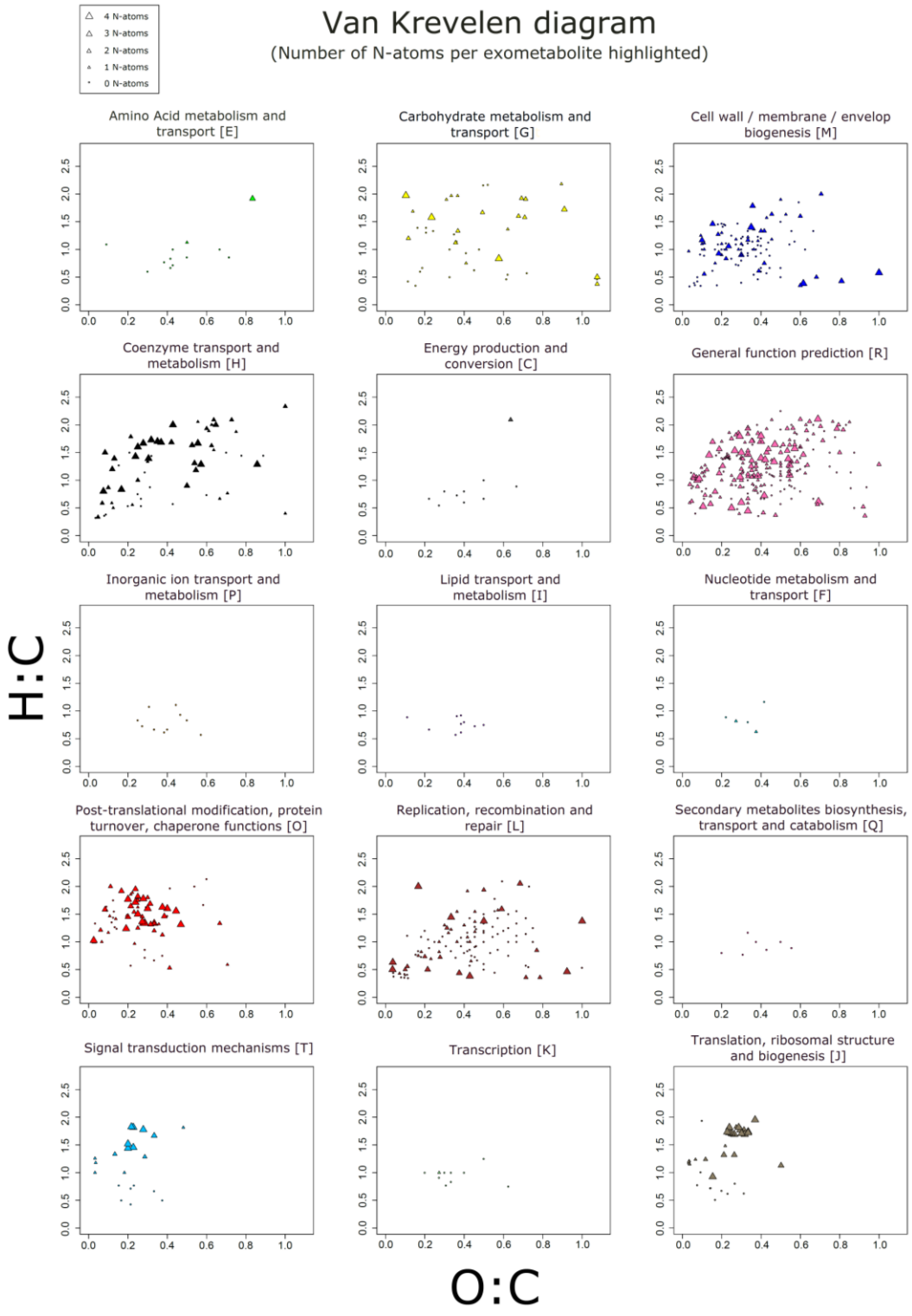
Supplementary information:



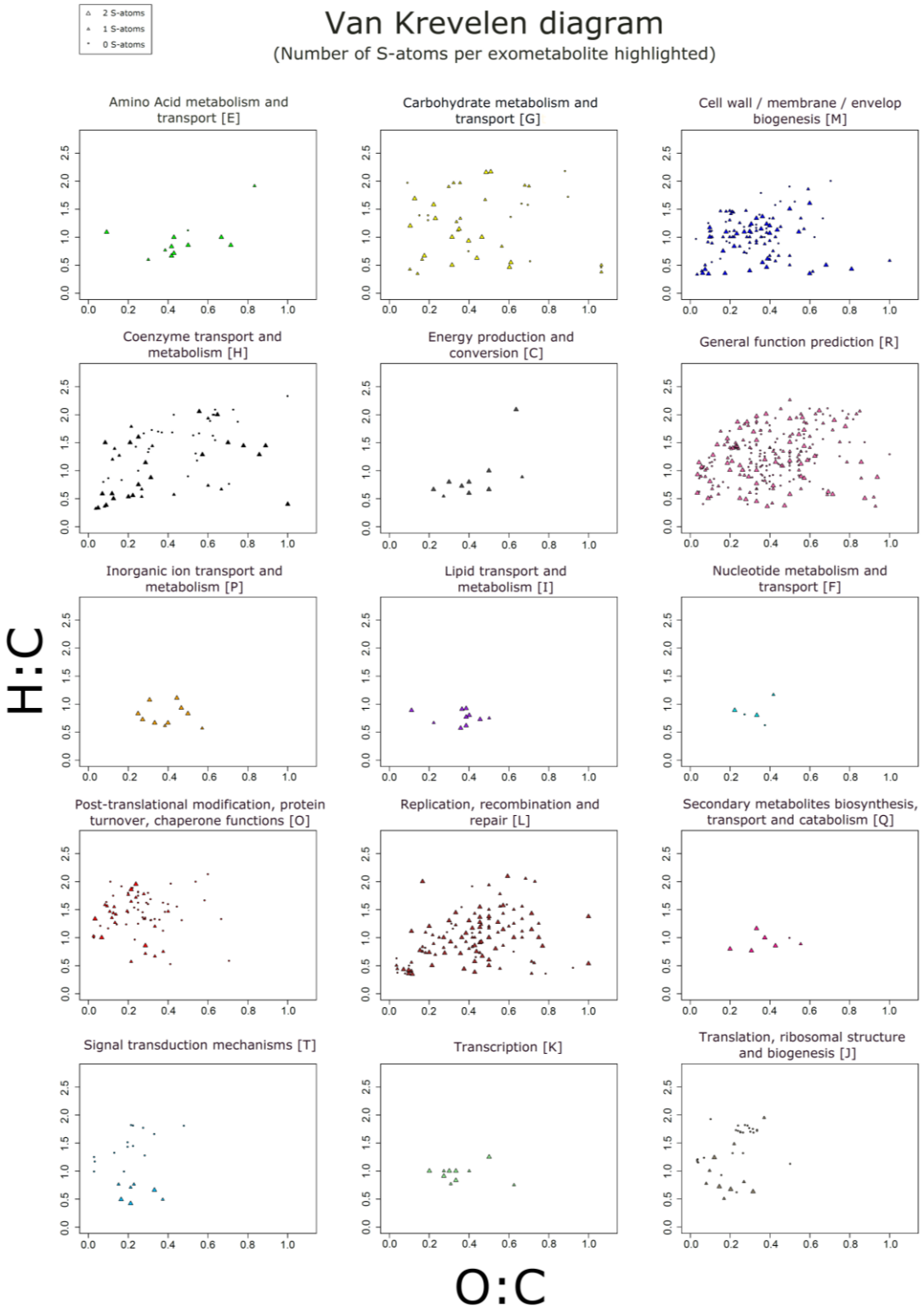
Supplementary figure S1 | Van Krevelen diagram (H:C / O:C) of a randomized transcriptomic dataset of exometabolite molecular formulae correlating with functional COG-groups. Color-codes indicate functional COG-groups each MF correlate to (see legend). In total 271 MF-correlated to the randomized dataset.



Supplementary figure S2 | Van Krevelen diagram (H:C / O:C) illustrates the correlation of exometabolite molecular formulae with the total ion current. The scale presents the range of correlation of individual molecules, in which blue shows a negative and red a positive correlation.



Supplementary figure S3 | Van Krevelen diagrams (H:C / O:C) of exometabolite molecular formula (MF) correlating with respective functional COG-groups. Triangle symbols show exometabolite MF with nitrogen-atoms and symbol size indicates the number nitrogen-atoms per molecule (see legend).



Supplementary figure S4 | Van Krevelen diagrams (H:C / O:C) of exometabolite molecular formula (MF) correlating with respective functional COG-groups shown. Triangle symbols show exometabolite MF with sulfur-atoms and symbol size indicates the number sulfur-atoms per molecule (see legend).

Supplementary table S1 | Number of genes associated to every functional COG-group used for the correlation analyses and the respective mean nucleotide activity per kilo base of exon model per million mapped reads (NPKM).

Functional COG-group	Number of genes associated to functional group	Mean NPKM
Amino Acid metabolism and transport [E]	264	1387
Carbohydrate metabolism and transport [G]	123	993
Cell wall / membrane / envelop biogenesis [M]	128	3576
Coenzyme transport and metabolism [H]	135	1535
Energy production and conversion [C]	145	2483
General functional prediction [R]	162	1465
Inorganic ion transport and metabolism [P]	264	1475
Lipid transport and metabolism [I]	104	1701
Nucleotide metabolism and transport [F]	66	1403
Post-translational modification, protein turnover, chaperone functions [O]	88	2257
Replication, recombination and repair [L]	60	1614
Secondary metabolites biosynthesis, transport and catabolism [Q]	61	1476
Signal transduction mechanisms [T]	90	2025
Transcription [K]	111	1827
Translation [J]	161	2437

Supplementary table S2 | Numbers and percentage of correlating exometabolites and exometabolites that share the identical MF compared to deep sea (NEqPIW) DOM-samples are listed (*=1911, **= respective number of correlating exometabolite MF).

Functional COG-groups	Correlating exometabolites		Exometabolite overlap to deep sea DOM	
	Number	% of (*)	Number	% of (**)
Amino Acid metabolism and transport [E]	13	0.7	1	7.7
Carbohydrate metabolism and transport [G]	44	2.3	2	4.5
Cell wall / membrane / envelop biogenesis [M]	116	6.1	12	10.3
Coenzyme transport and metabolism [H]	63	3.3	0	0.0
Energy production and conversion [C]	11	0.6	0	0.0
General functional prediction [R]	257	13.4	60	23.3
Inorganic ion transport and metabolism [P]	11	0.6	0	0.0
Lipid transport and metabolism [I]	15	0.8	0	0.0
Nucleotide metabolism and transport [F]	5	0.3	0	0.0
Post-translational modification, protein turnover, chaperone functions [O]	75	3.9	10	13.3
Replication, recombination and repair [L]	125	6.5	5	4.0
Secondary metabolites biosynthesis, transport and catabolism [Q]	7	0.4	1	14.3
Signal transduction mechanisms [T]	21	1.1	2	9.5
Transcription [K]	10	0.5	0	0.0
Translation [J]	37	1.9	0	0.0
Positive correlating exometabolites	819	42.9	93	11.4
All exometabolites	1911	100	196	10.3

Supplementary table S3 | Numbers of exometabolites correlating to functional COG-groups and the molecular chemical characteristics of every COG-group exometabolite cluster given as percentage. Highlighted in grey are the highest percentages of every COG-group exometabolite cluster.

Functional COG-group	Number of correlating exometabolites	Molecular chemical characteristics (given as percentage of respective No. of correlating exometabolites)						
		polyphenols	Highly unsaturated		Unsaturated aliphatic		sugars CHO	peptides
			Oxygen-rich	Oxygen-poor	Oxygen-rich	Oxygen-poor		
		(CRAM)	(Tanins)	(Condensed Hydrocarbon)	(Amino sugar)	(Lipids)	(Carbohydrates)	(Peptides)
Amino Acid metabolism and transport [E]	14	29	21	29	0	0	0	7
Carbohydrate metabolism and transport [G]	44	2	2	30	0	0	2	30
Cell wall / membrane / envelop biogenesis [M]	116	21	4	41	1	1	0	6
Coenzyme transport and metabolism [H]	63	10	8	14	3	2	0	30
Energy production and conversion [C]	11	45	9	9	0	0	0	0
General functional prediction [R]	257	19	12	27	4	3	0	18
Inorganic ion transport and metabolism [P]	11	45	0	36	0	0	0	0
Lipid transport and metabolism [I]	10	60	0	20	0	0	0	0
Nucleotide metabolism and transport [F]	5	40	0	20	0	0	0	0
Post-translational modification, protein turnover, chaperone functions [O]	75	15	0	37	3	8	0	32
Replication, recombination and repair [L]	125	14	14	32	4	1	0	6
Secondary metabolites biosynthesis, transport and catabolism [Q]	7	43	14	43	0	0	0	0
Signal transduction mechanisms [T]	21	29	0	29	0	0	0	29
Transcription [K]	10	40	0	60	0	0	0	0
Translation [J]	37	14	0	27	0	3	0	43

Chapter 5

Vitamin B1, B7 and B12 synthesis capabilities of major marine bacterial groups and circumvention of lacking genetic capabilities by metabolite allocation

Gerrit Wienhausen, Benedikt Heyerhoff and Meinhard Simon

Institute for Chemistry and Biology of the Marine Environment, University of Oldenburg, Oldenburg, Germany

Keywords: *Rhodobacteraceae*, genome streamlining, vitamin B1 and B12, thiamin moieties

Abstract

Vitamins function in myriad reactions as co-factors and are indispensable for all living organisms. Numerous organisms lack de novo synthesis of the two most pivotal vitamins, B1 and B12, possibly caused by genome streamlining. Interestingly, such synthesis limitations are partially only restricted to the lack of single genes and can be substituted by allocation of the absent metabolite precursor. In order to identify the relevance of such phenomena among roseobacters, a group of important marine bacteria, we screened 85 genomes for known B1 and B12 genes of respective bacterial strains. Further, we tested bacterial isolates in culture experiments, that were isolated of biofilms of eukaryotic organisms, whether they de novo synthesize vitamins B1, B7 and B12 or if B1-auxotrophy can be circumvented by thiamin precursor moieties. Our results show that vitamin B1, B7 and B12 auxotrophies are prevalent among the tested representative isolates, identified on genome and in culture surveys. For one bacterial isolate, we demonstrate that the lack of single genes causing vitamin B1 auxotrophy can be substituted by the allocation of single precursors, such as the thiamin moiety 4-amino-5-hydroxymethyl-2-methylpyrimidine. Overall, vitamin auxotrophy is seemingly ubiquitous among marine bacteria. Further, we highlight that vitamin auxotrophy in nature is not only encountered by the intake of final vitamin metabolites, but also by the allocation of vitamin precursors.

Introduction

Vitamins are essential metabolites for all living organisms, functioning as cofactors in various indispensable enzymatic reactions. The availability of such precious compounds, including even vitamin precursor metabolites, is often limited in nature by its allocation (Suffridge *et al*., 2017). In some cases, marine microbes release metabolites in mutual interdependent trades or as public goods and thereby guarantee the success and survival of the microbial community (Amin *et al*., 2015; Cruz-López and Maske, 2016; Fiore *et al*., 2015; Garcia *et al*., 2015; Wienhausen *et al*., 2017). The steady availability of metabolites is believed to facilitate genome streamlining among microbes and consequently enables a more efficient metabolism (Giovannoni *et al*., 2005; Giovannoni, 2012). Such genome streamlining phenomena were exemplarily demonstrated for specific genes in vitamin B1 (herein after called B1) and vitamin B12 (herein after called B12) pathways among various marine eukaryotes and prokaryotes (Croft *et al*., 2006; Sañudo-Wilhelmy *et al*., 2014). Gene deficiency simultaneously creates dependencies on the presence of microbes that conquer the lacking metabolic capabilities (Giovannoni, 2012; Morris *et al*., 2012). Consequently, essential metabolites hold the potential to alter the composition and dynamic of a marine microbial community, solely by their availability.

In order to examine the genomic capabilities of B1 and B12 biosynthesis of the important marine *Roseobacter* group, encompassing many members often associated to phytoplankton, we screened 85 genomes of this group for genes and pathways of B1 and B12 biosynthesis. Further we tested 26 bacterial isolates in batch culture for de novo B1, B7 and B12 synthesis. B1 auxotroph strains were further supplemented with both respective B1 moiety precursors, 4-methyl-5-(b-hydroxyethyl)thiazole (HET) and 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP), to test the circumvention of lacking genes.

Material and Methods

Gene analysis of B1 and B12 biosynthetic pathways in the *Roseobacter* group

This analysis was based on enzyme annotated genomes of 85 *Roseobacter* group members. For detailed methodological and bioinformatic information of the annotation of enzyme coding genes see Simon *et al*., (2017). Genomes were blasted for enzyme coding genes with the identified involvement in thiamin and cobamide biosynthesis pathways.

Growth experiments

Twenty six bacterial strains, isolated from eukaryotic macroorganism surfaces (crustacean and *Fucus Spiralis*) with highest similarities (16S rRNA blast) to *Flavobacteriaceae, Rhodobacteraceae* and *Pseudomonadaceae* strains were grown in artificial seawater (ASW)-medium and tested for B1, B7 and B12 auxotrophy. In subduction of each tested vitamin, both remaining vitamins were supplemented in concentrations of 100 nM. Growth was monitored as optical density against a positive control including all three vitamins at final concentrations of 100 nM each.

In order to test whether B1 auxotrophic isolates can be substituted by either thiamin moiety, HET (AstaTech inc., Bristol, PA, USA) or HMP (Sigma Aldrich, Munich, Germany), nine of thirteen strains were grown in ASW-medium and instead of B1, precursors were solely added at final concentration of 100 nM, together with B7 and B12 (100 nM each). Growth was monitored as optical density against a positive control including all three vitamins at final concentrations of 100 nM each and a negative control including only B7 and B12 at the same concentrations.

Results

Distribution of B1 and B12 synthesis genes in the *Roseobacter* group

Screening vitamin biosynthetic pathways of *Rhodobacteraceae* revealed that more than 35% of the *Roseobacter* group members presumably lack essential genes involved in the biosynthesis of the thiamin building blocks. Enzyme-coding genes for the synthesis of HMP and HET were detected in the genomes of only 44% and 64% of the roseobacters, respectively, whereas 93% retained the enzymatic ability to merge both moieties to thiamin (Fig. 1). Gene screening of all identified cobamide biosynthesis pathways suggested likewise a loss of single genes that presumably cause a B12

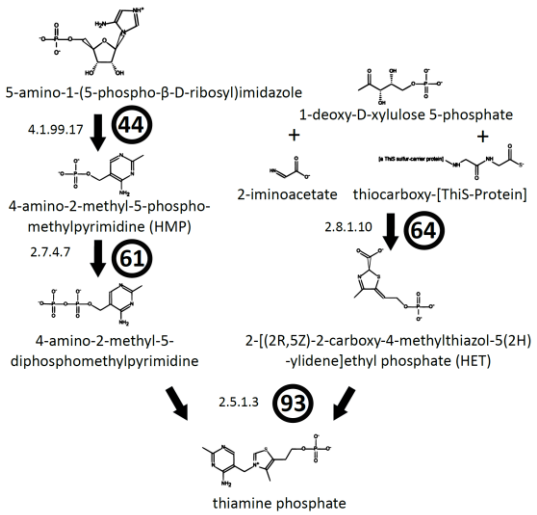


Figure 1 | Thiamin (B1) biosynthetic pathway elucidated for 85 marine *Roseobacter* group members. Percentage of enzyme coding genes (given as Enzyme Commission number) present in surveyed strains is shown in the adjacent black circles.

auxotrophy in single *Roseobacter* group members (Fig. S1).

Blasts for gene loss in both pathways indicated that the lack is not confined to single genes. Generally a large fraction of vitamin pathway genes were maintained. To draw general conclusions on gene streamlining with respect to B1 and B12 biosynthesis, erroneous enzyme annotation and the lack of knowledge on hypothetical genes has to be taken into consideration.

Vitamin auxotrophy and overcoming thiamin auxotrophy by HET/ HMP allocation

Auxotrophy for B1, B7 and B12 was detected among isolates of all prokaryotic families surveyed. In total, 50% of all tested strains revealed inefficiency of de novo B1 synthesis and 46% for the synthesis of vitamins B7 and B12 (see Fig. 2; Table 1). Nine thiamine auxotroph strains were again grown in ASW-medium, supplemented with HET or HMP to test whether the inefficiency of de novo B1-synthesis originated from the lack of single enzyme coding genes.

In fact, of all tested strains exclusively strain MA-E2-3, isolated of the shell of the crab *Cancer pagurus*, conquered B1 auxotrophy by artificial HMP supply (see Table S1). Growth detected by optical density was significantly induced even though with a lag of 24 h compared to growth with B1, peaking approx. at an OD of 0.25 when amended by HMP and approx. 0.35 when supplemented by B1. Strain MA-E2-3 seemingly maintained all other essential genes in the B1 pathway and exclusively lacks the phosphomethylpyrimidine synthase (EC: 4.1.99.17; see Figure 2).

Table 1 | De novo B1, B7 and B12 synthesis of selected bacterial strains tested in batch culture experiments. Bacterial strains were isolated from eukaryotic organisms and revealed closest 16S rRNA sequence alignment to representatives belonging to *Flavobacteriaceae*, *Rhodobacteraceae* and *Pseudomonadaceae* families.

Family (97 % identity)	Total number of strains	de novo B1 synthesis	de novo B7 synthesis	de novo B12 synthesis
Flavobacteriaceae	**(5)**	**2**	**1**	**1**
Rhodobacteraceae	**(18)**	**9**	**9**	**9**
Pseudomonadaceae	**(3)**	**2**	**2**	**2**

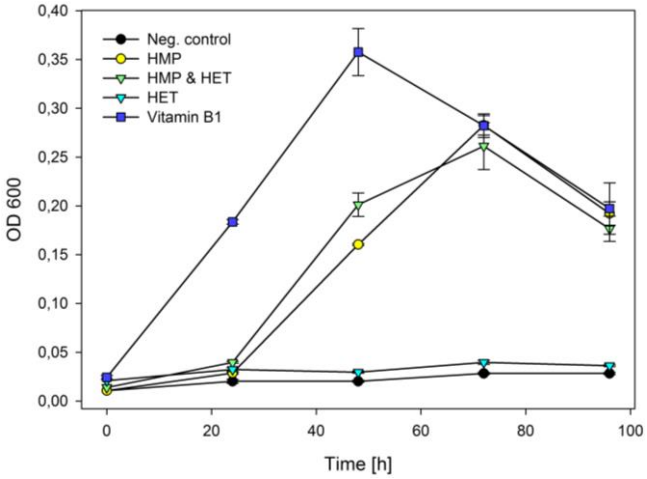


Figure 2 | Growth detected by optical density over time of a B1 auxotroph bacterial isolate (MA-E2-3), most closely related to a *Rhodobacteraceae* family strain. ASW-medium was enriched with HMP, HET, HMP & HET, B1 or used as control without any of these supplements.

Discussion

Vitamins are perhaps one of the most pivotal metabolite groups whose availability is largely controlled by microbes that assure the vitality of marine microbial communities. Our B1 and B12 gene screening analysis indicated the lack of individual genes in different enzymatic stages for both pathways in various members of the 85 *Roseobacter* group representatives. Thus, the phenomena of genome streamlining, in particular analyzing vitamin pathway genes, appears to be widespread among members of the *Roseobacter* group that commonly dwell in phytoplankton blooms and in association with vitamin dependent marine organisms (Dogs *et al*., 2017; Wagner-Döbler *et al*., 2009, Newton *et al*., 2010, Simon *et al*., 2017). Similar studies screening B1 and B12 synthesis genes and pathways in whole-genome-sequenced eukaryotic and bacterioplankton organisms likewise suggested a lack of individual genes, presumable interrupting entire pathways (Croft *et al*., 2006; Sañudo-Wilhelmy *et al*., 2014). Hence it appears that the exchange of vitamin pathway intermediates is beneficial and partially indispensable for marine bacterioplankton communities. In general, whole genome sequence based analyses need to be considered with caution because a large fraction of genes cannot be annotated and remain hypothetical or putative. Thus, the function of numerous enzyme coding genes remains yet unknown, erroneous gene annotation possibly leads to wrong prediction and genome sequencing can be incomplete. Thus, the existence of a given metabolic pathway in genomic studies is often based on the assumption that it is present

when >75% of the genes are present (Simon *et al*., 2017). However, Newton *et al*., (2010) concluded from genome analysis of 32 *Roseobacter* group isolates that gene assortment is unique for individual strains and reflecting its lifestyle and it is known that genome streamlining is a wide spread phenomena among marine bacteria. Hence, it is conceivable that members of the *Roseobacter* group lost single genes as adaptation strategy, saving metabolic cost.

As a consequence, we need to have a refined look at pathways and verify whole genome based auxotrophy observations experimentally. Such studies have for instance identified that the lack of single genes in essential vitamin pathways can result in a hindrance of synthesizing the final vitamin metabolite (Carini *et al*., 2014; Gray and Escalante-Semerena, 2010; Paerl *et al*., 2017). Also our finding, substituting the B1 auxotroph MA-E2-3 by HMP verifies our genome based assumption that the genome streamlining provokes not only dependencies on final vitamin metabolites, but also highlights the relevance of vitamin precursor availability. We demonstrate that numerous members of the relevant *Roseobacter* group presumable lack essential vitamin pathway genes and that vitamin auxotrophy is prevalent among this group and presumably also among other marine microbes. Thus, the exchange and availability of such precious vitamin metabolites is seemingly crucial for the well-being and functioning of marine microbial communities.

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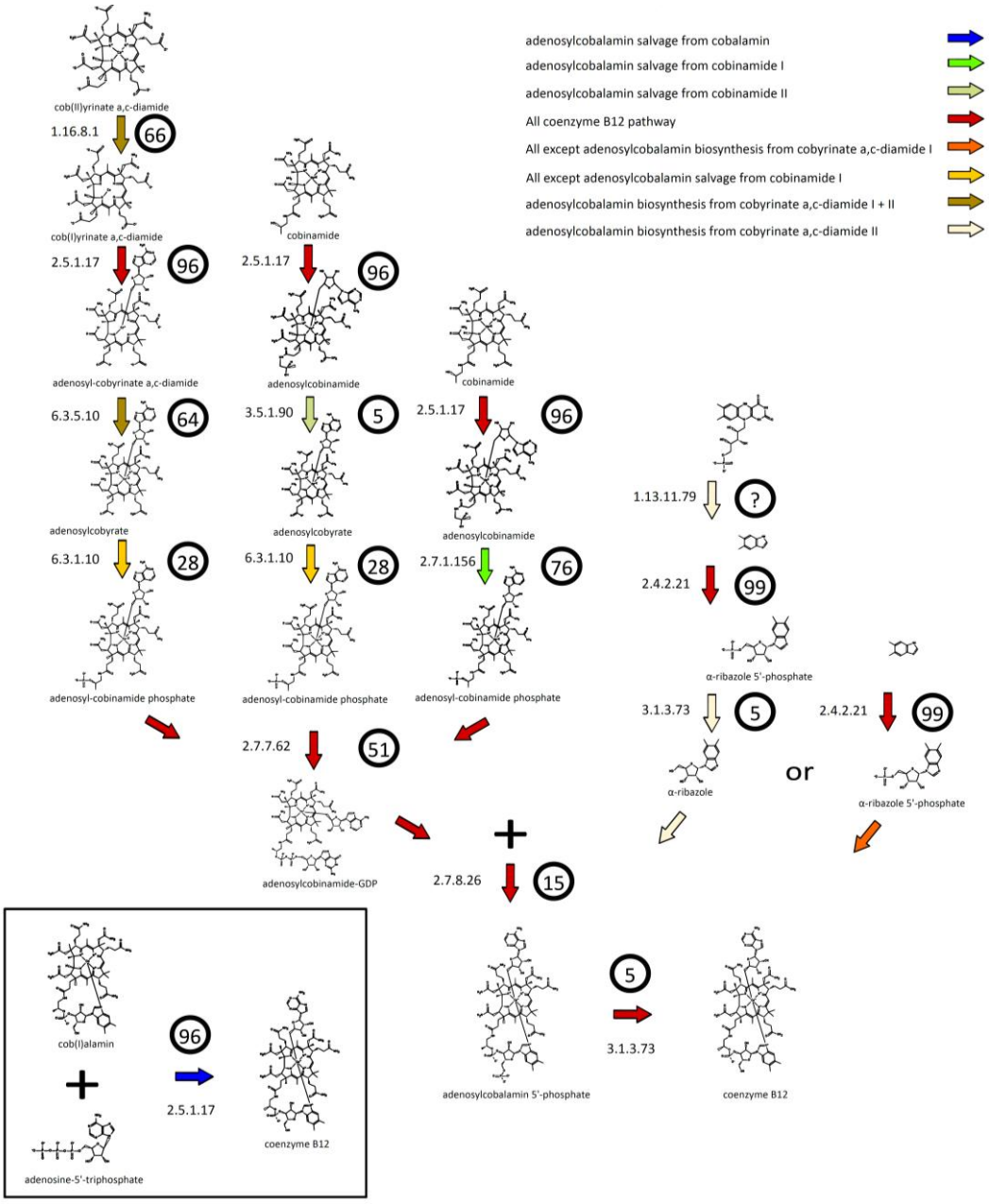
Supplementary information:

Supplementary table S1 | Cultures of nine B1-auxotroph bacterial strains were supplemented with either HET or HMP and both, to test whether the allocation of either thiamin moiety enables the lack of de novo thiamin synthesis. Growth (detected by OD600) after supplementation is presented as (+), no response as (-) Bacterial strains were isolated from eukaryotic organisms and revealed closest 16S rRNA sequence alignment to representatives belonging to *Flavobacteriaceae*, *Rhodobacteraceae* alignment to representatives belonging to *Flavobacteriaceae*, *Rhodobacteraceae.*

Strain identification code	Family (97 % identity)	Isolated of biofilm of	Growth after HET allocation	Growth after HMP allocation
MA-E2-3	*Rhodobacteraceae*	*Cancer pagurus*	(-)	(+)
Och 149	*Rhodobacteraceae*	*Cancer pagurus*	(-)	(-)
N° 156	*Rhodobacteraceae*	*Cancer pagurus*	(-)	(-)
N° 52	*Flavobacteriaceae*	*Cancer pagurus*	(-)	(-)
N° 32	*Rhodobacteraceae*	*Cancer pagurus*	(-)	(-)
N° 35	*Rhodobacteraceae*	*Cancer pagurus*	(-)	(-)
B3	*Rhodobacteraceae*	*Fucus spiralis*	(-)	(-)
LW-35	*Rhodobacteraceae*	*Fucus spiralis*	(-)	(-)
LW-41a	*Rhodobacteraceae*	*Fucus spiralis*	(-)	(-)



Supplementary figure S1 | Cobamide (coenzyme B12) biosynthetic pathways present in the *Roseobacter* group. By reason of the complexity and diversity of cobamide biosynthetic pathways, individual enzymes can be involved in different pathway successions. Each enzymatic step is depicted by arrows and its corresponding Enzyme Commission number. The arrow coloring refers to cobamide (coenzyme B12) biosynthetic pathway the respective enzyme is involved in (see legend). Percentage of enzyme coding genes present in surveyed 85 marine *Roseobacter* group members is shown in the adjacent black circles.

Chapter 6

Bacterioplankton growth in oceanic systems is controlled by vitamins B1 and B12 and biosynthetic precursors

Gerrit Wienhausen, René Jarling, Jutta Niggemann, Thorsten Dittmar and Meinhard Simon

Institute for Chemistry and Biology of the Marine Environment (ICBM), University of Oldenburg, Oldenburg, Germany

Keywords: Vitamin precursor, vitamin traffic, thiamin, cobalamin

Chapter 6

Abstract

Many marine prokaryotes and eukaryotic plankton cannot de novo synthesize the essential vitamin cofactors B1 and B12 and depend on the supply by prototrophic marine organisms. Therefore vitamin transfer and cycling is essential for the growth dynamics of microbial communities in marine pelagic ecosystems. Vitamin auxotrophy is not exclusively encountered by the exchange of final vitamin cofactors, but there is increasing evidence that also exogenously provided vitamin precursors can substitute a lacking genetic capacity. To shed more light on the complex exchange of vitamins in marine pelagic ecosystems we conducted five experiments in different biogeographic provinces in the Pacific. Mesocosms in the south Pacific subtropical gyre (SPSG), the equatorial upwelling and the north Pacific polar frontal region were supplemented with cobalamin (vitamin B12) and its lower ligand α-ribazole. Mesocosms in the SPSG and the New Zealand coastal province were supplemented with thiamin (vitamin B1), 4-methyl-5-(β-hydroxyethyl)thiazole (HET), 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP) and HET & HMP. The results show bacterial biomass production and growth rates were significantly enhanced not only upon the addition of vitamin B12, but also upon supplementation of α-ribazole and the two thiamin moieties HET and HMP relative to an unsupplemented control. Our findings provide evidence for the complexity of microbial vitamin cycling in the ocean and that single vitamin metabolites and precursors possess the potential to alter microbial activity.

Introduction

Vitamins are one of the most crucial and biologically active compound classes and essential growth factors with pivotal impact on marine pelagic microbial communities (Sañudo-Wilhelmy *et al*., 2014). In particular, thiamin (vitamin B1; called B1 herein) and cobalamin (generally known as vitamin B12; called B12 herein) are vital due to their function as cofactors for a broad variety of essential enzymes (see Figure 1; Jurgenson *et al*., 2009; Matthews *et al*., 2003; Dowling *et al*., 2012).

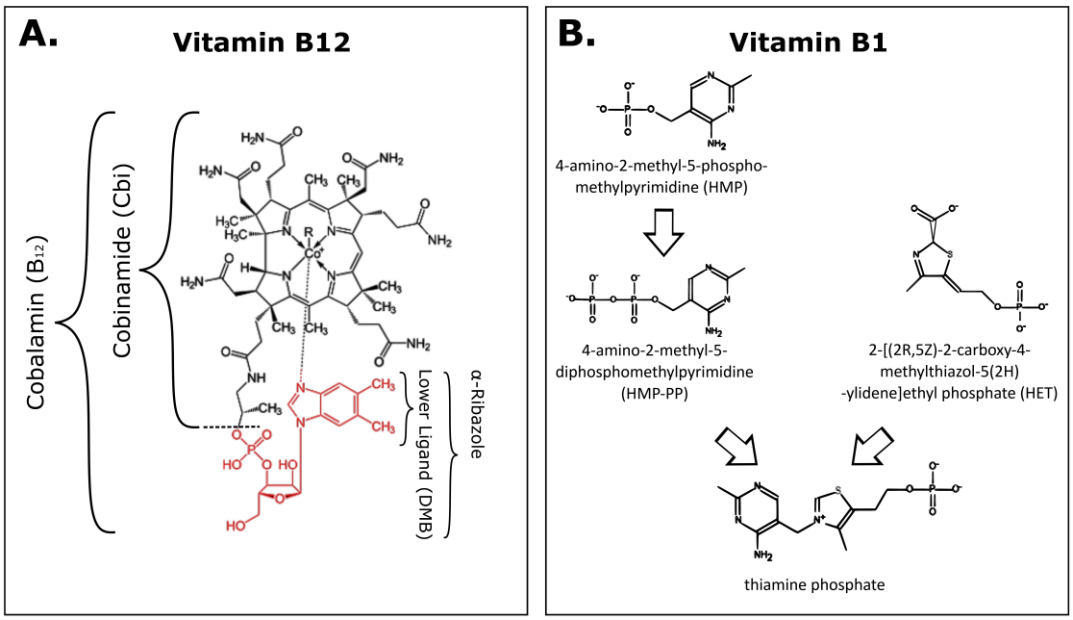


Figure 1 | A. Representative Example of corrinoid structures, presenting cobalamin in which α-ribazole is highlighted. B. Illustration of the simplified final thiamin pathway, depicting thiamin and the two moiety precursor, HMP and HET.

Despite their indispensability for life, quite a few groups of autotrophic and heterotrophic marine microbes are auxotrophic for one or more vitamins (Croft *et al*., 2005; Sañudo-Wilhelmy *et al*., 2014). Metagenome analysis for instance revealed the SAR11 clade to be auxotrophic for thiamin (Giovannoni *et al*., 2005; Carini *et al*., 2014). Similar findings of B1 auxotrophy have been reported for members of the order Rhodobacterales and class Flavobacteriia, both often associated to phytoplankton blooms (Sañudo-Wilhelmy *et al*., 2014). De novo B12 synthesis underlies vast variations among bacterial phyla, despite the fact that synthesis is exclusively performed by them and Archaea (Croft *et al*., 2005; Sañudo-Wilhelmy *et al*., 2006; Grant *et al*., 2014; Cruz-López and Maske, 2016). As shown by genome sequencing

the large majority of Rhodobacteraceae and Cyanobacteria possesses genes for de novo B12 synthesis, whereas most globally relevant and abundant SAR11 or Bacteroidetes strains disclose an obligate exogenous B12 requirement (Sañudo-Wilhelmy *et al*., 2014). Also more than half of all marine eukaryotic phytoplankton organisms exhibit B12 cofactor dependent enzymes (Croft *et al*., 2005) and hence rely on its exogenous supply. Consequently the production and supply of vitamins B1 and B12 is a significant factor in marine microbial interactions (Croft *et al*., 2005; Cruz-López and Maske, 2016; Grant *et al*., 2014; Provasoli, 1963; Sañudo-Wilhelmy *et al*., 2006).

Concentrations of vitamins B1 and B12 in seawater can vary greatly, ranging from 457 pM (B1) and 87 pM (B12) to nearly depletion in surface waters (Menzel and Spaeth, 1962; Sañudo-Wilhelmy *et al*., 2006, 2012, 2014; Suffridge *et al*., 2017). Differences in vitamin concentrations are believed to be a function of the trophic state of the water mass, depth and season, with highest concentrations in winter, followed by a decline during spring phytoplankton blooms (Menzel and Spaeth, 1962; Sañudo-Wilhelmy *et al*., 2012; Suffridge *et al*., 2017). Mesocosm amendment experiments by Bertrand *et al*., (2007) and Koch *et al*., (2011) demonstrated that B12 can be a limiting factor for growth of distinct phytoplankton groups and thus may be crucial in shaping phytoplankton community composition.

B12, more specifically defined as cobalamin and belonging to the class of cobamides, exhibits α-ribazole as lower ligand and is essential for most organisms (Crofts *et al*., 2013). Generally cobamides are specified by the attached lower ligand, a corrinoid cofactor, and possess differences in cofactor specificity. There is evidence that different B12 variants are used by different organisms (Helliwell *et al*., 2016) and that concentrations of dissolved exogenously available corrinoid cofactor variants vary greatly in seawater (Suffridge *et al*., 2017).

There is accumulating evidence from experimental studies and field observations that vitamin networks and resulting microbial interdependencies in marine pelagic ecosystems must be extended to vitamin precursors. Respective B1 and B12 precursors have been detected in natural seawater and in the exometabolome of representative bacterial strains. Four-amino-5-hydroxymethyl-2-methylpyrimidine (HMP), one of the two B1 moieties reached concentrations of 28 pM in Atlantic surface waters (Suffridge *et al*., 2017) and is known to be released by cyanobacteria, a marine Betaproteobacterium and the alga Dunaliella tertiolecta (Carini *et al*., 2014). The second B1 moiety, 4-methyl-5-(β-hydroxyethyl)thiazole (HET), was detected in the exometabolome of two *Roseobacter* group members(Wienhausen *et al*., 2017) . Recent studies detected α-ribazole, the lower ligand of the most relevant bioactive corrinoid coenzyme, within the exometabolome of several marine bacteria and its mass formula (MF) in dissolved

organic matter (DOM) of the Pacific and North Sea (Johnson *et al*., 2016; Romano *et al*., 2014; Wienhausen *et al*., 2017). The presence of vitamin precursor in seawater falls into place with the disability of most vitamin auxotrophic organisms to synthesize these vitamin building blocks and the lack of single genes (Carini *et al*., 2014; Gray and Escalante-Semerena, 2010; Paerl *et al*., 2017; Sañudo-Wilhelmy *et al*., 2014). Hence, vitamin precursors may function as crucial compounds in microbial metabolic networks and help to overcome auxotrophy. For instance, cosmopolitan members of the SAR11 clade require HMP for growth and similarly, exogenously provided HET complements the B1 auxotrophy of several green algae, including cryptophytes and dinoflagellates (Droop, 1958; Lwoff, 1947; Turner, 1979). Both moieties can not only substitute thiamin auxotrophy, their presence can also stimulate growth of vitamin-prototrophic diatoms (Wienhausen *et al*., 2017). The interaction of cobamides and their respective lower ligands appear to be even more complex. The attached lower ligand alters the B12 structure and thereby affects the cofactor binding and consequently the enzyme catalysis (Crofts *et al*., 2013; Koch *et al*., 2011; Mok and Taga, 2013; Stupperich *et al*., 1987; Yi *et al*., 2012). In particular, α-ribazole has been shown to be actively transported into the bacterial cell and enabling the synthesis of adenosylcobalamin (AdoCbl) thus complementing the lack of genes synthesizing α-ribazole de novo (Gray and Escalante-Semerena, 2010). The availability of lower ligands can even change the corrinoid synthesis (Keller *et al*., 2014). These findings indicate the existence of specific adaptation mechanisms by bacteria to low ambient bioactive cobamide concentrations and suggest a relevant function of lower ligands in the environment. Helliwell *et al*., (2016) demonstrated that representatives of major phytoplankton groups can remodel pseudocobalamin, commonly produced by cyanobacteria, by the aid of 5,6-dimethylbenzimidazole (DMB), a building block of the fundamental AdoCbl lower ligand α-ribazole. Corrinoid remodeling is also performed by bacteria, indicating an adaptation strategy to the presence of unsuitable cobamides in nature (Yi *et al*., 2012).

Life of the majority of marine microorganisms depends on the availability of vitamins B1 and B12, however only a minority holds the genetic apparatus for de novo synthesis of both of them. This makes B1 and B12 especially in oligotrophic waters a precious compound. To our knowledge, so far only B12 amendment experiments were performed in marine environments and solely in oceanic biogeographic provinces of rather high productivity, not yet in the oligotrophic open ocean with generally much more severe growth limiting conditions for microbes. Recent studies suggested much larger ramifications of vitamin precursors in microbial metabolic networks (see above), but the effect of these precursors and possible growth-limiting role has never been experimentally tested.

Consequently, in this study we conducted five mesocosm experiments in four different biogeographic provinces of the Pacific, distinct by their hydrography, trophic states and microbial communities, the oligotrophic south Pacific subtropical gyre (SPSG), the equatorial upwelling (EqUp), the north Pacific polar frontal region (NPF) and the New Zealand Costal Province (NZCP). In three experiments mesocosms were enriched with B12 or its lower ligand α-ribazole and in two experiments with B1, HET, HMP and HET+HMP. We assessed the effects of these additions by measuring bacterial cell numbers, bacterial biomass production, amino acid turnover and chlorophyll autofluorescence over a period of five to eight days.

Material and Methods

Water sampling and hydrographic parameter

Mesocosm water was collected in four different biogeographic provinces of the Pacific (see Fig. 2 and Table 1: SPSG (M1, M4); EqUp (M2); NPF (M3); NZCP (M5) during research cruises with RV Sonne SO248 on May 3rd, 2016 (M1), May 14th, 2016 (M2), May 24th, 2016, (M3; Badewien *et al*., 2016) and SO254 on Feb 1st, 2017 (M4) and Feb 11th, 2017 (M5; Zielinski *et al*., 2017). Hydrographic parameters (depth, temperature, salinity, fluorescence, turbidity and oxygen; Table 1) were recorded by a Sea-Bird Electronics Inc. SBE 911plus probe (SN 09-1266) attached to a conductivity temperature depth (CTD) rosette system as described by Badewien *et al*., (2016) at each mesocosm sampling station. For all experiments water was collected from 20 m depth using a SBE 32 Carousel Water Sampler (SN 32-1119) containing 24 20-liter Niskin bottles and transferred to 25 L Nalgene bottles.

Mesocosm incubation experiments

The Nalgene polycarbonate bottles were first washed with acidified ultrapure water (MilliQ, pH 2), rinsed with ultrapure water and again rinsed with the respective sample water. Mesocosm volume was set to 23 liter and mesocosms M1, M2 and M3 were amended with FeSO4 (5 nmol L-1), NaH2PO4 (1 µmol L-1), NaSiO3 (32 µmol L-1), NaNO3 (16 µmol L-1) and CoCl2 (500 pmol L-1) whereas mesocosms M4 and M5 were set up without any nutrient amendment. All mesocosms were conducted in triplicates with amendments of B12 (cobalamin; 100 pmol L-1; Sigma-Aldrich, St. Louis, MO, USA), α-ribazole (100 pmol L-1; self-manufactured; Fig. S1) and mesocosms M4 and M5 were supplemented with B1 (thiamin; 100 pmol L-1; Sigma-Aldrich, St. Louis, MO, USA), HET (100 pmol L-1; Sigma-Aldrich, St. Louis, MO, USA) and HMP (100 pmol L-1; astatechinc, Bristol, PA, USA). Every mesocosm setup was

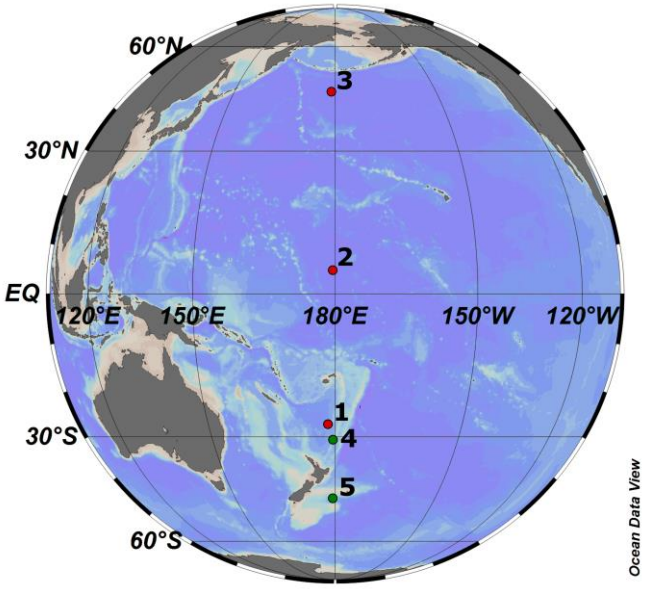


Figure 2 | Mesocosm water sampling stations during Sonne cruises SO248 and SO254 in the Pacific Ocean. Red dots denote mesocosms amended with B12 and α-ribazole, whereas green dots denote mesocosms supplemented with B1, HET and HMP.

accompanied by triplicate negative controls without vitamin amendment. Incubation for five to eight days was performed at the respective in situ temperature in a temperature controlled room (Table S1) and at constant illumination in a day:night rhythm of 12:12 hours (15-20 µmol photons m-2 s-1). Subsamples were withdrawn periodically to assess bacterial cell numbers, bacterial biomass production and turnover of dissolved free amino acids (DFAA).

Preparation and purity verification of α-ribazole

Alpha-ribazole was prepared by alkaline hydrolysis of cobalamin and purified according to (Gray and Escalante-Semerena, 2010). Purity of α-ribazole was validated applying nuclear magnetic resonance (NMR) spectra, recorded with a Bruker Avance DRX 500 MHz and a Bruker Avance III 500 MHz spectrometer at room temperature. 1H- and 13C-signals were assigned using DEPT, H,H-COSY and HMQC experiments. HPLC-UV-ESI-MS was carried out on a Waters Alliance 2695 system equipped with a NUCLEODUR C18 Pyramid column (Macherey-Nagel, particle size 3 µm, length 125 mm, inner diameter 3 mm), a Waters 996 PDA detector and a Micromass Q-ToF-MS (Waters). Water (solvent A) and methanol (solvent B), each acidified with 0.5% formic acid, served as eluents. Run conditions were initially 5% B at a flow rate of 0.8 ml/min. Concentration of B was raised linearly to 100% within 10 min and kept for further 6 min. UV-VIS detection was set in a wavelength range from 210 to 650 nm at a scan rate of 1 spectrum per second. The mass spectrometer was run in ESI positive mode covering a m/z range from 150 to 1400 at a scan rate of 1.7 spectra per second. High resolution (about 5 ppm) was achieved using lock spray (sodium formiate solution) for mass calibration (Fig. S1).

Bacterial cell numbers and phytoplankton autofluorescence

Live samples of 500 µl for enumerating bacterial cell abundance were stained with SybrGreen I (Invitrogen, United Kingdom) and counted aboard using a BD Accuri C6 cytometer (BD Biosciences, USA). Cell count calibration was performed using TruCount beads (BD) as described by (Osterholz *et al*., 2015). A second set of samples was fixed with 2% glutardialdehyde (final concentration, Carl Roth, Germany) and stored at -20°C. The size fractioned (0.2-200 µm) phytoplankton community was investigated by autofluorescence of live samples by flow cytometry.

Bacterial biomass production and growth rates

Biomass production of heterotrophic bacteria was determined by the incorporation of 14C-leucine as described in Lunau *et al*., (2006) and converted to carbon applying a leucine to carbon conversion factor of 3.05 kg C (mol leucine)-1 according to (Simon and Azam, 1989). Briefly, 10 ml subsamples and a formaldehyde-killed control were labelled in triplicates with 14C-leucine (10.8 GBq mmol-1, Hartmann Analytic, Braunschweig, Germany) at a final concentration of 20 nM, incubated in the dark at in situ temperature for 1-4 h, fixed with formaldehyde and further processed as described (Lunau *et al*., 2006). Bacterioplankton community growth rates (µ; day-1) were calculated as µ= ln(B1)-ln(B0), where B0 and B1 (B0+BP) are bacterioplankton biomass at T0 and one hour later. Bacterioplankton biomass was calculated from bacterial cell numbers, assuming a carbon content of 20x10-15 g C per cell (Simon and Azam, 1989) and BP is bacterioplankton biomass production as outlined above.

Results

Surface water temperatures and chlorophyll fluorescence reflected well the different hydrographic and trophic characteristics of the five locations of the mesocosm experiments. Highest temperature and lowest fluorescence occurred in SPSG and EqUp and lowest temperatures and highest fluorescence in NPF and NZCP (Table 1).

In the SPSG experiment (M1), bacterial cell numbers increased continuously until day 6 without significant differences in the treatments with additions of B12 and α-ribazole and the control (Fig. S2).

Bacterial production peaked at day 3 and decreased again thereafter. In contrast to cell numbers rates of BP yielded significant differences between the treatments and the control.

Table 1 | Biogeographic province, latitude, longitude, water depth, temperature, salinity and chlorophyll fluorescence of surface waters at mesocosm stations.

Mesocosm experiment (province)	Date	Latitude	Longitude	Depth [m]	Temp. [°C]	Salinity (PSU)	Chlorophyll Fluorescence [µg/l]
M3 (NPF)	24 May 2016	39.9706	177.3267	-5647	11.59	34.33	3.99
M2 (EqUp)	14 May 2016	4.6551	179.3978	-6230	28.84	34.32	0.05
M1 (SPSG)	04 May 2016	-26.9928	178.2133	-4197	25.22	35.64	0.15
M4 (SPSG)	02 Feb 2017	-30.0956	179.8220	-567	24.39	35.94	0.07
M5 (NZCP)	12 Feb 2017	-45.9518	179.3909	-3090	15.10	34.41	1.19

The addition of B12 resulted in BP rates twofold higher than in the control at days 3 and 6 and addition of α-ribazole resulted in a 1.5-fold higher rate of BP than in the control at day 3 (Fig. 3C). Bacterial community growth rates exhibited similar patterns as rates of BP and highest values of 1.2 and 0.9 d-1 at day 3 in the treatments with B12 and α-ribazole additions, respectively (Fig. 3H).

The EqUp experiment M2 was subsampled daily. The first subsampling, only 3 hours after adding B12 and α-ribazole, already yielded 4- and 3-fold higher BP rates in the treatments with B12 and α-ribazole additions, respectively, than in the control (Fig. 3B). After a decline on day 2, similarly high BP rates in the B12 and α-ribazole additions and still low rates in the control occurred. Thereafter no significant differences were recorded among the two treatments and the control and generally low rates of BP at days 5 and 6. Bacterial cell numbers increased until day 3 and remained constant until day 6 (Fig. 3 and S2). In the B12 treatment a trend of lower numbers occurred onwards from day 1 and numbers became significantly lower on day 6 than in the control and the α-ribazole addition, presumably as a result of intense grazing by mixotrophic small flagellates (G. Wienhausen *et al*., unpubl. results). Like BP bacterioplankton growth responded immediately to addition of B12 and α-ribazole by six- and twofold higher rates, respectively, three hours after addition of these compounds (Fig. 3G). As cell numbers were not recorded on day 2 no growth rates are available for this day but on days 3 and 6 they were low and not significantly different among the treatments and control.

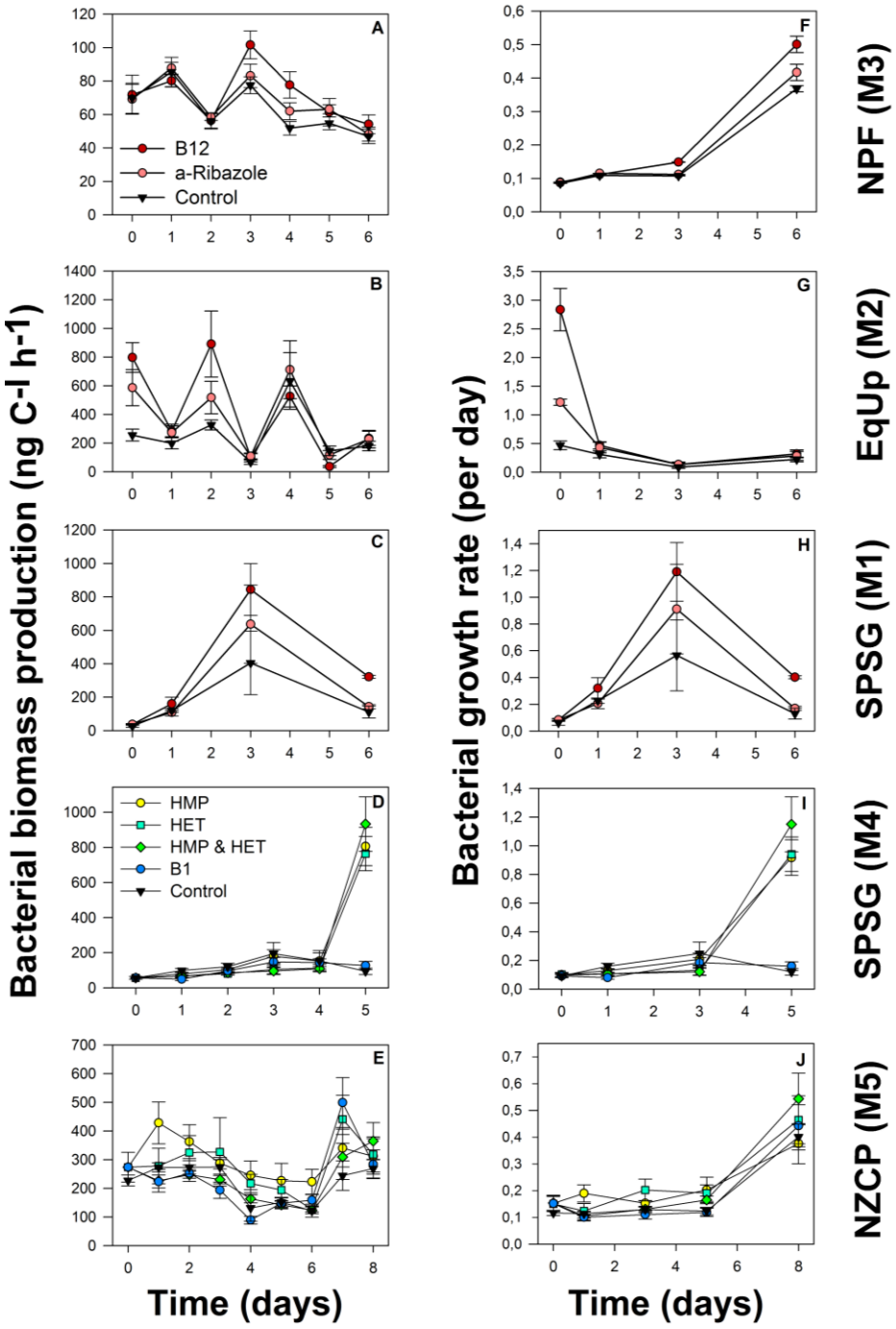


Figure 3 | Mean bacterial biomass production dissolved (A, B, C, D, E) and bacterial growth rate (F, G, H, I, J) over time. Mesocosms were either supplemented with vitamin-B12 (red), α-ribazole (pink) and unmodified (control; black) or enriched with HMP (yellow), HET (turquoise), HMP & HET (green), B1 (blue) and without any of these supplements (control, black). Mesocosms M1-M5 were conducted in four different oceanic provinces (NPF, EqUp, SPSG, NZCP).

In the M3 experiment in NPF the responses of BP to the B12 and α-ribazole additions were generally much lower than in the other two experiments (Fig. 3A). At day 3 the B12 addition

yielded a significantly higher rate than the control and the α-ribazole addition and at days 4 and 5 both additions yielded significantly higher rates than the control. Bacterial cell numbers, initially much higher than in the experiments M1 and M2, continuously decreased over the entire experiment and numbers in the B12 treatment and the control were significantly lower than in the α-ribazole treatment at day 6 (Fig. S2), presumably also a result of the high grazing pressure of small unidentified mixotrophic flagellates in these treatments. Bacterioplankton growth rates did not exhibit differences among the treatments and control at days 0 and 1 but at day 3 rates in the B12 treatment were 1.4-fold and significantly higher than in the α-ribazole treatment and the control (Fig. 3F).

The M4 experiment in SPSG with additions of B1 and precursors yielded significantly enhanced BP rates in the treatments with HMP and HET additions, separately or mixed, but the response occurred only at day 5, the last day of the experiment (Fig. 3D). Bacterial abundance generally increased until day 3 but most strongly in the treatment with the HMP addition and decreased again strongly at day 5 in all treatments and the control except in that with HMP addition (Fig. S2). Bacterioplankton growth rates showed similar patterns as BP with low values of all treatments and the control until day 4 and strongly increasing rates in the treatments with HMP and HET additions separately or together at day 5 (Fig. 3I).

In the experiment in NZCP BP was significantly enhanced in the HMP treatment relative to the others and the control at days 1 and 2 (Fig. 3E) whereas from day 3 to 5 there was no consistent difference among the various treatments. Interestingly, there was a general trend of low rates of BP in the B1 treatment relative to the other treatments and control from day 1 to 4. At day 7, however, rates of BP increased in all treatments and most strongly in that with the B1 and HET additions. Bacterial numbers exhibited an increase at day 1 and thereafter decrease until day 5. Numbers in the treatments were consistently lower than the in the control at days 1 and 3 (Fig. S2).

Discussion

Ramification of B1, B12 and respective precursors on bacterioplankton growth dynamics

Our mesocosm experiments were conducted in four biogeographic provinces of the Pacific and showed that vitamins B1, B12 and precursors stimulate bacterioplankton biomass production and growth, but to different extent. Vitamin B12 showed a stronger effect than α-ribazole in all three experiments but the magnitude and time course varied with the most rapid response in EqUp and a slower response in the NPF. In both experiments applying B1, HET and HMP the

vitamin did not exhibit a clear-cut superior effect on BP and bacterial growth relative to both precursors. It appears most remarkable that not only both vitamins but in particular the precursors had an effect on bacterioplankton biomass production and growth and thus on bulk properties of the bacterial communities. The different growth responses in the different biogeographic provinces are presumably a result of the different trophic states and distinct composition of the bacterioplankton communities in these provinces (Baldwin *et al*. 2005).

A requirement of B12 for phototrophic marine eukaryotes is well known and has been documented by genome analyses and shown experimentally (Bertrand *et al*., 2007; Croft *et al*., 2005; Cruz-López and Maske, 2016; Koch *et al*., 2011; Sañudo-Wilhelmy *et al*., 2014) A stimulating effect of B12 on growth of marine bacterioplankton, however, has only been shown in one study in the Gulf of Alaska (Koch *et al*., 2011). Such an effect can be expected, knowing that cobamides are only produced by roughly half of all Archaea and Bacteria in the marine environment, but all prokaryotes and half of the eukaryotic plankton contain cobamide-dependent enzymes (Martens *et al*., 2002; Sañudo-Wilhelmy *et al*., 2014). It is remarkable that B12 appears to be able to control growth of bacterioplankton communities in oceanic regions of very different trophic state. Our finding that α-ribazole, the lower ligand of B12, has also a stimulating effect on and thus appears to control growth of marine bacterioplankton communities is completely novel and documented for the first time in the present study. Hence, increased exogenous availability of α-ribazole has presumably ramifications on the bioavailability of cobinamides and affects the cofactor function. It is still open and needs further studies whether exogenously available α-ribazole has an effect on proto- and auxotrophic bacteria and how it affects the biosynthesis of B12.

As it has been shown that various *Rhodobacteraceae* bacteria release α-ribazole (Johnson *et al*., 2016; Romano *et al*., 2014; Wienhausen *et al*., 2017) and that these bacteria can constitute prominent components of the bacterioplankton community during phytoplankton blooms (Gifford *et al*., 2014; Segev *et al*., 2016) it appears likely that supply of α-ribazole by these and possibly other bacteria to bacterioplankton communities is more common than assumed. In fact, the MF of α-ribazole has been detected in DOM samples in the North Sea and a long-term mesocosm in which a phytoplankton blooms was stimulated (Wienhausen *et al*., 2017). Its release and supply presumably provides a further growth-promoting effect to bacteria and possibly to phytoplankton in addition to B12.

Results of our mesocosm experiments also demonstrate for the first time a stimulation of biomass production and growth of bacterioplankton communities by the supply of B1 and precursors HET and HMP. Interestingly, supply by B1 showed a positive effect only in the late

stage of M5 in NZCP and none at all in SPSG, whereas that by both thiamin moieties resulted in enhanced biomass production and growth in both provinces in which experiments were carried out. It is well documented that a large number of prokaryotic and eukaryotic microbes lack genes for de novo synthesis of B1 including representatives of the SAR11 and SAR86 clades and picoeukaryotes (Dupont *et al*., 2012; Carini *et al*., 2014; Sanudo-Wilhelmi *et al*. 2014, Paerl *et al*., 2015). There is also experimental evidence that SAR11, various microalgae and picoeukaryotes are auxotrophic for HMP (Carini *et al*., 2014, Paerl *et al*., 2015, Lwoff, 1947; Droop, 1958; Turner, 1979). However, auxotrophy for HET has been shown only for microalgae and not for bacteria (Lwoff, 1947; Droop, 1958; Turner, 1979). It has been shown recently, however, that HMP and HET can promote growth of two B1-prototrophic diatoms (Wienhausen *et al*., 2017). A growth stimulation of HMP and HET to B1-prototrophic bacteria, however, has not been tested, to the best of our knowledge. Our results, however, show for the first time a stimulating effect of B1 and in particular of both precursor moieties on biomass production and growth of bacterioplankton communities. Hence our results indicate that B1, but even more so, HMP and HET may be neglected controlling factors for growth of oceanic bacterioplankton communities and thus, together with the lower ligand of B12, α-ribazole, their availability may have implications for the global organic matter processing by oceanic bacterial communities. Our findings contribute to a better and refined understanding of the significance of trafficking of vitamins and precursors in marine microbial communities. Concentrations of B1, B12 and HMP have been assessed in oceanic waters and found to be in the pM range (Sanudo-Wilhelmi *et al*., 2014; Suffridge *et al*., 2017). Concentrations of HET, however, have not been analyzed and so far, only been assessed by bioassays (Pearl *et al*., 2015). However, HET, as well as HMP, has been shown to be released by two strains of the *Roseobacter* group (Wienhausen *et al*., 2017) which can be prominent members of bacterioplankton communities during phytoplankton blooms (Segev *et al*., 2016).

It was surprising that in the SPSG the B1 amendment did not promote growth of the bacterial community as observed for HET and HMP. It implies that B1 could not be taken up due to lacking B1 transport systems or that a sufficient concentration of B1 existed. The latter would imply further the existence of an unidentified function of both thiamin moieties in the metabolism of the ambient bacterial communities. We are unable to provide evidence for either of both possibilities and need to leave this question open for future studies.

Hence, the results of this study, that not only B12 and to a certain extent B1, but in particular the precursors α-ribazole, HPM and HET, can stimulate growth of marine bacterioplankton communities, have implications for a more refined understanding of a supply and cycling of

these growth factors among the various members of bacterioplankton communities and oceanic systems and possibly beyond to global scale oceanic microbial biogeochemical processes.

Marine microbial vitamin traffic

Vitamins are essential cofactors for all marine microbes, but the capability for their biosynthesis, i.e. the presence of complete biosynthetic gene clusters in their genome, is restricted to a minor fraction. The evolutionary loss of genes encoding single steps in or entire biosynthesis pathways arises by genome streamlining. This phenomenon, in particular of vitamin gene loss, was shown to be common among numerous marine pelagic bacteria and eukaryotic phytoplankton organisms (Carini *et al*., 2014; Giovannoni *et al*., 2005; Sañudo-Wilhelmy *et al*., 2014; Turner, 1979). As predicted by the black queen hypotheses, loss of favorable but costly functions will develop until public goods reach equilibrium in sustaining the community's survival (Morris *et al*., 2012). However, this hypothesis simultaneously implies a tight branching network of microbes exchanging public goods, which is especially true for essential vitamin cofactors.

In recent studies it has been observed that various vitamins and vitamin precursor are part of exometabolomes of marine bacteria and would therefore contribute to the marine geometabolome (Fiore *et al*., 2015; Johnson *et al*., 2016; Romano *et al*., 2014; Wienhausen *et al*., 2017). Besides pseudocobalamin, various cobalamins were detected in marine pelagic systems (Heal *et al*., 2017; Suffridge *et al*., 2017). The availability presumably guarantees the survival of the large fraction of B12-dependent and auxotrophic organisms or as seen in our experiment stimulates the bacterioplankton biomass production and growth. In several studies it has even been demonstrated that B12-supply provokes tight interdependencies between auxotrophs and provider organism (Cruz-López and Maske, 2016; Dogs *et al*., 2017; Wagner-Döbler *et al*., 2009). However, the cobamide cofactor effectivity varies with the lower ligand attached, hence not only its availability but also the cobamide class is essential for survival of individual microbes (Yan *et al*., 2012; Yi *et al*., 2012). Interestingly, various cobamides are available in marine ecosystems, but independent of their prevalence as cofactors in nature, highlighting the necessity for cobamide remodeling. In particular cyanobacteria are major cobamide suppliers, assumed to exceed heterotrophic bacterial contribution by 50 times (Bonnet *et al*., 2010). However, they exclusively release pseudocobalamin which is substantially less bioavailable for most marine microbes and requires remodeling of the lower ligands (Heal *et al*., 2017; Helliwell *et al*., 2016).

Yet the availability of lower ligands in marine ecosystems remains largely unknown, but several lower ligands were determined in non-marine host-associated and environmental samples (Crofts *et al*., 2014) and found in exometabolomes of strains of the *Roseobacter* group (Johnson *et al*., 2016; Wienhausen *et al*., 2017).

Further, it was demonstrated that the inability of the bacterial strain *Listeria innocua* of de novo α-ribazole synthesis was compensated by the active uptake and coupling to the incomplete cobinamide (Gray and Escalante-Semerena, 2010). Our mesocosm experiment results indicate that salvaging and remodeling of lower ligands is a more prevalent scenario in the marine microbial vitamin traffic as previously assumed. Triggered effects by α-ribazole likewise suggest the presence of sufficient concentrations of inactive cobamides for remodeling in nature (see Fig. 4A-E).

In figure 4 we aim to illustrate natural vitamin traffic scenarios among prokaryotic and eukaryotic organisms in nature that presumable explain to some extent our findings. Depicted are hypothetical and scientifically proven scenarios of vitamin supply and recycling, considering vitamins B1, B12 and respective precursor (see Tab S2 and S3). The availability of B1 is of significance for myriads of B1-auxotrophic eukaryotic and prokaryotic organisms, because of its function as essential coenzyme, catalyzing crucial transformations of carbon in all living systems (Bertrand and Allen, 2012; Cruz-López and Maske, 2016; McRose *et al*., 2014; Palenik *et al*., 2007). In our mesocosm experiments, we observed minor effects induced by the supply of B1, however, the fact that bacterial growth was predominantly enhanced by HET and HMP suggests a fundamental function in the bacterioplankton community uncoupled to B1 requirements. There is evidence that HET and HMP can be salvaged for the biosynthesis of B1, avoiding the lacking capability of de novo precursor synthesis (Carini *et al*., 2014; McRose *et al*., 2014; Paerl *et al*., 2015; Turner, 1979).

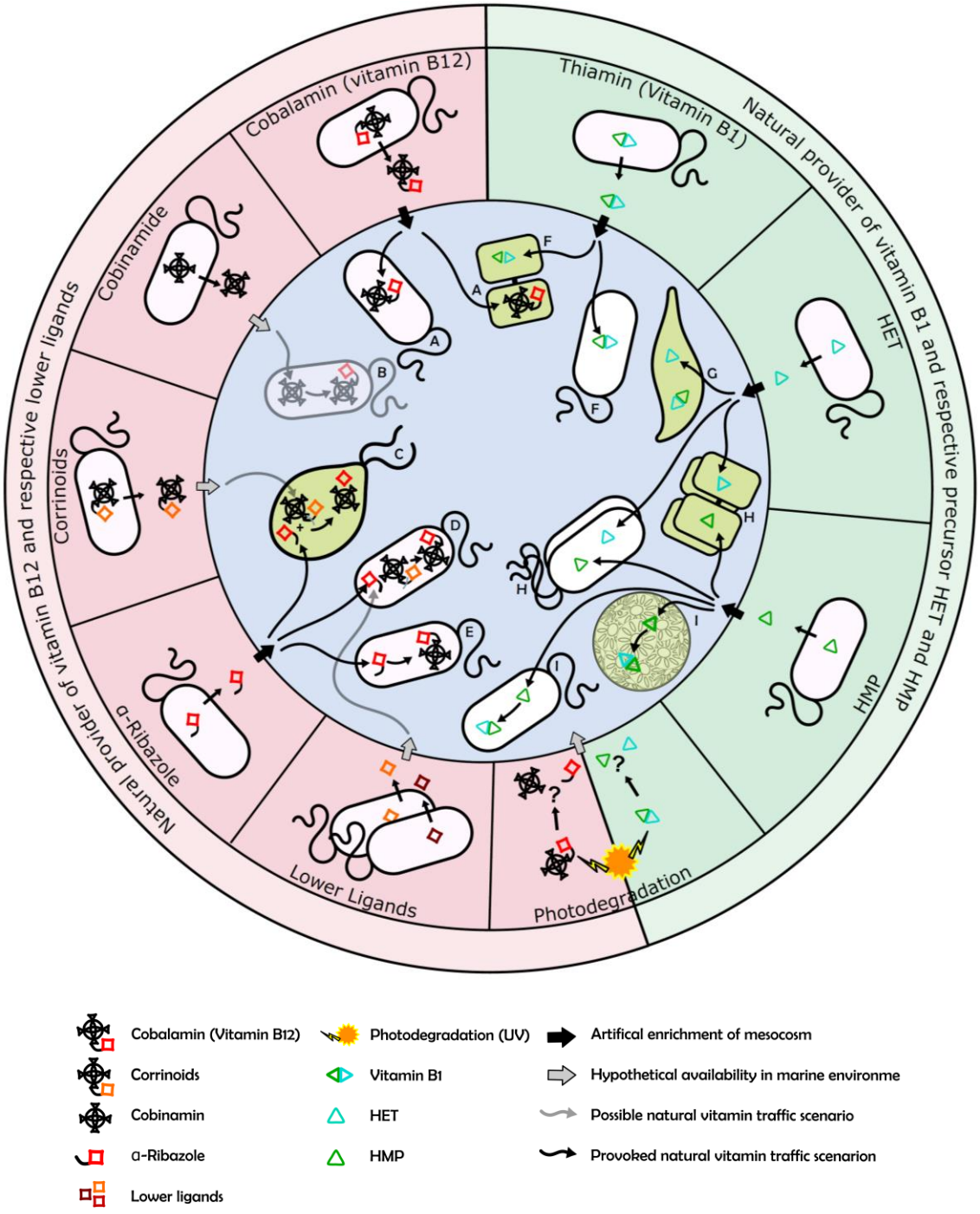


Figure 4 | Overview of natural marine vitamin traffic scenarios. Provider of vitamin and respective precursor (B1, green shading; B12, red shading) and known salvage and remodeling scenarios by microbes (blue shading). Experimental evidence for illustrated natural vitamin allocation and salvage as well as remodeling scenarios (labeled with respective letters) are listed in tables S2 and S3. Arrows crossing outer towards inner circle present the artificial enrichment of our experiments (black arrow) and supposable natural vitamin / precursor allocation scenarios (grey arrow). Faded schematics and arrows are scenarios that were not induced by our mesocosm, but presumably simultaneously occur in nature / our mesocosm experiment.

Even B1-prototroph organisms showed a stimulation by enhanced HET and HMP availability, possibly explaining the effects we observed by HET/HMP allocation for the bacterioplankton BP and growth rate (Wienhausen *et al*., 2017; see Fig. 4F-I).

Our findings add to the accumulating evidence that exchange of vitamins among members of marine bacterioplankton communities is an important feature in the interplay, structuring and possibly growth control of pelagic marine bacterial communities. Especially the vitamin precursors, α-ribazole, HET and HMP, until now rarely considered and obviously underestimated, seem to be essential elements in the interconnectedness of the marine microbial vitamin traffic. This feature may include the interplay between helpers and genome streamlined members of these communities but it seems that this supply may also have beneficial effects on the growth of prototrophic members, presumably saving biosynthetic energy under severe energy limitation. Hence it appears that the exchange of vitamins and respective biosynthetic precursors affects the major players in bacterioplankton communities and may generally enhance DOM turnover, bacterial growth and thus microbial biogeochemical processes.

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Conflict of interest

The authors declare no conflict of interest.

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Supplementary information:

Supplementary table S1 | Mesocosm experiment modifications are listed, including vitamin metabolite and nutrient amendments and general mesocosm parameter adjustment, such as temperature and mesocosm duration time.

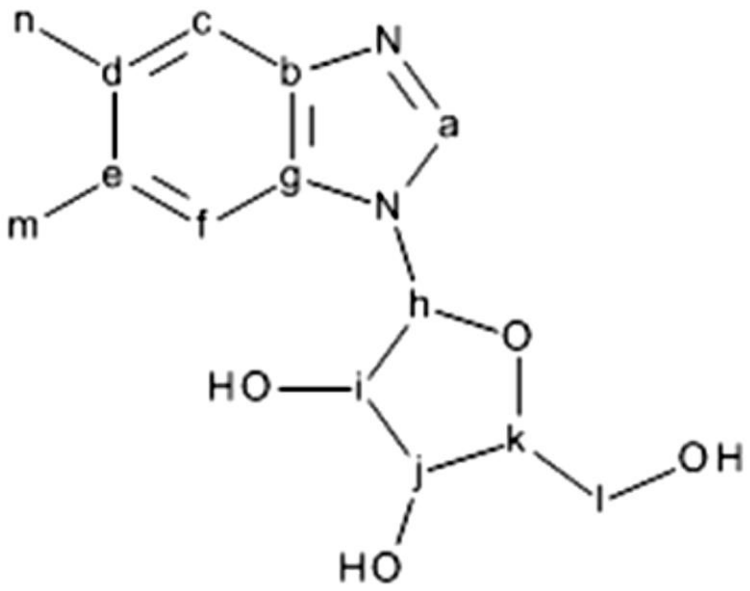
Mesocosm reference No.	Vitamin metabolites amendment	Nutrient amendment	Incubation temperature (°C)	Incubation time (days)
M1	B12, α-ribazole (100 pM)	$FeSO_4$ 5 nM, NaH_2PO_4 1 µM, Na_2SiO_3 32 µM, $NaNO_3$ 16 µM, $COCl_2 * 6H_2O$ 0.5 nM	25	6
M2			29	6
M3			12	6
M4	B1, HET, HMP (100 pM)	None	24	5
M5			13	8

Supplementary table S2 | Natural provider of B1, B12, B12-analogs and respective vitamin precursor. This table refers to figure 4, listing studies for the natural allocation of vitamins (B1, B12, B12-analogs and respective precursor). Superscripted stars elucidate the study field (* = marine microbial study; ** = microbial study, *** = hypothetical assumption).

Natural allocation scenarios	Literature
Cobalamin (B12)	Croft *et al*., 2005*
Cobinamide	Hypothetical***
Corrinoids	Hoffmann *et al*., 2000**; Heal *et al*., 2014**
α-ribazole	Johnson *et al*., 2016*; Wienhausen *et al*., 2017*
Lower ligands	Crofts *et al*., 2014**
Photodegradation (B12)	Carlucci *et al*., 1969***
Thiamin (B1)	Droop *et al*., 1958*
HET	Wienhausen *et al*., 2017*
HMP	Carini *et al*., 2014*
Photodegradation (B1)	Gold *et al*., 1966***; Okumura, 1961***

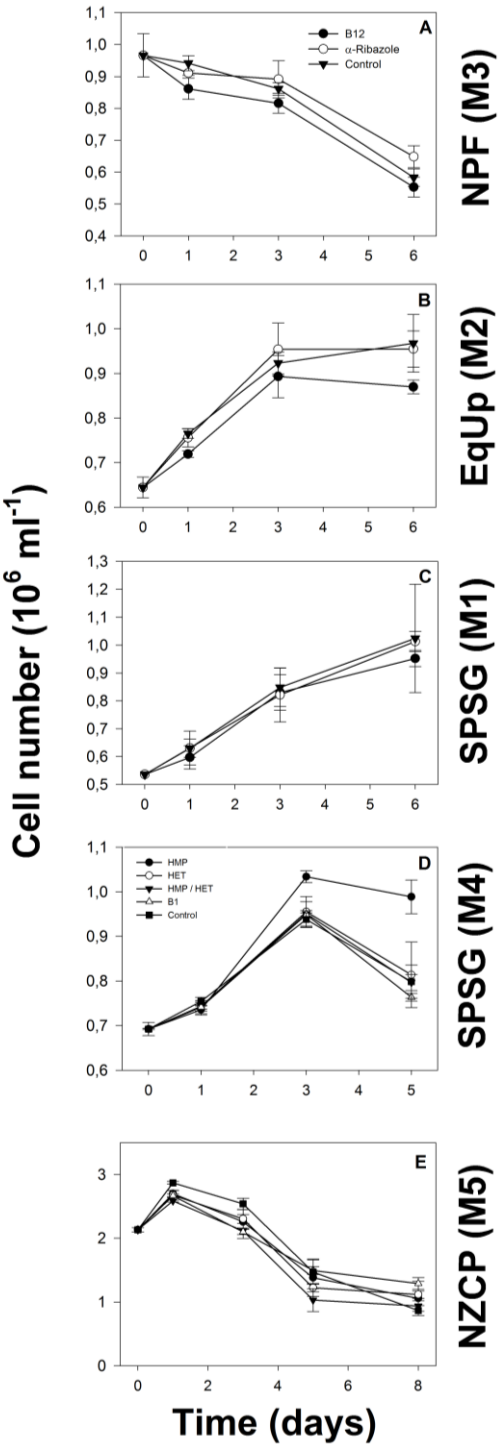
Supplementary table S3 | Natural marine vitamin traffic. Presented are natural vitamin and vitamin precursor salvaging and remodeling scenarios that are depicted in figure 4 and confirmed by experimental evidence (literature).

Figure reference	Natural vitamin traffic scenario	Literature
A	Vitamin B12 uptake (bacteria and eukaryotic algae)	Sañudo-Wilhelmy *et al*., 2014
B	Cobamid salvaging	Raux *et al*.; 1995
C	Corrinoid remodeling using lower ligands (eukaryotic algae)	Helliwell *et al*., 2016
D	Corrinoid remodeling using lower ligands (bacteria)	Yi *et al*., 2012
E	α-ribazole salvaging (bacteria)	Gray and Escalante-Semerena *et al*., 2010
F	Vitamin B1 uptake (bacteria and eukaryotic algae)	Cruz-López *et al*., 2016; Bertrand & Allen et al., 2012
G	HET salvaging (eukaryotic algae)	Lwoff 1947
H	HET and HMP induce growth stimulation (eukaryotic algae)	Wienhausen *et al*., 2017
I	HMP salvaging (bacteria)	Carini *et al*., 2014



1H NMR (500 MHz, methanol-d4): δ [ppm] 8.36 (s, 1H, a), 7.43 (s, 1H, c), 7.38 (s, 1H, f), 6.29 (d, 1H, J = 4.4 Hz, h), 4.88 (s[br], 3H, OH), 4.45, (dxd, 1H, J1 = 4.8 Hz, J2 = 4.4 Hz, i), 4.37 (dxd, 1H, J1 = 6.0 Hz, J2 = 4.8 Hz, j), 4.25 (dxdxd, 1H, J1 = 6.0 Hz, J2 = 4.0 Hz, J3 = 2.8 Hz, k), 3.87 (dxd, 1H, J1 = 12.2 Hz, J2 = 2.8 Hz, l1), 3.72 (dxd, 1H, J1 = 12.2 Hz, J2 = 4.0 Hz, l2), 2.41 (s, 3H, m), 2.38 (s, 3H, n). 13C NMR (125 MHz, methanol-d4): δ [ppm] 143.9 (1C, a), 142.1 (1C, g), 133.44 (1C, b/e), 133.37 (1C, b/e), 132.5 (1C, d), 119.9 (1C, c), 111.8 (1C, f), 87.3 (1C, h), 85.5 (1C, k), 73.0 (1C, i), 72.2 (1C, j), 62.8 (1C, l), 20.6 (1C, m), 20.3 (1C, n).

Supplementary figure S1 | Alpha-ribazole purity verification by NMR and HPLC-UV (Purity (HPLC-UV): >99%). HRMS (ESI-Q-ToF): found 279.1356, calculated for $C_{14}H_{19}N_2O_4$: 279.1345.



Supplementary figure S2 | Mean bacterial cell counts determined on board by flow cytometry. Upper panels (A, B, C) show mesocosms M1-M3 supplemented with B12, α-ribazole as well as a control without any of these supplements and both lower panels show mesocosms M4 and M5 (D, E) amended with 4-amino-5-hydroxymethyl-2-methylpyrimidine (HMP), 4-methyl-5-(β-hydroxyethyl)thiazole (HET), HET & HMP, B1 and a control without further manipulation.

7 Discussion

In the ocean, bacteria and DOM are mutually entailed in a tight interconnection. The complex molecular composition of DOM serves as main substrate and energy source for heterotrophic bacteria and vice versa bacteria encode the biosynthetic potential to produce myriads of complex organic molecules that contribute substantially to the global DOM pool. In addition, many of such DOM molecules fuel the complex microbial metabolic interaction network. This guarantees the viability of the microbial community and simultaneously sustains the DOM ramification cycle (see figure 1).

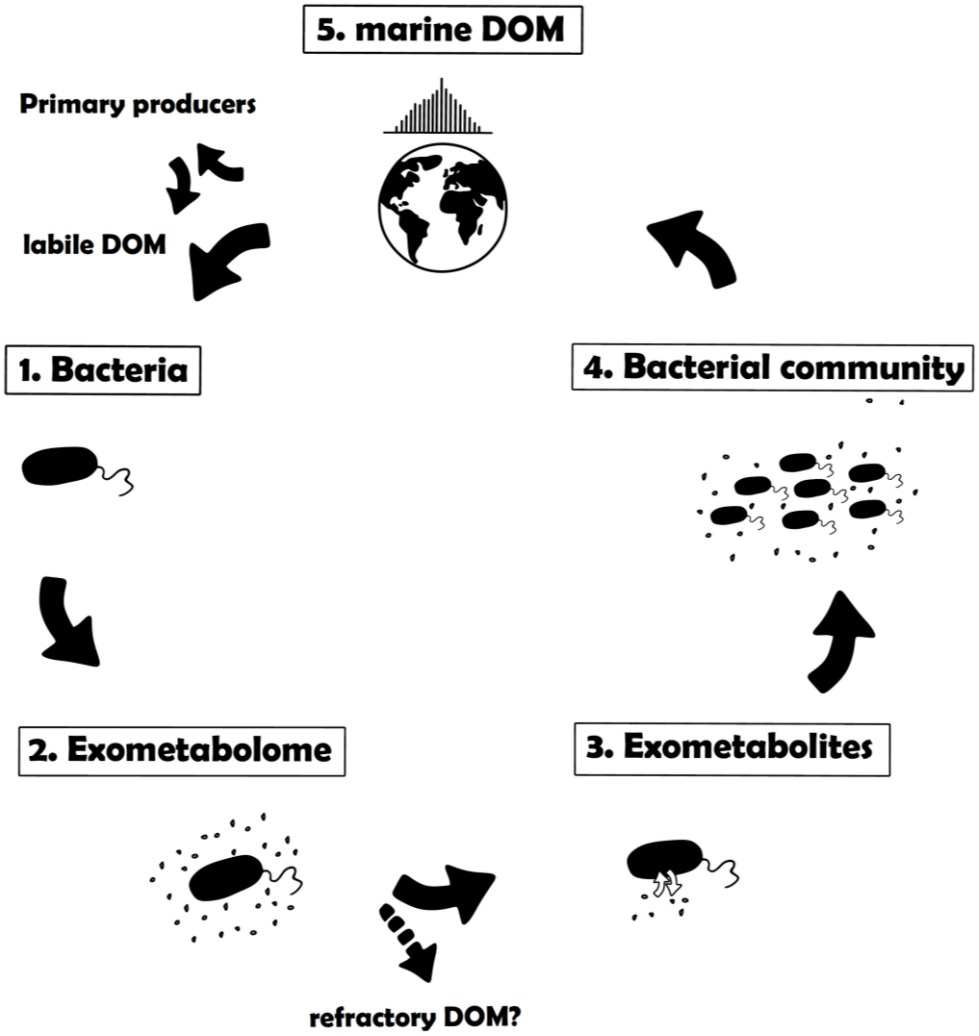


Figure 1 | Global bacterially mediated marine dissolved organic matter (DOM) cycle. This conceptual scheme depicts the marine global cycle of bacterially mediated DOM from a microbiological perspective. Labile DOM provided by primary producers is taken up by bacteria and assimilated as biomass as well as released as exometabolome. Various single exometabolites exhibit great benefit for the vitality of the bacterial community and therefore regulate the production of bacterial derived DOM.

Discussion

In this final chapter major findings and conclusions of my dissertation are discussed and embedded in the superordinate global perspective on the basis of each section depicted in figure 1. Finally, I recapitulate and summarize future scientific questions and research projects in the outlook, which may be of inspire and stimulate future scientific studies.

Bacteria – ubiquitous recycler of organic compounds

In the ocean, phytoplankton organisms are significant primary producers of marine DOM and by that fuel the heterotrophic bacterioplankton and constitute the basis of the microbial loop (Azam, F, 1983). Approximately half of the fixed carbon is released as extracellular compounds (Ducklow *et al*., 1993). Assessing diatom derived DOM we identified hundreds of variable, complex molecular formulas (Chapter 2). Yet, such an enormous number remains puzzling. While we cannot assign the large majority of organic compounds, we can speculate that the blend of exometabolites derives as by-products, secondary metabolites or waste products, but presumably large content results as overflow metabolism especially during late growth phases (Bertilsson and Jones, 2003; Bjørnsen *et al*., 1988; Barofsky *et al*., 2009). Once cell division is hindered but photosynthesis continues, energetic organic compounds accumulate and are released (Smith and Underwood, 2000). However, this suggests that a previously inconceivable blend of valuable compounds is provided for heterotrophic organisms. In fact, we know that phytoplankton-bacteria associations possess complex relationships, ranging from mutualistic to competitive and even parasitic interactions (Amin *et al*., 2015; Seyedsayamdost *et al.,* 2011; Sule and Belas, 2013). Heterotrophic bacteria are capable of transporting and metabolizing phytoplankton exudates (Matrai and Keller, 1994; Moran *et al*., 2004; Poretsky *et al*., 2010, Sarmento & Gasol 2012, Polimene *et al.* 2017, Horňák *et al.,* 2017) and occur in annual succession of algae blooms (Bell and Kuparinen, 1984; Matrai and Keller, 1994; Tada *et al*., 2017; Teeling *et al*., 2012, Teeling *et al*., 2016). Especially bacteria of the *Roseobacter* group were often identified as abundant and active members in plankton blooms (Buchan *et al*., 2005; Kirchman, 2002). The complexity and variability of phytoplankton derived exometabolome depends on the organism, its growth stage and on the present interacting planktonic community (Barofsky *et al*., 2009; Becker *et al*., 2014; Bjørnsen *et al*., 1988; Grossart and Simon, 2007; Wetz and Wheeler, 2007) and vice versa the molecular diversity of the algalexudate determines the diversity of bacterial communities (Sarmento and Gasol, 2012). As part of my dissertation, we assessed diatom derived DOM uptake and diversification patterns of different bacterial strains and identified remarkable differences. Each bacterial strain grown on the same diatom exudate exhibited unique utilization patterns and in return

also released distinct exometabolomes of different composition (Chapter 2). This observation can possibly be explained by differing physiological strategies, probably evolved in succession of continuous nutrient supply by phytoplankton associations during evolutionary adaptation. For instance, metabolic traits, such as degradation of variable aromatic compounds and the algal sulfonates 2,3-dihydroxypropane-1-sulfonate (DHPS) and DMSP indicate such specific evolutionary adaptation strategies by members of the *Roseobacter* group (Dickschat, 2010; González *et al*., 1999; Moran *et al*., 2004; Kiene 2000; Luo *et al*., 2013, Durham 2015). The large genome of most *Roseobacter* group members presumably accommodates specialized transporters that allow a fast response to the availability of organic compounds (Giovannoni *et al*., 2005; Moran *et al*., 2007; Newton *et al*., 2010). This suggests that bacteria evolved specialized traits for variable compound classes of the complex pool of phytoplankton derived DOM on an evolutionary timescale and engaged different ecological roles in the carbon turnover. Thus, the degree of bacterial DOM diversification in such small scale laboratory experiments lets us foreshadow the true complexity of DOM in the marine ecosystem.

Exometabolome – the puzzling bacterial extracellular link

Labile organic compounds are assimilated by bacteria and archaea as biomass and resupplied into the marine DOM pool as a diverse blend of complex exometabolite molecules. In the recent past, several microbial exometabolome studies were conducted, observing an unexpected large blend of exometabolites (Longnecker *et al*., 2015; Romano *et al*., 2014; Chapter 3). The physiological state, for instance driven by the substrate source or essential element availability, bears a meaning for the number and composition of released exometabolites (Romano *et al*., 2014; Chapter 2). Rosselló-Mora *et al*., (2008) demonstrated that bacterial strains isolated at varying geographical regions were distinguishable by their exometabolome composition. In fact, these findings indicate that the exometabolome is a reflection of metabolic processes. To prove this assumption we assessed correlations between intracellular processes, determined via transcriptome, i.e. COG categories, to the detected exometabolome. We demonstrated that roughly one third of all detected exometabolites correlate to the metabolism, and also showed that exometabolites with profound molecular characteristics are reflected by specific intracellular processes (Chapter 4). This allows us to attribute genetic or metabolic traits to bacterial exometabolomes and possibly in future projects to even interpret natural DOM formation processes.

Despite the fact that a large number of exometabolites appears to be associated to intracellular processes, yet more than 97% of the detected exometabolome MF remain

unknown (Romano *et al*., 2014; Chapter 3), not matching any known metabolite or secondary metabolite. This finding appears enigmatic, assuming that the exometabolome is a consequence of bacterial metabolism. However, a likely scenario explaining this paradox is the formation of metabolic waste, such as xenobionts or reactive oxygen species possible by malfunctioning enzymatic reactions and the cellular detoxification by the aid of Glutathione S-transferases (Fiore *et al*., 2015; Vuilleumier, 1997). Indeed, metabolic waste accumulation would explain low metabolite matching ratios and nevertheless explain a correlation appearance. On the other hand, studies by Ogawa *et al*., (2001) demonstrated that only a minor fraction of the microbial derived DOM escapes further microbial degradation. Also our findings demonstrated that bacteria render variable diversification patterns and already 3 tested bacterial organisms possess the ability to take up 75% of diatom-derived exudate. As a consequence in a complex environment, individual bacterial derived waste exometabolites would undergo further degradation progresses by the microbial community. Hence, the depiction of waste compounds is conditioned by the microbial point of view.

Exometabolites – creating metabolic networks

The wealth of exometabolites released by single bacterial strains is an enigmatic aspect in the study of bacterial DOM formation (Romano *et al*., 2014, Noriega-Ortega, in submission). At present, only a minor fraction (approx. 3%) is assignable to known annotated compounds, comprising a great blend of variable metabolites and metabolic precursor, such as amino acids, quorum sensing-related compounds, indole acetic acid and methyl-(indole-3-yl) acetic acid and diverse vitamin metabolites (Fiore *et al*., 2015; Johnson *et al*., 2016; Chapter 3). The identification of exometabolites can be hampered by various reasons. The function of roughly 30% of all protein coding genes in bacteria remains yet unknown, while for eukaryotes this accounts for up to 40% (Horan *et al*., 2008) Likewise, observations of previously unknown cross-enzymatic reactions that yielded in novel metabolites, foreshadow the potential of a broader metabolic range as known so far (Wang *et al*., 2016).

In nature, this complex mixture of organic compounds is believed to be essential for the microbial community viability (Garcia *et al*., 2015). As a consequence of genome streamlining, microbes lack metabolic traits, enforcing a dependency on an external resource (Giovannoni *et al*., 2005; Chapter 5). Our results and the work of others indicate that several bacterial exometabolites are present as DOM molecules (Johnson *et al*., 2016; Kujawinski *et al*., 2009; Chapter 3). Our current understanding of marine DOM, regarding bacteria, is its essential role as substrate and energy source. However, such findings presumably characterize the neglected

role of marine DOM as relevant storage and transmission body for essential metabolites. The exchange of such significant metabolites is known to occur in the phycosphere (Seymour *et al*., 2017), but as our findings in fact indicate, single metabolites in the DOM pool feature the momentousness to drive microbial community dynamics. In particular the exchange of vitamins and their respective precursor appear to have a profound relevance for the vitality of the microbial community (Chapter 6). In particular vitamins, their availability as enzymatic cofactor for essential metabolic pathways are crucial for the survival of all living organisms and thus have a pivotal impact on marine ecosystem dynamics (Bertrand *et al*., 2007; Heal *et al*., 2017; Koch *et al*., 2011). Our findings show that already marginal availabilities of even vitamin precursor, such as α-ribazole or both vitamin B1 moieties may significantly alter the bacterial community activity (Chapter 6). Thus marine microbial vitamin traffic appears essential for the microbial vitality, but nevertheless remains a small fraction in the complex microbial derived DOM pool. Overall, this example highlights and extends the key role of DOM for the marine microbial metabolic network.

At present, mechanisms of release and uptake of most (exo)-metabolites are largely unknown. Sporadic transporter systems that release single (exo)-metabolites that were also identified as part of the bacterial exometabolome are known (Gray and Escalante-Semerena, 2010), but in general, secretion systems for the large majority of compounds are still unidentified. . For the release of B vitamins no transporter has been found even though it is well known for a long time that these vitamins are released. The exchange of the hormone indole-3-acetic acid and tryptophan in a symbiotic bacterial diatom interaction (Amin *et al*., 2015) or the substitution of required vitamins for auxotrophic phytoplankton organisms by a bacterial community (Croft *et al*., 2005; Cruz-López and Maske, 2016) in fact reflects an active exchange of metabolites. Similarly, Heal *et al*., (2017) argue that abundant bacterial members play distinctive roles in providing differing forms of cobalamin and thereby sustain microbial interdependencies and community structures.

The inevitable release of essential metabolites by bacteria remains puzzling, since provider microbes cannot take an instantaneous advantage of such a costly loss. Results of Tianero *et al*., (2016) demonstrated that multiple metabolite products in a secondary metabolite pathway accumulate in succession of each ensuing decelerated metabolic step. This phenomenon, yet merely described for secondary metabolite production, sheds light on potential natural exometabolite acquisition processes. Analogous to the diversity-generating biosynthesis by Tianero *et al*., (2016), metabolite precursors presumably accumulate, not as a consequence of successional decelerating metabolic step, but simply as consequence of lacking genes that

interrupt a pathway. In the case of gene loss, metabolic precursors prior to the enzymatic lack would presumably accumulate. Many studies revealed the lack of essential genes as common genetic trait among marine microbes. However, all surveys solely focus on metabolite substitution and neglect the possible metabolite accumulation of discontinuous metabolic pathways (Croft *et al*., 2005; Sañudo-Wilhelmy *et al*., 2014; Chapter 5). Hence, if the BQH applies, gene loss of costly functions proceeds until the production of public goods is just sufficient. Consequently, individual microbes that lack genes would simultaneously supply the community with metabolic precursors.

Bacterial community – mirrors complexity of molecular DOM composition

Currently we define microbial communities as groups of microorganisms that share a common space. However, microbes are organized in complex ecological interaction webs, known as microbial networks. They are constructed of myriads of dependencies driven by seasonality, environmental parameters and microbial interactions and behavior including motility, chemotaxis, different substrate preferences and chemical cross talk (Falkowski *et al*., 2008; Fuhrman and Steele, 2008; Gilbert *et al*., 2012; An *et al*., 2006). In marine pelagic environments, microbes are constantly exposed to assemblage perturbations and shifts in geometabolome availability, even in scales of microns (Seymour *et al*., 2017). Hence, a clear delineation of microenvironments and by those defining microbial communities remains challenging.

However, current concepts and hypotheses that aim to understand microbial biogeochemical interrelationships and dependencies are based on the consideration of defined and isolated microbial communities, possibly creating a bias in our current interpretation. The widely accepted BQH predicts the loss of genes as a consequence of the continuous metabolite supply within an “entire community” (Morris *et al*., 2012). Marine free living microbes live in patchy and dynamic environments and encounter waterbodies with variable biogeochemical conditions. As our work emphasized, already single metabolites within the geometabolome possess the scope to affect phytoplankton growth or the activity of a microbial community (Chapters 3 and 6). In such perturbed environments, public goods will unlikely remain at states of equilibrium. Hence, microbial dependencies and networks will constantly rearrange. Dissenting as predicted by the BQH, which acts on the assumption of an enclosed community, provider microbes will encounter other provider microbes with the same leaky metabolic trait. Thus, in succession of altering microbial community compositions, the loss of selectively favored metabolic trait continues unlimited.

Also, the accentuated advantage of genome streamlining by the BQH becomes a burden, "you can't catch a fish without its bones". As argued by the BQH, genome streamlining reduces the metabolic costs, but concurrently provokes a spatial dependence towards providing microbes. The many of microbial metabolites, for instance released by roseobacter model strains *P. inhibens* and *D. shibae* and likely others making it a market place of microbial metabolites, supposable enables the survival of genome streamlined microbes (Chapter 3). As a consequence, the stigmatized providing black queens (BQ), termed after the homonymous hypothesis, are possible less abundant but render a vital contribution for the environed microbial community. For example, genome streamlined members of the SAR11 clade are abundant and ubiquitous, but require for instance the thiamin precursor HMP for growth. Laboratory experience has also shown that bacterial isolation often yields rare members of whole-community identified by culture-independent approaches (Fuhrman, 2009), possibly reflecting the BQ.

Our findings are consistent with other studies, indicating that a majority of dependencies in microbial networks are likely to be driven by microbial metabolic cross feeding (Carini *et al*., 2014; Garcia *et al*., 2015; Heal *et al*., 2017; Chapters 3 and 6). The degree of vitamin traffic in the marine environment for instance, determines the vitality and dynamics of microbial communities. Such dependency scenarios presumably also exist for various other metabolite groups exchanged among the microbial community (Chapter 3). At present, microbial network inference are conducted by either Pearson or Spearman correlations for abundance data (Barberán *et al*., 2012; Zhou *et al*., 2012) or the hypergeometric distribution for presence–absence data (Chaffron *et al*., 2010; Freilich *et al*., 2010) and often correlated to seasonality or environmental parameters (Falkowski *et al*., 2008; Fuhrman *et al*., 2006; Fuhrman and Steele, 2008; Gilbert *et al*., 2012). Such approaches cannot distinguish between true ecological interactions, such as the neglected metabolic cross feeding, but rather render equal occurrence patterns. Indeed metabolic potentials, comprised as pangenome or metabolic activities, assessed by pathway regulations, shed more light on metabolic dependencies (Freilich *et al*., 2011; Gómez-Consarnau *et al*., 2012), but cannot yet depict the true metabolic cross feeding driven microbial networks. Likewise, DOM, largely composed of microbial exometabolome buildups, renders the microbial transmission of (exo)-metabolites (Jiao and Zheng, 2011; Osterholz *et al*., 2015; Zweifel *et al*., 1995). Hence, microbial metabolic dependencies are presumably maintained over great distances and might even outlive microbial lifetimes. However, temporal, as well as spatial distance dependencies are at present only considered in microbial population succession studies and within a given sampling scale.

Additionally, microbial networks underlie continuous alterations and are substantially more complex as our present models allow us to predict. Currently we can conclude that DOM diversity is the foundation for a prosperous microbial community and vice versa, the variety of metabolic traits within the pan-genome of a microbial community determines DOM diversity.

Marine DOM – fuel for micro- and global scale processes

Marine DOM is an enigmatic mixture of organic molecules with myriad origins and various age classes. The primary base for the overwhelming majority of the manifold dissolved organic molecules evolves by photosynthesis. As a result of multitudinous organic matter ramification by heterotrophic microbes, the ocean accumulates bioresistant refractory DOM. As our findings demonstrated, individual microbes reveal remarkable variable ramification patterns utilizing and transposing algal exudates (Chapter 2). So far we are aware that marine DOM is at the same time direct source for and cause of microbial metabolism (Chapter 4). However, marine DOM conceals an aspect that received little notice until now. On grounds of its ubiquity in the ocean, DOM is not only one of the largest carbon storages in the world, but likewise connects the entire ocean down to remotest spots. This suggests that the ocean, accommodating DOM, is an ideal transmission body not only for organic molecules as substrate, but also for the microbial metabolic cross-feeding.

The success of microbial communities is constricted to the availability of metabolites, distributed as dissolved molecules. Evolutionary driven genome streamlining created a tight net of metabolite dependencies among microbial organisms (Giovannoni *et al*., 2005; Morris *et al*., 2012). However, the exchange of metabolites is a consequence of the degree of metabolite privatization of a microbe and can be mediated within the phycosphere (Seymour *et al*., 2017) or as dissolved metabolites in large distances across the ocean (Heal *et al*., 2017; Chapter 6). The awareness that microbial communities are highly dependent on the exchange of metabolites supports the environment hypothesis that is based on a metabolite-limited causation for the DOM biodegradation resistance (Hansell and Carlson, 2014). While essential metabolites are absent in the environment, microbes supposedly remain unresponsive to a surrounding complex mixture of DOM molecules.

Particularly puzzling is the unanticipated high number of bacterial and diatom-derived exometabolites, leading to the speculation of metabolic waste formation. In such a hypothetical scenario, microbes release waste products in succession of presumably malfunctioning enzymatic reactions. If microbes release such waste compounds, also the

intrinsic stability hypothesis applies, implying the synthesis of molecules resistant to further biodegradation (Hansell and Carlson, 2014).

It appears that the huge marine DOM buildup is presumably in large parts a reflection of myriad microbial processes (Chapter 4) and the fuel for the underestimated metabolic cross-interlocking of the microbial world (Chapters 3 and 6). Our perception of the unknown is always hampered by our acquired scientific focus. In future studies we need to change our constricted perspective and disassemble the enigmatic pool of marine DOM in its constituent parts.

Outlook

The study of bacterial exometabolomes has improved our understanding of large scale global phenomena, such as DOM diversification processes and bacterial carbon sequestration, and likewise provided novelties on the chemical cross feeding of microbes. Hence, novel perspectives and questions arose and require our dedication in future scientific projects.

Bacteria release an unexpected and unintelligible diverse blend of organic compounds, even under starvation (Romano *et al*., 2014; Schwedt *et al*., 2015; Chapter 3). So far, the ecological function or formation processes for the majority of compounds remains unknown. Whether such compounds are waste products of malfunctioning enzymatic reactions or unassignable active metabolites is currently debated and requires more experimental emphasis (Fiore *et al*., 2015; Chapter 3). This goal can be achieved by simplifying the metabolite genesis to a single unmated enzymatic reaction level. The number of resulting molecules, detectable by FT-ICR-MS, will reveal the efficiency of enzymatic reactions and give a general conspectus on the ratio of waste and metabolite products. Additionally, to better understand natural diversification processes and formation of refractory organic compounds, we could use defined bacterially derived exometabolomes as carbon source for bacteria to identify compound classes that are resistant to further microbial degradation.

The large quantity of bacterial exometabolites remains a riddle and might hold back answers for the study of the global marine DOM diversity. In a complex modelling approach, we predict that the accumulation of bacterial exometabolomes is a reflection of the global quantitative molecular DOM diversity (Noriega-Ortega in submission). To further support this assumption, exometabolomes of various bacteria or even archaea, comprising distinct and variable metabolic traits and genome sizes, should be surveyed in future projects. In the ocean, bacterial organic molecule sequestration is presumably far more complex as our simplified batch culture experiments can reflect. Microbial interactions are believed to be an effective

driving force for marine DOM diversification. Recently, we designed a 2- and 5-chamber-coculture-flask separated by a 0.2 µm membrane, which allows the defined study of microbial interaction ramification on molecular exometabolome diversity formation processes.

Microbes interact in a complex web of metabolite cross feeding mediated by dissolved molecules in the marine environment. Therefore, the identification of individual bacterially-derived exometabolites can provide substantial information on microbial metabolite interrelationships. In our study we were able to assign a relatively small fraction of exometabolites to known metabolites, still comprising more than thirty molecular formulas per bacterial strain. The tested exometabolites revealed remarkable effects on tested microbes and foreshadowed an involved web of exometabolite exchange among a natural community (Chapter 3). By reason of the large number of interesting exometabolites, we were only able to demonstrate the notable effect on the microbial community using selected vitamin-metabolites (B1, B12, HET, HMP, α-ribazole). Hence, as indicated by literature for the untested bouquet of (exo)-metabolites, a great potential for defined microbial metabolite exchange experiments is available for promising future projects (See Chapter 3). The overarching long term goal is to substitute the lack of metabolic traits induced by genome streamlining to improve marine microbial isolation. Therefore, it would be of great advantage to heighten the number of identified exometabolites and to improve the methods to verify our findings. So far, applying the untargeted FT-ICR-MS approach, the assignment of MF to metabolites is limited by the decryption of unknown protein coding enzymes and their functions in metabolic pathways. It has also been proposed that enzymes might be involved in multifunctional reactions, producing a larger blend of metabolites as known so far (Wang *et al*., 2016). Thus, the yield of identifiable exometabolites is coherent to the state of knowledge of metabolites and therefore should be repeated in the near future. To further validate resulting MF matches, besides the already applied MS-MS fragmentation, a LC-MS could be applied.

A great emphasis of my work was on the study of vitamin traffic among microbes. Connatural to the synthesis of most metabolites, limitations of de novo vitamin synthesis is widespread among marine microbes by reason of genome streamlining (Croft *et al*., 2006; Giovannoni *et al*., 2014; Sañudo-Wilhelmy *et al*., 2014). The essentiality of vitamins as cofactors for all living organisms makes them an ideal metabolite class for the study of microbial metabolic interaction webs. As part of my dissertation, I tested the effect of vitamins and respective precursor on either bacterial or phytoplankton batch cultures and mesocosm microbial communities. It would be of great interest to continue this work using different microbes that

are dominant in variable ecological niches or manifest different metabolic traits and to quantify such vitamin metabolites in nature. In addition, the establishment of a validation method applying an LC-MS-MS would be of great advantage. Furthermore, using the other identified but not yet tested vitamin (exo)-metabolites will give an overview of the natural relevance of vitamin traffic. To examine whether the release of such precious vitamin exometabolites is of commensalism or mutualism interaction nature, co-culture experiments should be set up. In particular vitamin B12 is an interesting metabolite candidate to study the vitamin traffic. Variable corrinoid B12-analogs exits with differing co-enzymatic functions (Banerjee, 1999; Lengyel *et al*., 1960; Stupperich *et al*., 1987; Yi *et al*., 2012). We demonstrated that the availability of the crucial lower ligand (α-ribazole) in nature supposable enables B12-remodeling by bacteria. The broad variety of alternative attachable lower ligands discloses great opportunities for future projects. A scenario in which two bacterial mates produce different building blocks of one metabolic pathway and perform a co-synthesis via the exometabolite exchange are imaginable for both, vitamin B1 and B12. Indications for such defined microbial metabolic interactions can presumably be identified by screening selected genomes for specific vitamin metabolic functions. In spite of our extensive research, we still gain more questions than answers and thus the erstwhile statement by Newton (1583), “what we know is a drop, what we don't know is an ocean”, is still valid.

List of references

(Introduction and discussion)

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Erklärung

Hiermit versichere ich, dass ich diese Arbeit selbständig verfasst und kein anderen als die angegebenen Quellen und Hilfsmittel benutzt habe. Zudem versichere ich, dass diese Dissertation weder in ihrer Gesamtheit noch in Teilen einer anderen wissenschaftlichen Hochschule zur Begutachtung in einem Promotionsverfahren vorliegt oder vorgelegen hat. Außerdem versichere ich, dass ich die allgemeinen Prinzipien wissenschaftlicher Arbeit und Veröffentlichung, wie sie in den Leitlinien guter wissenschaftlicher Praxis der Carl von Ossietzky Universität Oldenburg festgelegt sind, befolgt habe.

Oldenburg, den 07. Februar 2018

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Gerrit Wienhausen

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Curriculum Vitae

Gerrit Wienhausen

Wiesenstraße 36

26135 Oldenburg

Germany

Nationality: German

25.09.1986, Braunschweig / Niedersachsen

Since 01/2014	PhD student at the Carl-von-Ossietzky University Oldenburg, Germany. Supervisor: Prof. Dr. Meinhard Simon
10/2013 – 12/2013	Scientific assistant at the Institute for Chemistry and Biology of the Marine Environment (ICBM) in the working group "Biology of geological processes"
10/2013	Master in Microbiology the Carl-von-Ossietzky University Oldenburg, Germany
10/2011 – 10/2013	Graduate student at the Carl-von-Ossietzky University Oldenburg, Germany
09/2012 – 11/2012	Research project at the Scripps Institution of Oceanography, San Diego, CA, USA
06/2011	Bachelor in Environmental Science at the Carl-von-Ossietzky University Oldenburg, Germany
10/2007 – 06/2011	Undergraduate student at the Carl-von-Ossietzky University Oldenburg, Germany
08/2009 – 01/2010	Semester abroad at the Hawaii Pacific University, Waikiki, HI, USA
08/2006 – 08/2007	Alternative civilian service at the Camphill Special School, Philadelphia, PA, USA
2006	High school "Hildegardisschule Münster", Germany, graduating with „Abitur"- certificate