**High-throughput cultivation of heterotrophic bacteria along a spring
phytoplankton bloom in the North Sea**

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20

21 **Abstract**

22 On-going studies of phytoplankton-bacterioplankton interactions at the long-term
23 ecological research site Helgoland Roads have indicated that many of the heterotrophic
24 bacterial taxa have not yet been cultivated. A high-throughput approach combining whole
25 cell matrix assisted laser desorption ion - time of flight mass spectroscopy with 16S rRNA
26 gene sequencing was applied to the spring bloom of 2016. Aiming at an assessment of
27 cultivability along a spring bloom we had to use cultivation on solid marine media as a
28 dilution to extinction would not be doable in a high-throughput approach as performed in
29 this study. A total of 5,023 isolates were obtained from nine weekly samples, from early
30 bloom to post bloom, on eight different solid media. Most of the 4,136 identified strains
31 affiliated with *Bacteroidetes* (13.3%), *Gammaproteobacteria* (26.9%),
32 *Alphaproteobacteria* (40.6%) and *Actinobacteria* (6.7%). Of 271 operational phylogenetic
33 units (OPUs) identified, 13 likely represent novel genera and 143 novel species. A
34 comparison with 16S rRNA gene tag data indicated that most of the isolates were rather
35 rare in surface waters, with the exception of five OPUs affiliating with *Rhodobacteraceae*,
36 *Polaribacter*, *Psychromonas* and *Pseudoalteromonas*. The effort yielded many novel
37 isolates, yet most of the abundant heterotrophic bacteria still remain elusive. The large
38 strain collection obtained will not only provide insights into the succession of the
39 cultivable fraction of the bacterioplankton, but also enable fine-tuned taxonomic and
40 physiological follow-up studies to improve our knowledge on heterotrophic bacteria in the
41 North Sea waters.

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Introduction

Cultivation-independent molecular techniques are providing unprecedented insights into the microbial diversity and genomic potential of *Bacteria* and *Archaea*. They often result in hypotheses on the physiology and ecology of microorganisms that have so-far not been cultivated. As much as this is true for extreme and remote habitats, it also applies to readily accessible sites which have been studied for decades due to their importance in global element cycling. This includes coastal marine surface waters where aerobic heterotrophic *Bacteria* and *Archaea* remineralize terrestrial and phytoplankton derived organic matter on a Gt/yr scale. It has been estimated that heterotrophic bacteria process about half of the oceanic net primary production [2,3], thus contributing importantly to the carbon cycle [29,38].

One such site is the long-term ecological research station Helgoland Roads in the southern North Sea with its pronounced seasonal dynamics characterized by annual diatom blooms in spring and summer [18,63]. Phytoplankton blooms are ephemeral events which break down due to grazing, nutrient limitation or viral infections [7,64]. This releases huge amounts of diverse high and low molecular weight organic compounds which are utilized by marine heterotrophic *Bacteria* and *Archaea* [7]. The time-restricted pulses of organic matter result in rapid substrate-driven successions of recurrent clades mostly from *Bacteroidetes*, *Gammaproteobacteria* and *Alphaproteobacteria* [7,33,56]. These fluctuations in nutrient availability result in the coexistence of different physiological types of microorganisms referred to as oligotrophic and copiotrophic [51].

Ever since the seminal study of Claude Zobell [68] there have been attempts to isolate and identify the aerobic heterotrophic bacteria dwelling in marine surface water. This resulted in much frustration since cultivability calculated as the ratio of colony forming units divided by microscopic cell counts ranged from 0.001% to 1% [10,27]. Along those lines, a cultivation study at Helgoland Roads yielded dominantly strains of the *Roseobacter* clade and the genera *Vibrio*, *Alteromonas*, *Marinomonas* and

70 *Pseudoalteromonas* [14,16]. Over the years, there have been breakthroughs such as the
71 isolation of “*Pelagibacter ubique*”, the first representative of the SAR 11 clade [47],
72 strains of the OM43 clade [11] and the marine group I archaeon *Nitrosopumilus*
73 *maritimus* [30]. These were enabled by high-throughput cultivation and the application of
74 the dilution-to-extinction method in liquid media [11,54]. Past targeted efforts for
75 obtaining the dominant heterotrophic bacteria degrading algae-derived polysaccharides
76 at Helgoland Roads resulted in a collection of several hundred isolates of mostly
77 *Bacteroidetes* and *Gammaproteobacteria* [21,22].

78 Recent molecular studies at Helgoland Roads confirmed that important clades of
79 the *Bacteroidetes* but also of the *Gammaproteobacteria* and *Alphaproteobacteria* have
80 still not been cultivated [8,31]. This had been indicated before by various studies applying
81 cultivation-independent techniques such as DGGE, T-RFLP, CARD-FISH, 16S rRNA
82 gene amplicon sequencing and metagenomes [8,14–16,50,52,55,56]. The purpose of
83 this study was to use a massive high-throughput cultivation effort on solid media (i) to
84 gain further knowledge on the cultivable diversity of heterotrophic bacteria in the North
85 Sea along a spring bloom, (ii) to generate a large collection of new strains for further
86 taxonomic, physiological and/or genetic studies, and (iii) to evaluate different media to
87 be used in future studies. Other techniques targeted at the retrieval of abundant
88 oligotrophic bacteria, such as dilution-to-extinction, could not be considered in this study
89 as they are much more laborious and handling such a great number of isolates would
90 have not been feasible. Here, the isolates obtained on solid media were characterized
91 by a tandem approach consisting of Whole Cell Matrix-Assisted Laser Desorption
92 Ionization – Time Of Flight mass spectrometry (WC MALDI-TOF MS) and 16S rRNA
93 gene sequencing of cluster representatives. This approach had been successfully used
94 to identify thousands of strains isolated from solar salterns [61] and hot springs [67]. In
95 this study, we obtained on a purely statistical basis more than 5,000 heterotrophic strains
96 by a weekly sampling along the 2016 Helgoland spring phytoplankton bloom. In doing
97 so, we also evaluated eight different media varying in carbon content and the chemical

98 nature of the organic matter. It was anticipated that the sheer size of the effort would
99 result in many novel isolates potentially including missing dominant taxa.

100

Materials and methods

Sample collection

At Helgoland Roads (54° 11' 17.88" N, 7° 54' 0" E) surface seawater was pumped from within the first meter into 10 L containers and processed within two hours in the laboratories of the Biologische Anstalt Helgoland (Germany). During nine weeks from March to May 2016, samples for cultivation were taken weekly (samplings S1 to S9). Relevant physicochemical parameters such as water temperature and chlorophyll concentration as well as plankton data were measured as part of the Helgoland time series [63] are available online as part of the Helgoland Roads LTER time series (www.pangaea.de).

Growth media, plating and isolation

Eight different solid media were used: Marine Agar 2216 (**ZB**) [68] for marine heterotrophs from Difco Laboratories (Detroit, USA). It is composed of peptone, 5.0 g/L; yeast extract, 1.0 g/L; ferric (III) citrate, 0.1 g/L; NaCl, 19.45 g/L; MgCl₂, 5.9 g/L; Na₂SO₄, 3.24 g/L; CaCl₂, 1.8 g/L; KCl, 0.55 g/L; NaHCO₃, 0.16 g/L; KBr, 0.08 g/L; SrCl₂, 34 mg/L; H₃BO₃, 22 mg/L; sodium silicate, 4.0 mg/L; NaF, 2.4 mg/L; (NH₄)NO₃, 1.6 mg/L and Na₂HPO₄, 8.0 mg/L; final pH 7.6. Modified seawater medium (**SW**) [49] at 3% w/v salts concentration contained pyruvate, 1.5 g/L; yeast extract, 0.5 g/L; NaCl, 23.4 g/L; MgSO₄ × 7H₂O, 5.94 g/L; MgCl₂ × 6H₂O, 4.15 g/L; KCl, 0.6 g/L; CaCl₂ · 0.09 g/L; NaBr, 0.08 g/L and NaHCO₃, 0.02 g/L; adjusted to pH 7.5. Artificial sea water (ASW) [22] without magnesium ions was prepared with NaCl, 26.37 g/L; CaCl₂ × 2H₂O, 1.47 g/L; KCl, 0.72 g/L; NaHCO₃, 0.19 g/L; KBr, 0.1 g/L; H₃BO₃, 0.02 g/L and SrCl₂, 0.02 g/L and used to four different dilutions of Reasoner's 2A medium (R2A) (Difco Laboratories, Detroit, USA) [48] to account for bacteria adapted to different nutrient concentrations. The media dilutions contained yeast extract, proteose peptone no. 3, casaminoacids, dextrose and soluble starch (each 0.05, 0.1, 0.2 or 0.36 g/L, depending on the dilution), sodium

pyruvate and dipotassium phosphate (each 0.03, 0.06, 0.12 or 0.21 g/L) and MgSO_4 (0.005, 0.01, 0.02 or 0.036 g/L), so the final concentrations of organic matter in the media were 0.28 g/L (**R2A028**), 0.56 g/L (**R2A056**), 1.12 g/L (**R2A112**) and 2 g/L (**R2A2**), final pH was adjusted to 7.5.

Lastly, medium HaHa proven to be suitable for the isolation of *Bacteroidetes* [22] was used with 1 g/L glucose and 1 g/L casaminoacids (**HaHa**) and, alternatively, with 2 g/L laminarin (**HL**). For this, agar was washed with MilliQ water, and Tris (50 mM, pH 7.5) and 350 mL of twofold aforementioned ASW without magnesium ions were added. This mixture was cooled to 55°C after autoclaving and supplemented with the following stock solutions: 7.9 mL $\text{MgCl}_2 \times 6 \text{ H}_2\text{O}$ (500 g/L; autoclaved); 9.5 mL $\text{MgSO}_4 \times 7 \text{ H}_2\text{O}$ (500 g/L; autoclaved); 1.4 mL trace-element solution (per L: $\text{FeSO}_4 \times 7 \text{ H}_2\text{O}$, 2.1 g; $\text{Na}_2\text{-EDTA}$, 5.2 g; H_3BO_3 , 30 mg; $\text{MnCl}_2 \times 4 \text{ H}_2\text{O}$, 100 mg; $\text{CoCl}_2 \times 6 \text{ H}_2\text{O}$, 190 mg; $\text{NiCl}_2 \times 6 \text{ H}_2\text{O}$, 24 mg; $\text{CuCl}_2 \times 2 \text{ H}_2\text{O}$, 10 mg; $\text{ZnSO}_4 \times 7 \text{ H}_2\text{O}$, 144 mg; $\text{Na}_2\text{MoO}_4 \times 2 \text{ H}_2\text{O}$, 36 mg; pH adjusted to 6.0 with 5 M NaOH; autoclaved); 1.4 mL of Se-W solution (per L: NaOH, 200 mg; $\text{Na}_2\text{SeO}_3 \times 5 \text{ H}_2\text{O}$, 18 mg and $\text{Na}_2\text{WO}_4 \times 2 \text{ H}_2\text{O}$, 18 mg; autoclaved); 1.4 mL of KH_2PO_4 -solution (50 g/L; autoclaved); 1.4 mL 7-vitamin-solution (per 100 mL: 3 mM NaH_2PO_4 , 7 mM Na_2HPO_4 , pH ~ 7.1; pyridoxine hydrochloride, 15 mg; nicotinic acid, 10 mg; Ca-D-(+)-pantothenate, 5 mg; 4-aminobenzoic acid, 4 mg; folic acid, 4 mg; D-(+)-biotin, 2 mg and lipoic acid 2 mg/L; sterilized through filter); 1.4 mL vitamin-B12-solution (per 100 mL: cyanocobalamin, 5 mg; sterilized through filter), 1.4 mL riboflavin-solution (per 100 mL: 25 mM NaH_2PO_4 , pH ~ 3.4; riboflavin, 5 mg; sterilized through filter), 1.4 mL thiamine-solution (per 100 mL: 25 mM NaH_2PO_4 , pH ~ 3.4; thiamine-hydrochloride, 5 mg; sterilize through filter) and 0.7 mL NH_4Cl -solution (50 g/L; autoclaved). Final pH was 7.5 for both media. ZB, SW and HaHa media were used for all samples collected and **HL in all but S3 and R2A028 in all but S1**. The media R2A056, R2A112 and R2A2 were only used three times (S3, S6 and S9) to check for the effect of varying concentrations of organic matter on cultivability.

One millilitre seawater samples were diluted in artificial seawater (ASW) to the 10^{-3} at 10^{-1} intervals and each dilution, including the undiluted sample, was plated in triplicates of 100 μ L. Inocula were spread with sterile glass Drygalski spatula. Plates were incubated at 12 °C for 30 days and colonies were counted every 10 days.

Approximately 150 randomly selected strains per sampling and medium were isolated from the plates of the highest dilution. Random selection was performed by isolating all colonies that grew on a single quarter. In some cases complete plates needed to be harvested. Each colony was re-streaked twice on the isolation medium to obtain pure cultures which were subsequently grown in the media of isolation. Isolates were stored at -80°C in a cryopreservation solution consisting of K_2HPO_4 , 12.6 g/L; KH_2PO_4 , 3.6 g/L; $(NH_4)_2SO_4$, 1.8 g/L; sodium citrate, 0.9 g/L; $MgSO_4 \times 7 H_2O$ and 13% w/v (v/v) of glycerol.

WC MALDI-TOF MS analysis

All isolates were initially characterized with WC MALDI-TOF MS as previously described [61] using freshly grown biomass. Spectra were manually inspected with Biotyper software v 3.2 (Bruker Daltonics, Germany) and used to create similarity dendrograms if their baseline was below 2,000 in relative intensity, and showed no protein degradation. To define mass spectrometric operational taxonomic units (MS-OTUs) in the dendrograms a cut-off criterion of 750 distance level was applied to group related spectra.

PCR amplification and 16S rRNA gene sequencing

Taking the MALDI-TOF MS-OTUs as a reference and depending on the number of strains in the OTU, one to ten strains were selected to sequence their complete 16S rRNA gene. DNA extraction was performed by osmotic shock using MilliQ water followed by a thermal shock consisting in three cycles of 100°C to -20°C for 5 minutes. The extract was used to amplify the 16S rRNA gene using the Bioline MyTaq protocol (2X MyTaq

Mix, 10 µM of each primer, DNA ≤ 250 ng/reaction and distilled water) in a final volume of 50 µl. The amplification was performed using the universal primers for *Bacteria*: GM3 (5'-AGAGTTTGATCATGGCTCAG-3') [42] and 1492R (5'-GGTTACCTTGTTACGACTT-3') [58] following previously reported conditions [39]. PCR products were visualized in 1% w/v agarose gels with ethidium bromide. Amplicons were purified with MSB® Spin PCRapace (Invitek GmbH, Berlin) and sequenced by Secugen S.L. (Madrid, Spain).

Phylogenetic reconstruction and creation of OPUs

Sequencher v4.8 (Gene Codes Corporation, USA) was used for trimming, error removal and assembling of 16S rRNA gene sequences. In the ARB v6 program package [34], sequences were aligned to the SILVA SSURef_NR99_128 [46] and LTPs128 [65] databases using the SINA tool (SILVA Incremental Aligned) [46]. After manual curation of the alignment, the phylogenetic reconstructions were performed using the almost complete sequences to reconstruct trees *de novo* using the neighbour-joining algorithm with the Jukes-Cantor correction and a phylogenetically broad supporting set of high quality almost complete sequences [41], and by using different filters available in the respective databases. Partial sequences were added to the reference tree using the Parsimony tool. Sequences were grouped in Operational Phylogenetic Units (OPUs) as previously reported [61]. An OPU is considered as the smallest monophyletic group of sequences containing at least one sequence from our cultured strains with the closest affiliated reference sequence from the SILVA SSURef_NR99_128, including the sequence of a type strain when possible. All 16S rRNA gene sequences were deposited at the EMBL repository under the accession numbers LR722694-LR722963 and LR732071-LR732073. Representative strains from each OPU were deposited in the Spanish Type Culture Collection (CECT; Valencia, Spain) under the reference number CECT BD002.

Statistical analyses

Based on OPUs (i.e. unique phylogenetic clades) and OTUs (i.e. unique MALDI-TOF similarity clusters), diversity and richness (Shannon and Chao-1 indexes, respectively), evenness and rarefaction curves were calculated using the PAST software v3.15 [23]. To compare the bacterial communities present at different times during spring, a non-metric multi-dimensional scaling (NMDS) based on the Bray-Curtis dissimilarity and a Jaccard similarity cluster were calculated with the software R Studio v1.1.447 using the vegan package [44].

V4 16S rRNA gene amplicon sequencing

Over the sampling period the microdiversity of 16S rRNA genes in the 0.2–3 µm fraction of seawater was determined work-daily as described by Chafee [8]. The V4 region of the 16S rRNA gene was amplified using primers 515 F (5'-GTGCCAGCMGCCGCGGTAA-3') and 806 R (5'-GGACTACHVGGGTWTCTAAT-3') and sequenced by Illumina MiSeq 2 × 250 bp chemistry as described previously [33]. These were correlated with the OPU 16S rRNA gene sequences via megablast [1]

Total cell counts and CARD-FISH

The bacterioplankton community was quantified as described previously by Teeling [55]. *Roseobacter* clade, *Gammaproteobacteria* and *Bacteroidetes* were quantified by catalysed reporter deposition fluorescence in situ hybridization (CARD-FISH) using appropriate probes [55].

Results and discussion

Environmental data and abundance of colony forming units (CFU)

The spring bloom of 2016 developed as expected from long-term observations. Chlorophyll concentrations along the bloom fluctuated between 2.12 µg/L and 11.8 µg/L, with maxima in March and early April. We refer to this period as bloom and separate it from the post-bloom samples taken in May (Figure 1 and Supplementary Table 1). The water temperature increased from 5.8 °C in late March to 11.5 °C at the end of May (Figure 1A). The bacterioplankton counts increased along the sampling period. Microscopic counts in S1 to S4 were around 5×10^5 cells/mL before fluctuating between 9×10^5 and 1.7×10^6 cells/mL in S5 to S9 (Figure 1A). The number of colony forming units per millilitre (CFU/mL; Figure 1B) ranged from 2.4×10^2 ($\pm 2 \times 10^1$) CFU/mL of seawater in HL medium in S1 to 1.8×10^4 ($\pm 2.9 \times 10^3$) CFU/mL of seawater in SW medium in S7. CFU counts are low during the early phase of the phytoplankton bloom (S1 and S2). Starting at S3 the CFUs abundance increased by about four times, fluctuating around 1×10^4 per mL during bloom and post-bloom. The highest cultivability (CFU/mL)/(TCC/mL) of 3% in S3 on ZB (Figure 1C) occurred in a phase of the phytoplankton bloom when the bacterioplankton counts were still low (Figure 1A). The post-bloom phase was dominated by decreasing chlorophyll levels together with an increase of water temperature and high bacterioplankton cell counts. In this second phase cultivability fell to 0.1% in S9 R2A2 (Figure 1B-C; for detailed data see Supplementary Table 1).

Characterization of the isolates by MALDI-TOF and 16S rRNA gene sequencing of MS-OTU representatives

A total of 5,023 strains were randomly isolated. Of those, 4,136 yielded MALDI-TOF MS profiles with a stable baseline and a good signal quality. These clustered into 661 MS-OTUs. The results were visualised in nine sample-specific dendrograms

(Supplementary Figure 1). The number of spectrometric profiles ranged between 260 in S1 to a maximum 745 in S3 (average: 459.5 spectra $\pm$ 158.8). The number of MS-OTU per sample ranged from 88 in S8 to 256 in S3.

16S rRNA gene sequencing of 468 strains representing 365 MS-OTUs assigned the strains into 271 OPUs based on a threshold of 98.7% 16S rRNA gene identity (Supplementary Table 2). As we wanted to check the dynamics of the more abundant cultivable prokaryotes, we did not sequence 484 rare strains that made 11.7% of the total collection. These, represented 296 MS-OTUs of which 86% were isolated only once or twice and 14% were represented by three to six strains, which occurred at seemingly random time points.

The individual sampling depth did not cover the total CFU diversity, as rarefaction curves for MS-OTUs and OPUs demonstrated (Supplementary Figure 1). Individual samples yielded between 71 and 205 OPUs (Supplementary Figure 1) while our extrapolations with Chao-1 index [9] ranged from 106 to 467 OPUs. Collectively, we covered about two-third of the taxa (567 of 858) estimated by Chao-1 to be cultivable by our approach (Supplementary Figure 2A). The Chao-1 index for OPU richness indicated that we have covered 271 of an extrapolated 308 OPUs (Supplementary Figure 2B) assessable by our solid-media based approach during the whole observation time. The evenness and Shannon diversity were higher during the phytoplankton bloom. Some abundant OPU reduced the cultivable diversity and evenness in the post-bloom samples (Supplementary Figure 1).

Applying the 16S rRNA gene sequence divergence thresholds for species and genus level (<98.7% and <94.5%, respectively) [53,66] the 271 OPU were identified as members of the *Alphaproteobacteria* (100), *Gammaproteobacteria* (82), *Bacteroidetes* (70), *Actinobacteria* (15), *Epsilonproteobacteria* (1), *Firmicutes* (1) and *Verrucomicrobia* (2) (Supplementary Table 2). The identification indicated that our strain collection encompassed 13 new genera and 143 new species. It was dominated by *Proteobacteria*,

with 40.6% of the strains affiliating with *Alphaproteobacteria*, 26.9% with *Gammaproteobacteria* and 0.46% *Epsilonproteobacteria*. *Bacteroidetes* had a share of 13.3% of isolates, *Actinobacteria* 6.7%, *Firmicutes* 0.29% and *Verrucomicrobia* 0.05%. More frequently retrieved OPUs affiliated with families *Rhodobacteraceae*, *Flavobacteriaceae*, *Sphingomonadaceae* and *Pseudoalteromonadaceae* (Figure 2). Family level dynamics revealed that *Flavobacteriaceae* showed higher cultivability during the early bloom stage. *Sphingobacteriaceae* were cultivated during the bloom and post bloom samples. *Rhodobacteraceae* increased during the bloom period. *Pseudoalteromonadaceae* were high in early and post bloom samples. Other recognizable trends of less abundant families were for *Alteromonadaceae* and *Psychromonadaceae* which were more present in the early and post bloom samples. *Colwelliaceae* peaked in the early bloom and bloom samples. *Caulobacteraceae* and *Rhodospirillaceae* showed a sudden increase in the bloom period. Lastly, *Nocardiaceae* showed higher abundances during bloom and post bloom phases. These ten most abundant families covered 78.8% of total cultivated diversity.

Overall, the distribution of isolates was as expected. It has been observed that the cultivability of *Proteobacteria*, *Bacteroidetes* and *Actinobacteria* is higher than that of all other phyla [66].

The effect of media composition on strain isolation

In this study we compared eight different media. These were either composed of complex ingredients such as yeast extract or defined additions of trace elements and vitamins. Growth on plates started after two days of incubation on most of the plates. The slowest growth was observed in the oligotrophic R2A028. We found that CFU abundance on R2A112 and R2A2 was clearly lower than on R2A028 and R2A056. The highest cultivabilities were observed for SW and ZB, two media with yeast extract and either 1.5 g/L pyruvate or 5 g/L peptone as major carbon source. HaHa and HL yielded slightly less CFU/mL (Figure 1B), although they also had 2 g/L organic carbon. Shannon

diversity and evenness values demonstrated a larger diversity among the strains obtained on HaHa and SW, in comparison to ZB (Supplementary Table 3).

In order to discriminate the effects of bacterioplankton dynamics from media specificity, we performed non-metric multidimensional scaling (NMDS) based on a dataset combining the OPU relative abundances obtained on the three media HaHa, SW and ZB (Figure 3A) and on the individual media HaHa, SW and ZB (Figure 4). The NMDS plots of the combined dataset and individual media revealed a high similarity of adjacent samples. The relatedness of samples in the NMDS plot coincided with the branching in dendrograms obtained using Jaccard distance indices (Figure 3B; Supplementary Table 4). These findings demonstrated that the differences in the cultivable diversity between the time points were larger than the diversity differences due to media compositions. The robustness of this finding was confirmed in an NMDS plot using all media which gave a very similar picture of the differences (Supplementary Figure 3).

Only a few OPUs were very frequently isolated with over 100 strains and allowed to investigate whether they formed more colonies on other media than on ZB, widely considered as the standard medium for marine microorganisms. The most abundant OPUs were two *Sulfitobacter*-affiliated ones: OPU006 preferred ZB, and OPU001 grew on ZB, but had more CFU on HaHa (Supplementary Table 5). *Sphingomonas* sp. (OPU074) were preferentially isolated HL and HaHa, whereas *Pseudoalteromonas aliena* (OPU100) showed no preferences. *Rhodococcus cerastii* (OPU260) and *Brevundimonas vesicularis* (OPU069) were a bit more abundant on R2A media. In summary, there was a degree of media-specific selectivity in the isolation of strains, which did however, not completely mask the sample-specific composition of the bacterioplankton.

Temporal dynamics of strain isolation

While most strains were found only transiently at few of the sampling times, there were five species from which strains were cultured at all time points (Figure 5A): *Sulfitobacter brevis*, *Altererythrobacter* sp. (corresponding to a yet unclassified species of the genus identified as the OPU086), *Sphingopyxis baekryungensis*, *Vibrio splendidus* and *Maribacter lutimaris*. This suggested that these species are generalists [6] capable of bearing environmental changes as they appear along the bloom. On the genus level *Sulfitobacter*, *Roseovarius*, *Sphingomonas*, *Pseudoalteromonas*, *Psychromonas*, *Marinobacter*, *Winogradskyella*, *Polaribacter* and *Rhodococcus* were present all over the bloom but not always represented by the same species (Figure 5B).

Abundances of *Thalassotalea* were high during early bloom (Figure 5C), while *Psychrobacter*, *Algibacter* and *Cellulophaga* abundances were high in both, early bloom and bloom (Figure 5D). It is plausible to assume that they were among the first degraders of complex organic matter derived from the bloom [55,62]. From the peak of the bloom we isolated strains of *Hyphomonas*, *Hoeflea*, *Brevundimonas*, *Rhodobiaceae*, *Colwellia* and *Pseudomonas* (Figure 5E). Interestingly, *Marinobacter* strains were isolated both in early bloom and post-bloom samples (Figure 5F). The finding of this genus in both stages of the bloom also suggested that temperature was not a limiting factor. Detailed information for the dynamics of all isolated OPUs can be found in Supplementary Table 6 (heatmap columns C-K).

In the following we hypothesize physiological capabilities of our isolates based on their next relatives, well aware that this has to be taken with a grain of salt. *Sulfitobacter* and *Roseovarius*, members of the *Roseobacter* clade, are ubiquitous marine bacteria and one of the two marine lineages capable of degrading the widespread algal osmolyte dimethylsulfoniopropionate (DMSP) [35] thus playing an important role in the sulphur cycling [25]. Furthermore, algicidal effects have been reported for the genus *Sulfitobacter* [4]. Members of the genus *Roseovarius* might be mutualistic pathogens [20]. Strains of *Sphingomonas*, *Psychromonas*, *Marinobacter* and

Winogradskyella have been reported to be associated with phytoplankton [26,57], likely utilizing glycans and proteins. Selected strains of all four genera have also been shown to degrade hydrocarbons [13,17,60]. The ubiquitous genus *Pseudoalteromonas* encompasses diatom-epibionts [12] and macroalgae-associated strains [37] which frequently can produce antibacterial metabolites [5]. As expected we also found the well-known phytoplankton-bloom associated genus *Polaribacter* [36], which dominates spring blooms at Helgoland [56]. Finally, many of the strains isolated fell in genera well known for the degradation of algae derived polysaccharides such as agar, carrageenan and alginate. This included many *Gammaproteobacteria* and *Bacteroidetes* [24,37,43,59].

Comparison between culture-dependent and culture-independent observations

In order to assess the representativeness of our isolates we compared their 16S rRNA gene sequences with PCR-amplified 16S rRNA gene fragments obtained in parallel from environmental samples of the same sampling days. Along the 2016 spring bloom analysis of amplicon sequences yielded a total of 6,362 minimum entropy decomposition (MED) nodes in the 3-0.2 μm fraction of surface water (Supplementary Table 7). We identified MED nodes matching OPU sequences with identity percentages $\geq 98.7\%$, since these could be from the same species [66]. Of the 271 OPUs retrieved by cultivation, 64 did not match any of the 16S rRNA gene amplicons and, hence, may represent rare species not detected by oligotyping. The remaining 207 OPUs matched with 170 MED nodes at the species level (Supplementary Table 6). Of those, 142 OPUs matched single MED nodes, and 65 OPUs did not have one-to-one assignments and clustered at $\geq 98.7\%$ with only 28 nodes, meaning that single OPUs clustered to more than one MED node. NMDS analysis of MED nodes that had a match with OPUs (Supplementary Figure 4) revealed a similar temporal dynamic to the one found by NMDS of OPUs, once again indicating that the bacterial succession was recorded by cultivation.

For none of the very frequent MED nodes, which accounted for >10% of the total reads in an individual sample (max. 25.4%, NODE 12007, a so-far-uncultured *Rhodobacteraceae* bacterium) we could obtain an isolate. The numerically most relevant hit was NODE 2246 $\geq 98.7\%$ identical with OPU022, a *Rhodobacteraceae* bacterium which based on 95.18% 16S rRNA gene identity with its closest type species *Planktotalea frisia* (at) would represent a new genus and species within this family. This node represented 8.2% of the 16S rRNA gene amplicon reads in S4. We also isolated strains $\geq 98.7\%$ identical with three MED nodes accounting for 1-2% of all reads in single samples: NODE 7072 clustered with OPU214 (*Polaribacter* sp.), NODE 7298 was covered by OPU101 and OPU153 (*Pseudoalteromonas* sp. and *Pseudoalteromonas undina*, respectively), and NODE 17033 affiliated with OPU106 and OPU108 (both *Psychromonas* sp.).

The thirty most frequently cultured OPUs were represented in 16S rRNA gene MED nodes in abundances below 0.6% (Supplementary Table 7). These OPUs affiliated with the following 17 genera (Figure 6): *Sulfitobacter*, *Roseovarius*, *Lentilitoribacter*, *Litorimonas*, *Andersenella*, *Sphingomonas*, *Sphingopyxis*, *Altererythrobacter*, *Thalassospira* (all *Alphaproteobacteria*), *Psychromonas*, *Colwellia*, *Vibrio*, *Marinobacter*, *Pseudomonas*, *Pseudoalteromonas* (all *Gammaproteobacteria*), *Rhodococcus* (*Actinobacteria*) and *Maribacter* (*Bacteroidetes*). Interestingly, of these more abundant isolates, OPU127 and OPU162, affiliating with *Vibrio splendidus* and *Pseudomonas* sp., respectively, did not match any of the 16S rRNA gene amplicon reads. As reported before cultivable bacteria compose often only a minor part of the bacterioplankton [10,16,27]. Our data confirmed earlier observations that isolates may not necessarily be detected by molecular techniques in environmental samples [45]. Besides a few of the environmentally abundant bacteria, most of our isolates represent minor abundant taxa and the rare biosphere [45]. Our isolation of rare taxa may be caused by short-lasting

blooms of these rare bacteria, also called pulse-populations, in response to the environmental dynamics of the habitat [32].

In addition to the total cultivability, we determined with group-specific CARD-FISH probes the abundances of *Roseobacter* clade (*Alphaproteobacteria*), *Gammaproteobacteria* and *Bacteroidetes* at the sampling times. We derived the taxon-specific cultivability on the media HaHa, SW and ZB (Supplementary Figure 5). Two different phases were observed, S1 to S4 and S5 to S9. The first phase had a high cultivability, especially for *Gammaproteobacteria*, and low CARD-FISH and total cell counts. The second phase had a lower cultivability and a higher abundance of cells. This suggested that the increase in bacterial abundance occurred mainly by bacteria which did not grow on our solid media. Surprisingly, the increased organic matter levels of the bloom and post-bloom phase did not select for cultivable bacteria. Rather, the opposite seems to be true. The most competitive species in such a setting are less cultivable, one explanation being that they have streamlined genomes relying on compounds provided by co-occurring organisms which absent from our media [40]. Overall, *Gammaproteobacteria* showed the highest cultivability with a maximum of 1.75% in S2 on HaHa, in agreement with observations reported before [16,27]. Among the three taxa, *Bacteroidetes* were the most abundant population, however, their cultivability was below that *Proteobacteria*. These asks for the development of a better media for *Bacteroidetes*.

Conclusions

In this high-throughput cultivation study we have obtained more than 5,000 bacterial isolates by weekly sampling of a spring phytoplankton bloom on eight different solid media. By combining MS-based typing with 16S rRNA gene sequencing we identified 82% of the strains in a time- and cost-efficient manner. We found that the cultivable fraction of bacterioplankton follows a successional dynamic reminiscent of the swift changes observed by cultivation-independent methods. The vast majority of the isolates were *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes*, known as “heterotrophic master recyclers” [7], as well as *Actinobacteria*. Since the exact composition of the media had little effect we recommend that future cultivation studies apply only one medium, and rather invest in obtaining more isolates per sample. We would like to recommend at least 500 isolates, if possible even 1,000, which should be sufficient to acquire most of the abundant cultivable strains. One could select either SW or HaHa medium. Both consistently yielded high numbers of CFU, often in the range of $1 \times 10^4/\text{mL}$, and highly diverse collections of strains.

An alternative to up-scaling of cultivation on solid media would be dilution-to-extinction techniques applying liquid media. These are more laborious, yet they have proven to recover another segment of the bacterial diversity [19,21], potentially also including *Aurantivirga*, *Cryomorphaceae*, SAR92 clade or the NS3 and NS5 marine groups known to dominate spring blooms at Helgoland [31,56]. Nevertheless, the value of this large strain collection should not be underestimated. Recent studies suggested that although a collection of 53 *Flavobacteriia* isolated from the North Sea was phylogenetically incomplete [28], their genomes encoded examples of all relevant polysaccharide utilization loci indicated by metagenomics [31]. Although not representative of the winning ecotypes the respective strains may therefore serve as a basis for meaningful physiological and biochemical studies.

456 The numerous new isolates furthermore will enable fine scale taxonomic studies.
457 This said, it has to be noted that it is a major challenge to make collections of several
458 thousand strains available for the scientific community. Current high-quality deposit
459 controls used by public culture collections are not compatible with large collections as
460 ours. However, there are ongoing efforts of collections such as CECT (Valencia, Spain)
461 or DSMZ (Braunschweig, Germany) to increase public availability of large collections.
462 Thus, bulk submissions may in the future become excellent resources for the scientific
463 community, although they require specific quality controls when retrieving particular
464 strains from the repositories.
465

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