

**Diversity and abundance
of Gammaproteobacteria during the winter-spring
transition at station Kabeltonne (Helgoland)**

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Abstract

Recent evidence has suggested that Gammaproteobacteria are strongly associated with phytoplankton blooms and may play a role in the processing of the algal derived organic substrate. Therefore, the succession of specific groups within the Gammaproteobacteria may be strongly influenced by the availability of this organic matter. Other factors such as the increase of temperature during the winter-spring transition time may also be important for such a succession as it promotes the bacterial growth rate.

The aim of this thesis was to study the population dynamics of the gammaproteobacterial groups caused by a massive spring algal bloom in the North Sea. Accordingly, surface seawater was investigated, that had been taken twice weekly in years 2009 and 2010 from the long-term ecological research station Kabeltonne located at the Helgoland Roads. Bacterial diversity was examined in samples of 11.02.2009 (winter) and 14.04.2010 (spring) by comparative sequence analysis of two full-length 16S rRNA gene clone libraries. Around 800 sequences were obtained from each library and revealed that Bacteroidetes and Gammaproteobacteria comprised the major fraction of the bacterial community in the coastal North Sea in April in contrast to February when Alphaproteobacteria was retrieved in high frequency. Within the Gammaproteobacteria the SAR86 clade dominated the community in the winter library while the SAR92 clade was the dominating group in the spring one. *Reinekea*, *Balneatrix*-related clade and *Glaciecola* were genera only occurring in the spring library.

For the newly occurring gammaproteobacterial groups oligonucleotide probes were designed. The full sequences of both winter and spring clone libraries were used for the *in silico* validation of the probe specificities. Subsequently, the abundances of the gammaproteobacterial subgroups were monitored for the years 2009 and 2010 using catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) and a probe set consisting of the newly designed probes and previously designed ones. The sum of all counts as detected by this set of probe covered almost 100% of cells detected by the general probe GAM42a.

In the year 2009, a tight succession of members of the genera *Reinekea*, the SAR92, OM182 and NOR5/OM60 clades, *Balneatrix*-related clade, *Vibrio*, *Glaciecola* and *Pseudoalteromonas* was observed about one month after a discrete spring algal bloom in late March which mainly consisted of diatoms. These groups seem to respond to the substrate generated by the bloom (bottom-up effect). A succession of *Reinekea* spp. was observed reaching up to 1.4×10^5 cells ml⁻¹. This was followed by a particular succession of SAR92 clade showing the strongest increase within the Gammaproteobacteria, up to total abundances of 3.0×10^5 cells ml⁻¹. OM182 and NOR5/OM60 clades subsequently reached total abundances of 1.2×10^5 cells ml⁻¹ and 9.0×10^4 cells ml⁻¹, respectively. These larger groups likely play a major role in the degradation of the organic matter produced by the diatoms spring bloom. Although the abundance of other gammaproteobacterial

groups such as *Balneatrix*-related clade, *Glaciacola*, *Vibrio* and *Pseudoalteromans* remained lower, they also participated into the upshift with maximum abundances of 3.6×10^4 cells ml⁻¹, 1.0×10^4 cells ml⁻¹, 3.0×10^4 cells ml⁻¹ and 9.0×10^3 cells ml⁻¹ respectively. Members of the SAR86 clade were no major players in the succession directly after the bloom, yet, they dominated later in May reaching up to 2.0×10^5 cells ml⁻¹.

In year 2010, a similar succession pattern was observed for the so far investigated groups *Reinekea*, SAR92, SAR86 and OM182 clades, *Balneatrix*-related clade and *Glaciacola* also within one month after the phytoplankton blooming peak. This indicates that this is an annual phenomenon occurring in the North Sea during the winter-spring transition period and further suggests substrate specialization for those groups.

Zusammenfassung

Aktuelle Forschungsergebnisse wiesen im Vorfeld darauf hin, dass das Auftreten von Gammaproteobakterien stark an Phytoplanktonblüten gekoppelt ist, da sie vermutlich eine wichtige Rolle beim Abbau der Algenbiomasse spielen. Daher sollte die Sukzession von bestimmten Gruppen innerhalb der Gammaproteobakterien stark von der Verfügbarkeit dieser organischen Materialien beeinflusst werden. Für die Sukzession können aber auch andere Faktoren wie die Erhöhung der Temperatur während der Winter-Frühling-Übergangszeit wichtig sein, da sie die bakterielle Wachstumsgeschwindigkeit fördert.

Das Ziel dieser Doktorarbeit war es, die Populationsdynamik von den Gruppen der Gammaproteobakterien, die durch eine massive Frühjahrsalgenblüte in der Nordsee verursacht war, zu studieren. Dementsprechend wurde Oberflächenseewasser untersucht, das in den Jahren 2009 und 2010 zweimal wöchentlich an der langfristigen ökologischen Forschungsstation 'Kabeltonne' genommen worden war, die an der Helgoländer Reede liegt. Zunächst wurde die bakterielle Diversität in zwei Proben vom 11.02.2009 (Winter) und 14.04.2010 (Frühling) durch vergleichende Sequenzanalyse von jeweils mehr als 800 fast vollständigen 16S rRNA-Genen untersucht. Dabei zeigte sich, dass Bacteroidetes und Gammaproteobakterien die Frühjahrsklonbank dominierten, im Gegensatz zur Februarprobe, wo die Alphaproteobakterien in hohe Häufigkeit gefunden wurden. Innerhalb der Gammaproteobakterien dominierte die SAR86-Gruppe die winterliche Klonbank, während SAR92 die dominierte Gruppe in der Frühlingsklonbank war. Sequenzen aus den Gattungen *Reinekea* und *Glaciecola*, sowie von einer *Balneatrix*-verwandten Gruppe wurden nur in der Frühjahrsklonbank gefunden.

Für einige Gruppen der Gammaproteobakterien wurden hier neue Oligonukleotidsonden entwickelt. Dabei wurden die Sequenzen der beiden Klonbanken für die *in silico* Validierung der SONDENSPEZIFITÄT verwendet. Anschließend wurden die Abundanzen der Gammaproteobakterienuntergruppen für die Jahre 2009 und 2010 mittels der katalysierten Reporter Deposition-Fluoreszenz *in situ* Hybridisierung (CARD-FISH) und eines umfangreichen SONDENSATZ quantifiziert, der aus den neuen und bereits vorher entwickelten Sonden bestand. Die Summe aller Zählungen, wie mit diesem SONDENSATZ erzielt wurden, erreichte für fast alle Zeitpunkte ca. 100% der Zellen, die durch die allgemeine gammaproteobakterielle Sonde GAM42a erfasst wurden.

So konnte in dieser Doktorarbeit für das Jahr 2009 eine hoch-dynamische Sukzession der Gattung *Reinekea*, der SAR92-, OM182- und NOR5/OM60-Gruppen, sowie von *Balneatrix*-verwandte Bakterien und den *Vibrio*, *Glaciecola* und *Pseudoalteromonas* beobachtet werden. Diese begann ungefähr einen Monat nach einer diskreten Frühjahrsalgenblüte im späten März, die hauptsächlich aus Diatomeen bestand. Die Untergruppen der Gammaproteobakterien scheinen im Sinne eines bottom-up-Effekts auf die durch die Blüte generierten Substrate zu reagieren. *Reinekea* spp. erreichten dabei Zahlen von bis zu $1,4 \times 10^5$ Zellen ml⁻¹. Darauf folgte für die SAR92-Gruppe ein

Anstieg auf bis zu $3,0 \times 10^5$ Zellen ml^{-1} folgte. Die OM182- und NOR5/OM60-Gruppen erreichten anschließend Häufigkeiten von $1,2 \times 10^5$ Zellen ml^{-1} und $9,0 \times 10^4$ Zellen ml^{-1} . Diese größeren Gruppen spielen wahrscheinlich eine wichtige Rolle beim Abbau der organischen Substanz der Kieselalgen-Frühjahrsblüte. Obwohl die Zahlen der anderen gammaproteobakteriellen Gruppen wie der *Balneatrix*-verwandten Gruppe oder der Gattungen *Glaciecola*, *Vibrio* und *Pseudoalteromonas* insgesamt niedriger bleiben, stiegen auch hier in Folge der Algenblüte die Zahlen auf bis zu $3,6 \times 10^4$ Zellen ml^{-1} , $1,0 \times 10^4$ Zellen ml^{-1} , $3,0 \times 10^4$ Zellen ml^{-1} und $9,0 \times 10^3$ Zellen ml^{-1} . Die SAR86-Clade wurde hingegen nicht direkt von der Blüte beeinflusst und dominierte erst später im Mai 2009, mit Zahlen von bis zu $2,0 \times 10^5$ Zellen ml^{-1} .

Für das Jahr 2010 wurde in dieser Studie eine ähnliche Sukzession beobachtet. Erneut zeigten Vertreter der Gattung *Reinekea*, der SAR92-, SAR86- und OM182-Gruppen, der *Balneatrix*-Verwandten und von *Glaciecola* einen Anstieg innerhalb eines Monats nach der Phytoplankton-Frühjahrsblüte. Zusammenfassend wurden also deutliche Hinweise dafür gefunden, dass die Frühjahrsalgenblüte in der Nordsee zu einer jährlich wiederkehrenden, hochdynamischen Sukzession von definierten Untergruppen von Gammaproteobakterien führt, die vermutlich auf eine Substratspezialisierung zurückzuführen ist.

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1.1 Station Kabeltonne, Helgoland, North Sea

The marine sampling site “Kabeltonne” is located at the Helgoland Roads between the main island of Helgoland and the Düne (54°11.3'N, 07°54.0'E) (**Figure 1**). It has been a permanent biological station for marine research since the 19th century (48). For over 40-years, also microbial parameters and an archive of contextual data have been collected here. The island of Helgoland is characterized by a high diversity of marine life and a broad array of habitat types. The island is located about 60 km off the estuaries of the rivers Elbe and Weser in the German Bight of the North Sea, which makes it an ideal representative site to study bacterioplankton diversity in an open coastal area with occasional exchange of the water body.

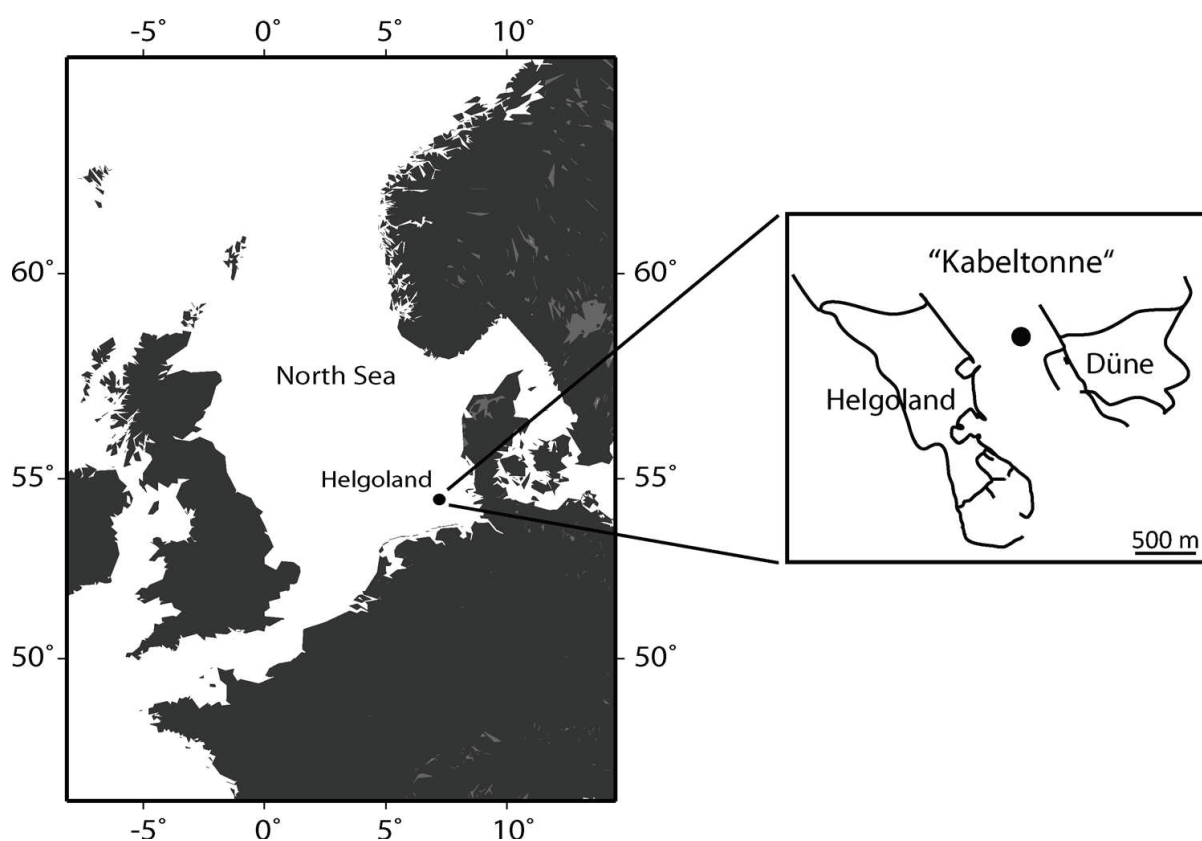


Figure 1 Location of Helgoland island in the North Sea (91).

Biodiversity studies have been conducted in Helgoland to investigate the effect of seasonal changes in temperature, salinity, nutrients and phytoplankton dynamics on bacterioplankton dynamics. A substantial temperature increase of 1.13°C for the 40 years since 1962 and an average salinity rise by 1.0 PSU over the same period of time was recorded (48). Minimal temperatures ranged between 1°C and 4°C and maximal values were between 17°C and 20°C in this time period (132) while salinity values varied from 24 to >34 PSU (95). The salinity at Helgoland Roads is mainly influenced by the

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mixture of coastal (riverine) water masses and water of central North Sea origin, controlled by hydrological and meteorological forces as well as by the river discharges (80).

At Helgoland Roads, a steady decrease in all nutrients since the early 1980s has been observed due to a reduction in nutrient input (69) and due to the fact that the current direction is less influenced by coastal waters, and more open North Sea water reaches the site than 45 years ago (130). In particular, there was a significant decrease in annual phosphate concentrations. According to data extracted from the database 'PANGAEA' (www.pangaea.de), hosted by the Alfred-Wegener-Institut (AWI, Bremerhaven), February median concentrations of phosphate rose from 0.8 to > 1 μM from 1962 to 1985 (95). From 1985 to 2004 they significantly decreased to <0.8 μM . In the late 1970s, a significant upward shift in the annual silicate concentrations were observed which perhaps was related to a combination of increased runoff from rivers and the input from highly saline Atlantic water (95). Since then, the silicate concentrations has steadily decreased (130).

February median concentrations of silicate significantly decreased from almost 15 μM down to <5 μM from 1966 to 1986. In 1987, they increased culminating in a peak of >25 μM in 1988. However they slowly decreased again, but did not reach the low level of <5 μM that had been registered, before 2003. Nitrite February medians varied between 0.5 and 2 μM over all years showing a significant decreasing trend from 1990 to 2002, apart from 1999, and they strongly increased again in 2003 and 2004. February medians for nitrate were mostly below 20 μM before 1981, whereas the levels ranged from 20 to 40 μM after 1981 showing a peak of >70 μM in 1994, and generally decreasing to <20 μM till 2004 (95).

A rigorous analysis of the Helgoland phytoplankton series showed that there has been a warming-related shift in the spring diatom bloom over the past 40 years (Wiltshire and Manley 2004) (132). It is well known that the phytoplankton succession is the main driving factor for ecosystem seasonality in marine temperate zones (48). Thus, changes in the timing phytoplankton blooms will inevitably affect the performances of other members of both the pelagic and benthic food webs.

There are a growing number of studies dealing with the seasonality of bacterioplankton community composition. They show a strong association of bacteria with phytoplankton (103). Thus phytoplankton blooms impact the composition of the heterotrophic bacterioplankton community. Phytoplankton blooms dominated by diatoms typically occur in coastal seas such as the North Sea when high nutrient concentrations coincide with increased solar irradiance (90).

Phytoplankton releases a fraction of its photoassimilated carbon as DOC during its growth. The collapse of phytoplankton blooms can be sudden, with viral cell lysis potentially being an important source of mortality. According to the 'microbial loop' hypothesis, phytoplankton biomass is partly converted into dissolved and colloidal matter through cell lysis, and subsequently converted back to particulate organic matter by uptake and cell growth of heterotrophic prokaryotes and thereby, not completely lost from transfer to higher trophic levels (9). The greatly increased availability of algal-

derived particulate and dissolved organic carbon results in an increase in the abundance and production of specific bacteria, thereby causing strong changes in the microbial community composition (1).

For instance, Sapp et al. 2006 has shown that the increase in temperature and the appearance of the phytoplankton species *Phaeocystis* spp., *Guinardia delicatula* and *Chattonella* spp. contributed to shifts in the bacterial community in year 2004. The occurrence of members belonging to the Flavobacteria and the Gammaproteobacteria in April and May 2004, respectively, was strongly correlated with the bloom of *Phaeocystis* spp. (103).

A parallel study was done during the spring and summer season of the same year to determine the dynamics of the abundance and activity of some selected heterotrophic prokaryotic groups in the coastal regions of the North Sea during the phytoplankton spring bloom period. This study also showed the dominance of bacteria belonging to the Bacteroidetes, particularly during the collapse of *Phaeocystis globosa* spring bloom and suggested their involvement in the degradation of mucopolysaccharides produced by the phytoplankton (1).

The substantial increase of bacterial abundance during phytoplankton bloom senescence is coupled with large increases in per-cell activity in hydrolytic enzyme activity and growth rates (111). Few studies have investigated the composition of bacterioplankton assemblages in relation to naturally occurring or experimentally induced blooms of phytoplankton (90). However, it appears that changes in bacterial activity are associated with significant shifts in bacterioplankton species composition (88), (98). Bacteria shown to increase in abundance during the decay of algal blooms belong to the phylogenetic groups Bacteroidetes, Alpha- and Gammaproteobacteria (98), (106). Considering the ample phylogenetic diversity within each of these groups, it is expected that they contain substantial physiological and metabolic variability that contribute to the marine microbial processes.

1.2 Marine Gammaproteobacteria

Gammaproteobacteria represents one of the major heterotrophic bacterioplankton groups in oceans and coastal seas (50), (46), (1), (32), (42), (110). Most of the readily culturable bacteria isolated from marine water belong to the Gammaproteobacteria. 16S rRNA gene sequence analyses revealed that this group not only dominated the water column in a wide range of oceanic environments, but that it is also abundant in coastal seas such as the North Sea and to take part in the carbon cycle pathway. Many Gammaproteobacteria were found in association with surfaces in the marine environment. It was reported that the Gammaproteobacteria constituted about one fourth of the bacterial assemblage and contributed to the microbial glucose uptake in late winter and during spring phytoplankton bloom in coastal North Sea waters (5).

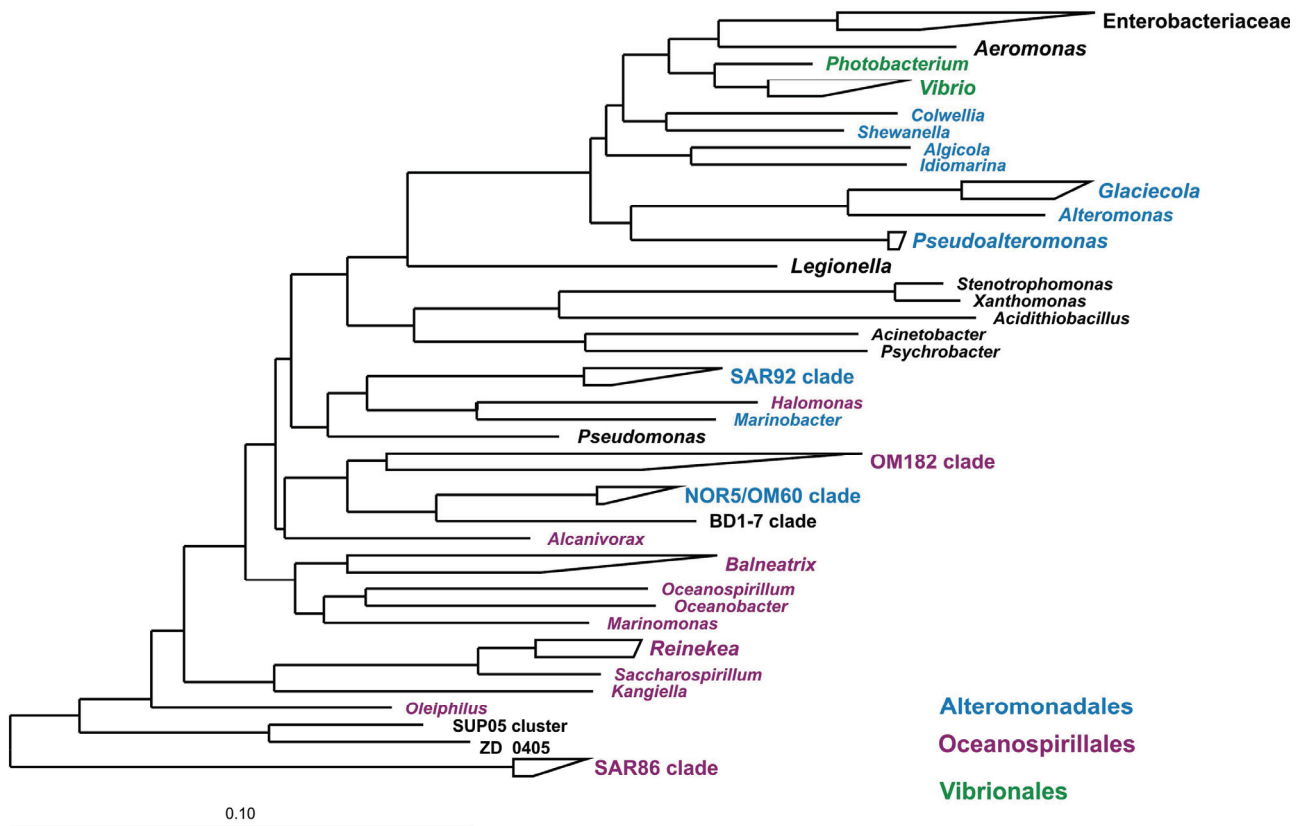


Figure 2 A general phylogenetic RAxML tree based on comparative sequence analysis of 16S rRNA gene showing the major members of Gammaproteobacteria. Each group is represented by few or one species for simplification. The colour coding reflects the SILVA taxonmy of major marine groups. The bar indicates 10% sequence divergence.

Before the advent of culture-independent methods for the investigation of microbial communities, cultivation-dependent approaches restricted our knowledge of microbial diversity since the standard cultivation conditions were highly selective for particular groups of organisms (8), (35). Thus, the knowledge about the diversity of the Gammaproteobacteria was limited to those strains which could be readily cultivated such as representatives of e.g. the genera *Vibrio*, *Photobacterium*, *Alteromonas* or *Pseudoalteromonas*. In the past decade additional Gammaproteobacteria have been cultured by the application of new high throughput cultivation methods (31). Phylogenetically, the majority of the named species of culturable heterotrophic marine bacteria fell within three orders of the Gammaproteobacteria which are paraphyletic: *Alteromonadales*, *Oceanospirillales* and *Vibrionales* (**Figure 2**).

Many marine isolates were initially classified as *Alteromonas*, which encompassed diverse gram-negative aerobic chemoorganotrophic marine bacteria with a single polar flagellum. Although these isolates did not closely match the 16S rRNA gene sequences of characterized strains, indicating that there was much diversity, most isolates matched closely enough to conclude that they belong to this

branching of the evolutionary tree. Thus, studies based on cultivation-based methodologies led to an overestimation of their abundance in the environment due to the apparent ease of their cultivation (54).

Since then the former genus *Alteromonas* has been subdivided into several distinct clades. Today, the genera *Alteromonas*, *Marinobacter*, clades SAR92 and NOR5/OM60 and the newly formed genus *Glaciacola* were encompass the family *Alteromonadaceae*. The genera *Pseudoalteromonas* and *Shewanella* are placed in two further separate but closely related families (*Pseudoalteromonadaceae* and *Shewanellaceae*) which also belonged to the same order of *Alteromonadales*.

Members of the *Oceanospirillum* subcluster have been traditionally distinguished from other easily cultivated species by their unique morphology of helical cells rather than rod-shape. However, this morphology showed to be polyphyletic among marine bacteria. They form several separate lineages belonging to the genera *Oceanospirillum*, *Marinomonas*, *Balneatrix*, *Oceanobacter* and the newly isolated *Reinekea* species. *Oceanospirillum* spp. has been more difficult to isolate than other Gammaproteobacteria and their occurrence in the oceans is still under exploration (54).

Several cultivation studies revealed that sporadically detected gammaproteobacterial 16S rRNA gene clones from seawater are part of a phylogenetically diverse constellation of organisms mainly composed of oligotrophic and ultraoligotrophic lineages that are culturable under specific cultivation conditions. For example, the oligotrophic marine Gammaproteobacteria (OMG) group was isolated from coastal and pelagic regions of the Pacific Ocean, using high-throughput culturing methods that rely on dilution to extinction in very low nutrient media. The isolates belonged to five rRNA clades, all of which contained rRNA gene sequences previously reported from seawater environmental gene clone libraries (SAR92, OM60, OM182, BD1-7, and KI89A) (31). These isolates were physiologically diverse heterotrophs. They are distantly related to the other major marine Gammaproteobacteria lineages, such as the SAR86 cluster, *Vibrio*, *Alteromonas*, and *Oceanospirillum*. Connon and Giovannoni also cultivated several strains of the OM60/241 and SAR92 clades from the coast of Oregon by HTC methods, which allow large numbers of microbial isolates to be recovered by dilution to extinction in natural seawater media (34). In addition, Eilers et al. isolated strain KT71 in the NOR5/OM60 lineage (a part of the OM60 clade) from a coastal region of the North Sea by plating methods using inorganic N and P compounds at concentrations that were reduced by several orders of magnitude (47).

The family *Vibrionaceae* encompasses a phylogenetically related group of bacteria that includes many characterized bacteria of the genera *Vibrio* and *Photobacterium*. These organisms are able to grow both aerobically and in the absence of oxygen using fermentations for energy-yielding metabolism. This family represents one of the most important groups of bacteria in the marine environment. Members of this family were thought to dominate the heterotrophic bacterioplankton

and to interact with other marine organisms in relationships ranging from symbiotic to pathogenic (54).

With the advent of culture-independent approaches and molecular identification based on 16S rRNA gene sequences, the limited knowledge about the diversity of Gammaproteobacteria was extended to include uncultured strains. The widespread cloning of 16S rRNA genes from marine bacterioplankton has resulted in high numbers of sequences in public databases. Unexpectedly, the majority of 16S rRNA genes recovered from marine bacterioplankton populations belonged to novel clades with no close relatives in culture collections (118) showing that many of the most abundant phylogenetic groups of marine bacterioplankton still remain uncultivated (31).

One clade of the Gammaproteobacteria 16S rRNA genes has been repeatedly recovered in culture-independent studies of marine bacterioplankton, the SAR86 clade. This clone cluster was named after one of the first clones recovered from the Atlantic Oceans in the Sargasso Sea and represents a phylogenetically unique clade within the Gammaproteobacteria (50), (81). SAR86-affiliated clones have dominated 16S rRNA gene libraries at Kabeltonne before (46), strikingly emphasizing the limitation of isolation techniques to recover unculturable marine bacteria. Based on 16S rRNA gene sequence comparisons, the SAR86 cluster is clearly unrelated to the cultivated species belonging to the Gammaproteobacteria and it has evolved from a separate clade within the subphylum. A closely related, cultivated relative has not yet been isolated, and all available 16S rRNA genes sequences from cultivated microorganisms are less than 90% similar to full-length gene sequences from members of this cluster.

1.3 Gammaproteobacterial community in the North Sea

Members of the Gammaproteobacteria are considered to represent a large fraction of the marine surface water bacteria that are able to grow and to degrade rapidly the more easily accessible fraction of organic matter (22). Such a role has been assigned to groups which are found abundantly in marine and estuarine environments, such as members of the genus *Rheinheimera* which was isolated from the Baltic Sea (22). The enrichment of members of the readily culturable copiotrophic gammaproteobacterial genera *Vibrio*, *Alteromonas* and *Pseudoalteromonas* in the southern basin of the Lagoon of Venice was also detected (110). Based on ribosomal intergenic spacer analysis (RISA) and denaturing gradient gel electrophoresis (DGGE) analysis the Gammaproteobacteria constituted about one fourth of the bacterial assemblage in the coastal North Sea waters and the succession of specific members within this subphylum was found in strong association with spring phytoplankton bloom (103). Genera such as *Alteromonas*, *Pseudoalteromonas* and *Vibrio* were frequently isolated from the North Sea (46). Despite being readily culturable, they were found to form low density populations in the German Bight (14).

Other genera such as members of the SAR86 clades and the NOR5/OM60 cluster were found in higher densities. Eilers et al. reported that members of the SAR86 cluster constituted a prominent fraction of the bacterioplankton community at station Kabeltonne in the North Sea with up to 10% of the total cells in August 1998 (46), while members of the NOR5/OM60 cluster formed up to 8% of DAPI counts (31) and up to 61% of the Gammaproteobacteria (47). Members of the SAR92 clade were found in high abundances up to 10% of the total bacterial population in coastal surface waters (117). The abundance of this group in the North Sea has not yet been examined. Physiology, genes, and functions known so far about those groups will be discussed below in detail.

1.3.1 *Reinekea*

The genus *Reinekea* (99) belong to the order *Oceanospirillales*. It consists of chemoheterotrophic, aerobic or facultatively anaerobic, motile or non-motile and rod-shaped bacteria. At present, three species belonging to the genus *Reinekea* have been recognized: *Reinekea marinisedimentorum* (99), *Reinekea blandensis* (89) and *Reinekea aestuarii* (33).

A strain of *Reinekea marinisedimentorum*, the type species of the genus, was the first cultured representative for this group. It was isolated from coastal marine sediment. It stands phylogenetically isolated and is remotely related to members of a broad range of taxa comprising *Oceanospirillum*, *Oleiphilus*, *Marinobacter*, *Alcanivorax* and *Vibrionaceae* with less than 90% sequence similarity. The strain is heterotrophic, rod-shaped and motile by single polar flagella. Cells are 0.4–0.5 μm in diameter and 1.5–1.7 μm in length. The strain showed to be facultatively anaerobic and could utilize several carbohydrates and alcohols to form acids under anaerobic and aerobic conditions (99). Another species, *Reinekea blandensis*, was isolated from a seawater sample collected at the Blanes Bay Microbial Observatory in the northwestern Mediterranean Sea. It differs from *R. marinisedimentorum* by being strictly aerobic, utilizing much broader range of sugars, alcohols and amino acids as sole carbon and energy sources. In addition, it is able of hydrolyzing some polymeric compounds such as casein and chitin. A whole genome shotgun sequence was obtained for *R. blandensis* and phylogentic analysis revealed its relatedness to the species *R. marinisedimentorum* and *Saccharospirillum impatiens* (89). Enzymes putatively involved in cell adhesion and polysaccharide degradation have been previously identified in this strain (27). However, the abundance of genes involved in attachment and the range of hydrolytic enzymes is still not well known for that genus and needs to be further investigated.

Two other strains were further isolated from a tidal flat sediment of the Yellow Sea, represented the third species *Reinekea aestuarii* and were differentiated from the type strains of the two recognized *Reinekea* species by several phenotypic properties. Based on 16S rRNA gene sequence comparisons, both isolates were most closely related to *R. blandensis* with 98.7–98.8% 16S rRNA gene sequence

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similarity, *R. marinisedimentorum* with 95.3–95.4% sequence similarity and *Saccharospirillum impatiens* with 93.7–93.8%. In contrast to *R. marinisedimentorum*, it can hydrolyze chitin and gelatin, while the hydrolysis of starch and casein was negative for that group in contrast to *R. blandensis* which indicates the existence of a diverse potential of metabolism for that genus.

1.3.2 SAR92 clade

Sequences of the SAR92 clade were among the first 16S rRNA gene of yet uncultivated marine bacteria to be recovered (23). Members of this clade are organoheterotroph able to degrade sugars and amino acids as carbon and energy sources. In addition, they harbour the proteorhodopsin (PR) protein which is a light-dependent proton pump. The PR protein is creating an additional proton motive force without the cost of oxidizing carbon compounds. This would theoretically lead to a more effective use of organic carbon at limiting concentrations, as it is the case of many marine environments. This makes bacteria bearing such PR protein more competitive in oligotrophic waters where carbon concentrations are scarce. Although the SAR92 clade is such able to grow in low-nutrient conditions, its abundance increases in high nutrient concentrations as previously reported (117). Initial genome sequencing was obtained for HTCC2207, an isolate of this clade from the Oregon coast, and revealed an operon with genes encoding proteins hypothesized to be involved in the biosynthesis of retinal, the chromophore of PR, besides the PR gene (117). No genome has been further sequenced for isolates of that group.

1.3.3 SAR86 clade

The uncultivated SAR86 cluster of the Gammaproteobacteria is not only present in neritic seas such as the North Sea and the Mediterranean but also dominates open oceans such as the Pacific and Atlantic (46), (4). It is one of the most common groups observed in clone libraries made from surface water samples. SAR86 clade cells have a size of around 0.5 μm in width and 1.0 μm in length (45) and have low ribosomal content (87). It was previously linked to extreme oligotrophic environments (123). In an earlier study, SAR86 constituted 8.1% of the total bacterial population but approximately 50% of all DNA-synthesizing bacteria in coastal North Sea surface water in one early autumn sample, thus substantially contributing to the active fraction of the bacterioplankton during a season with generally declining bacterial activity (87). Furthermore, it often peaked as a highly seasonal clade in the summer (123), (1). In another study, 39% of the SAR86 cells were found to contribute to the leucine uptake in the North Sea (1). Different SAR86 subgroups are known to possess divergent types of proteorhodopsins (15), (102), a feature that this clade shares with

members of the SAR92 clade (117) and which supports survival during carbon starvation. However, not much more is known about other genomic features for this clade so far.

1.3.4 OM182 clade

The OM182 clade is a typical example of oligotrophic bacteria. Strains of this clade grow optimally in low-nutrient media. In contrast to the cultivated strains of the SAR92 clade, the growth rate of the OM182 strains is reduced by high DOC concentrations (31). 16S rRNA gene sequences of the OM182 clade were previously found in seawater clone libraries. They were retrieved from different marine environments such as coastal areas, the Arctic Ocean and marine sediments. A previously reconstructed 16S rRNA phylogenetic tree showed that this clade was clearly separated into two independent lineages with 100% bootstrap support, indicating that these two lineages may represent two separate genera within the class Gammaproteobacteria. Although sequence similarities between the two lineages were very low, in the range of 88.3 to 91.0%, they were defined as a single clade due to the high level of bootstrap support (88%) between the two lineages. The OM182 clade comprised 1.3 to 3.7% of total DAPI counts in the Oregon coast throughout the year (31). Not much is known about the abundance of this clade in the North Sea.

1.3.5 NOR5/OM60 clade

The first related strain isolated for this clade was “*Congregibacter litoralis*” strain KT7 from marine surface water at the “Kabeltonne” station (47). Several related strains were isolated by a novel high-throughput culturing method, and were placed in the OM60/OM241 clade (34), which was later referred to as the OM60 clade. Due to the large redundancy of all these clade names both clades were brought together and referred to as the NOR5/OM60 clade (134) which forms a part of the oligotrophic marine Gammaproteobacteria (63). Thirteen subclades were identified for the NOR5/OM60 clade so far, some of which are currently restricted to discrete habitat types. Almost all sequences in the largest subclade NOR5/OM60-1 and related subclade NOR5/OM60-4 originated from marine surface water samples. Overall, most of the NOR5/OM60 sequences were retrieved from marine coastal setting and fewer from open-ocean surface waters, deep-sea sediment, freshwater, saline lakes and soil (134). Based on comparative 16S rRNA sequence analysis, the NOR5/OM6 clade is most closely related to the genera *Endobugula*, *Microbulbifer*, *Teredinibacter* (all *Alteromonadales*), *Cellvibrio* (*Pseudomonadales*) and several other groups of oligotrophic marine Gammaproteobacteria, including the clades BD1-7, KI89A, OM182 and SAR92 (31).

A nearly complete genome sequence was obtained for KT71 strain and unexpectedly revealed a complete photosynthesis superoperon, including genes for accessory pigments. According to the

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genome analysis of that strain, it was identified as the first cultured representative of marine aerobic anoxygenic phototrophic (AAnP) Gammaproteobacteria. Like other AAnPs, KT71 is a photoheterotroph. It is neither able to grow anaerobically nor photoautotrophically. Cultivation experiments and genomic evidence showed that this strain needs organic substrates like carboxylic acids, oligopeptides, or fatty acids for growth, so it is chemo-organotrophic (49), (114).

Primarily, AAnP bacteria appear to play an important role in marine carbon cycling since they are able to metabolize organic carbon and they can fulfill part of their energy requirement by photosynthetic light utilization (67). However, genome analyses of another strain of the NOR5/OM60 clade, HTCC2148, a member of the NOR5/OM60-8 subclade, showed the absence of photosynthetic gene clusters in the genome, indicating that not all members of the NOR5/OM60 clade are AAnPs (122).

Another strain, IMCC3088 strain, was recently isolated from a surface seawater sample collected from the Yellow Sea off the coast of Incheon, South Korea and belonged to the NOR5/OM60-4 clade. In contrast to *C. litoralis* KT71 and HTCC2080, there are no predicted *puf* genes, which are characterizing the AAnP bacteria, in the genome of IMCC3088. Instead, the IMCC3088 genome was recently predicted to encode proteorhodopsin, which is commonly found in SAR92 and SAR86 clades, followed by the gene clusters for the biosynthesis of the retinal chromophore of rhodopsin (*crtEIBY*), suggesting that proteorhodopsin is functional. Herewith, it could be the first bacterium that harbors proteorhodopsin among the members of the NOR5/OM60 clade, confirming the potential of metabolic diversity among the members of this marine clade (63).

1.3.6 *Balneatrix*-related clade

A marine gammaproteobacterium HTCC120 [AY386340] has been previously identified (116) which showed a quite distant relationship with the closest cultured reference strain *Balnetarix alpica*. This bacterium was firstly reported as being associated with a case of pneumonia and meningitis in a spa therapy center in southern France in year 1987 (36). This bacterium constitutes the single species discovered so far for this genus, which is close to *Oceanospirillum*. Its pathogenicity seems to be weak but it could be enhanced by inhalation of concentrated steams by susceptible individuals (83). *Balneatrix alpica* is strictly aerobic, having the shape of straight or curved rods and a size of 0.5 μm to 0.7 μm by 2.8 to 5 μm . It can utilize some sugars, alcohols and organic acids (36). So far, nothing is known about the distribution of this group in marine environment.

1.3.7 *Glaciecola*

The genus *Glaciecola* was originally described to accommodate aerobic, psychrophilic, halophilic bacteria and initially comprised two species, *Glaciecola punicea* and *Glaciecola pallidula*. Both species were isolated from sea-ice diatom assemblage samples collected from coastal areas of eastern Antarctica (29) and seemed to be restricted to sea-ice habitats (100). Since then, several other species have been further isolated from marine environments and described within the genus of *Glaciecola*: *Glaciecola mesophila* (100) from marine invertebrates, *G. polaris* (Van Trappen et al., 2004) from Arctic Ocean seawater, *G. nitratireducens* (Baik et al., 2006) from coastal surface seawater, *G. chathamensis* (Matsuyama et al., 2006) from Pacific Ocean floor sediment, *G. psychrophila* (Zhang et al., 2006) and *G. agarilytica* (Yong et al., 2007) from East Sea sediments (29). The genus *Glaciecola* forms a separate lineage within the Gammaproteobacteria which is phylogenetically closely related to *Alteromonas macleodii* (100). Species belonging to this genus show diverse physiological and biochemical properties, such as psychrophily (*G. punicea*, *G. pallidula* and *G. psychrophila*), agar digestion (*G. mesophila* and *G. agarilytica*), nitrate reduction (*G. nitratireducens*) and polysaccharide formation (*G. chathamensis*). Their wide distribution in marine environments and the diversity of physiological properties suggest that they may play important roles in processes within marine ecosystems such as the degradation of polysaccharides and biogeochemical cycles of elements such as carbon and nitrogen.

1.3.8 *Vibrio* and *Pseudoalteromonas*

In marine surface waters *Vibrio* spp. are among one of the most commonly isolated microorganisms. Over thirty *Vibrio* species have been validly described, one third of which are known to be pathogenic for humans. The halophilic *Vibrio* species, *V. parahaemolyticus*, *V. alginolyticus* and *V. vulnificus* are known to be involved in intestinal as well as in extra-intestinal human infections. *V. parahaemolyticus* and *V. alginolyticus* strains were previously isolated from seawater samples and from inshore water collected along the Dutch, the Belgian and the British coasts and their occurrence showed a seasonal effect (125). Members of this genus show a broad spectrum of microbial activities and seem to exhibit a surface-associated life style (93), (94). For instance, species *Vibrio cholerae*, *V. vulnificus* and *V. parahaemolyticus* showed the ability to acquire virulence genes by the uptake of extracellular DNA in the presence of the polysaccharide chitin (16). It was earlier reported that 50% of the *Vibrio* strains isolated from a coastal lagoon are able to use chitin as a sole carbon source (51).

At the present time the genus *Pseudoalteromonas* contains 27 validly described species including reclassified former *Alteromonas* species and recently described *Pseudoalteromonas* species such as *P. antarctica* (21), *P. prydzensis* (19), *P. bacteriolytica* (105), *P. tunicate* (29), *P. peptidolytica* (126) and *P. ulvae* (43). On the basis of the phylogenetic study of Gauthier et al. (1995),

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Pseudoalteromonas haloplanktis ATCC 14393T was described as a type strain and type species of this genus (53). Bacteria of the genus *Pseudoalteromonas* are widespread inhabitants of marine environments (41). They have been isolated from diverse marine sources from various geographical regions of coastal and open water areas (41). *Pseudoalteromonas* strains are aerobic, straight or slightly curved rod-shaped bacteria (0.2–1.5 μm \times 1.8–4.0 μm) and most of them are motile by means of a single unsheathed polar flagellum. They utilize a variety of organic compounds as sole sources of carbon and energy, including carbohydrates, monocarboxylic acids and amino acids. Utilization of carbon sources varies between the different species and for the strains within some species. The majority of *Pseudoalteromonas* species is characterized by high hydrolytic activities. Many strains can utilize D-glucose as a sole carbon source. This newly created genus has attracted significant interest. This is firstly because *Pseudoalteromonas* species are frequently found in association with eukaryotic hosts in the marine environment such as sponges, mussels, pufferfish and a range of algae. Secondly, because the production of biologically active metabolites targeting a range of organisms by many of the species appears to be a unique characteristic for this genus and seem to be greatly aiding *Pseudoalteromonas* cells in their competition for nutrients and colonization of surfaces (60).

The closely related genus *Colwellia* was proposed by Deming et al. (1988) for curved or straight rod-shaped (0.5–1.0 μm in diameter and 2.5–5.0 μm in length), motile by polar flagella, chemoorganotrophic, microorganisms, associated with deep marine environments and often with diatom algae assemblages (20). *Colwellia psychrerythraea* was described as the type species of this genus (38). Currently the genus *Colwellia* encompasses species *Colwellia psychrerythraea*, *C. hadaliensis* (38), *C. demingiae*, *C. rossensis*, *C. hornerae*, *C. psychrotropica* (20) and *C. maris* (136). *Colwellia* strains have been isolated from organic materials and coastal marine water samples collected offshore areas (37), (120), Antarctic sea ice and water samples (20) and cold coastal waters near Hokkaido, the Sea of Japan (136). Thus, they are common inhabitants of the cold sea environments and have not been isolated from any other environments. *Colwellia* seems to be a psychrophilic genus and all known strains reduce nitrate to nitrite as well as they can degrade chitin and starch under aerobic conditions. Carbohydrate oxidation, fermentation of *N*-acetylglucosamine, chitin and D-glucose, and hydrolysis of other substrates vary between *Colwellia* strains (41).

The genera *Vibrio*, *Pseudoalteromonas* and *Colwellia* are readily culturable marine coastal bacteria. They are copiotrophic, such dwelling under nutrient-rich conditions, in contrast to the OMG group (110). They can grow rapidly at high substrate concentrations with high cellular rRNA contents. However, these bacteria can also survive carbon starvation for extended periods of time using the strategy of “feast and famine”. This is because they can maintain their ribosomal content for several days in large excess over the apparent demand for protein synthesis upon the onset of carbon starvation (46). These bacteria are known to resist nutrient deprivation for long periods of time and to regain active metabolism quite rapidly. Members of both genera typically form larger cells than

the oligotrophic marine isolates and are particle attached where the particles are sites of higher nutrient availability (46). This in addition to the large size and high ribosome content of the cells detected could be an indication of recent metabolic activity (46). Both genera were once claimed to be major components of the bacterial flora of the sea and to account for a relatively high abundance of the bacterial community in surface waters (46). However, FISH results later showed their rare presence as part of the North Sea bacterioplankton community (46). It was previously suggested that their rapid growth is counteracted by their higher grazing mortality which explains their low abundances, despite of being actively growing in their respective habitats (14).

1.4 Aims and motivation of this study

This doctoral thesis was performed in the context of the interdisciplinary joint BMBF (Bundesministerium für Bildung und Forschung) project MIMAS (Microbial Interactions in Marine Systems). This 3-years project started on October 1st, 2008 and was conducted with the following goals:

- > Development of new molecular and genomic techniques for metagenomic, -transcriptomic and proteomic analysis of marine microbial assemblages and their validation.
- > Combination of the developed metagenomic, metatranscriptomic and metaproteomic approaches for comprehensive, cultivation-independent determination of the composition and function of marine microbial communities.
- > Comprehensive analysis of the genomic diversity and activity of marine microorganisms.
- > Strict monitoring of physico-chemical parameters to track the effect of climate changes and other environmental influences (e.g. seasonality) on metabolic cycles and community dynamics.
- > Integration of microbial diversity studies with metagenomic, -transcriptomic, -proteomic data as well as physico-chemical parameters and interpretation with the final aim of better understanding the microbial complexity in marine ecosystems and to monitor the seasonal impact on such system.

Within this framework, an in-depth investigation of the marine bacterioplankton diversity at the Long Term Ecological Research (LTER) – Station, Kabeltonne, Helgoland was conducted in the winter-spring transition phase for two consecutive years 2009 and 2010. Previously established molecular techniques, such as 16S rRNA gene clone library construction and CARD-FISH were used to strictly monitor the community dynamics of dominant groups and to deduce a link between identity and role of the chosen groups at particular time points (blooming versus non-blooming phase).

Gammaproteobacteria was chosen as the group of interest for this doctoral thesis since this subphylum comprises a major fraction of the prokaryotic community in the coastal North Sea (46), (1). Furthermore, there were indications for a strong association with phytoplankton blooms (5) (103).

This shaped the following specific goals for this thesis:

- 1) The construction and comparative sequence analysis of two large 16S rRNA gene clone libraries with almost full-length sequences. Around 900 independent clones should be analyzed from each library, one representing a late winter setting (February 2009) and another after the first pronounced spring algal bloom (April 2009).
- 2) Construction of a probe set. This is based on the sequence information obtained on Gammaproteobacteria from these two libraries and 16S rRNA gene tags information obtained from the doctoral thesis of Anna Klindworth, Microbial Genomics Department, Max Planck Institute for marine Microbiology, Bremen, Germany.
- 3) Subsequently, the novel probes and previously designed probes would be applied to the winter and spring samples of the years 2009 and 2010 in order to quantify the abundances of the various gammaproteobacterial subgroups in the context of various environmental parameters.

Thus, this study formed an important part to a larger seasonal diversity analysis of North Sea bacterioplankton community with the specific aim to reveal the complexity of the marine prokaryotic system and the effect of seasonal changes on the cellular abundances of specific gammaproteobacterial clades.

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2.1 Study site, sample procedure and biomass archiving

Surface water samples were taken once weekly from a depth of 1 m from February to May 2009 at Helgoland Roads (54° 11.3'N, 7°54.0'E), North Sea by the motor boat Aade. Water samples were collected in water-rinsed containers and immediately transferred to the laboratory. The sampling period covered the change of seasons from winter to spring, and included a phytoplankton bloom with a peak of (28 mg m⁻³) consisting mainly of centric diatoms on 23rd March 2009.

For DNA analysis, 100 L water sample were first passed through 10 µm pore size polycarbonate filters (type TCTP, 142 mm diameter, Millipore, Eschborn, Germany) and 3 µm filters (type TSTP, 142 mm diameter, Millipore, Eschborn, Germany) in succession to remove most of the eukaryotes. The filtrate was subsequently filtered onto 0.2 µm polyethersulfon membrane filters (GPWP, 142 mm diameter, Millipore, Eschborn, Germany). Filters were immediately frozen, and kept at -80 °C until processed for nucleic acid extraction. The same sampling procedure was repeated from February to July in the year 2010. However, sampling has not taken place during the month of June.

2.2 Measurement of physico-chemical and biological parameters

Temperature and salinity were measured as part of the ongoing long-term observations of the Biological Station Helgoland on a daily basis (131). Salinity was determined using an inductive salinometer (GDTAutosal8400B Salinometer, Guildline, Ontario, Canada) followed by conversion to a salinity value using UNESCO table (58). In order to monitor the concentration of nutrients, silicate, phosphate, nitrite, nitrate, ammonium and dissolved inorganic nitrogen were measured photometrically as previously described (58). In parallel, the chlorophyll content was also determined photometrically. For the enumeration of phytoplankton, samples were preserved with Lugol's solution and counted using the Uthermöhl method and an inverted microscope (131).

The species lists and quality control used were described in Wiltshire and Dürselen 2004. Permanent slides have been prepared approximately every month and when a new species has been identified. Over 360 phytoplankton species are listed for Helgoland, which is regularly updated and taxonomic images are stored in an open-access taxonomic database Plankton* Net [<http://planktonnet.awi.de>]. Counts of diatoms (Bacillariophyceae) and dinoflagellates (Dinophyceae) were taken into account for this study since they have been the most reliable algal counts (48).

2.3 Study of bacterial diversity and seasonal succession

2.3.1 DNA extraction and 16S rRNA gene amplification

Total nucleic acids were extracted from 0.2 μm pore size polyethersulfone membrane filters (type GPWP, 142 mm diameter; Millipore, Eschborn, Germany) using the SDS-based DNA extraction method according to Zhou et al. (1996) (137). For cell lysis, sections of half of the filters were incubated with 13.5 ml extraction buffer (100 mM Tris-HCl (pH 8.0), 100 mM EDTA (pH 8.0), 100 mM Na-Phosphate (pH 8.0), 1.5 M NaCl and 1% CTAB [Hexadecyltrimethylammonium-bromide] to remove protein and polysaccharide complexes) and 50 μl 20 mg ml^{-1} Proteinase K for 30 min at 37 °C on a shaker. 1.5 ml 20% SDS was added to the filtrate and further incubated for 1-2 h at 65°C under mild shaking. The DNA extract was centrifuged at 6000 x g for 10 min at RT. The supernatant was transferred to a fresh 50 ml tube. In order to break more resistant cell walls, the filter sections were again incubated with 6.75 ml extraction buffer and 25 μl 20 mg ml^{-1} Proteinase K for 15 min at 37°C. 0.75 ml 20% SDS was then added to the DNA extract and further incubated at 65°C for 30 min the centrifuged as mentioned before. For DNA purification, supernatants of all extractions were combined together and an equal volume of chloroform/isoamylalcohol was added, mixed thoroughly by careful shaking, and centrifuged at 10.000 x g for 10 min. The aqueous upper phase was collected and transferred to a new tube. The chloroform/isoamylalcohol extraction step was repeated and all aqueous phases were combined together. DNA was precipitated by addition of 0.6 volume isopropanol and incubation at RT for 1 h. The extract was centrifuged at 50.000 x g and at RT for 30 - 45 min. Supernatant was decanted and the pellet was washed by addition of 10 ml cold 70% (v/v) ethanol, centrifugation at 16.000 x g and at RT for 10 min and careful decantation of the supernatant. The pellet was air dried for about 15 min at RT and resuspended in 100 μl 1x TE overnight and stored at 4°C. The concentration of the environmental genomic DNA was checked on a 1% agarose gel with 2 μl , 4 μl and 8 μl of 25 ng μl^{-1} lambda/Hind III fragments and by using the nanodrop.

The 16S rRNA genes were amplified by PCR (polymerase chain reaction) using the general bacterial primers GM3 (5'-AGA GTT TGA TCM TGGC-3') and GM4 (5'-TAC CTT GTT ACG ACTT3') (82). In parallel, the PCR amplification was done using a modified GM3 forward primer (5'-AGA GTT TGA **TYM** TGG **CTC** AG-3') (24) and the usual reverse primer GM4. The 50 μl PCR reactions contained 0.3 mg ml^{-1} BSA, 250 μM total dNTPs, 1x Taq buffer in addition to 0.5 μM of each primer, and 0.2 U of Master Taq Polymerase (5 PRIME GmbH, Hamburg, Germany). The amplification was carried out with a Thermocycler Mastercycler (Eppendorf) as follows: an initial denaturation step at 95 °C for 10 min, followed by 20 cycles

of 1 min denaturation at 95 °C, 1.5 min annealing at 48 °C, and 2 min elongation at 72 °C. There was a final extension step of 15 min at 72 °C.

2.3.2 Clone library generation, sequencing and phylogenetic analysis

In order to concentrate the PCR product yield, the products were pooled together and speed vacuumed to evaporate water till about 40 µl were obtained. To avoid cloning of small and non-specific PCR products, fragments of the correct size of 1.5 kb were excised from 1% agarose gel, and subsequently extracted and purified using the QIAquick Gel Extraction Kit (QIAGEN, Hilden, Germany). Cleaned PCR products were cloned into the pCR4 TOPO vector (Invitrogen, Groningen, the Netherlands), according to the manufacturers' instructions. The recombinant vector was transformed into chemically competent top 10 *E. coli* cells, (Invitrogen, Groningen, the Netherlands) by heat-shock, plated on selective LB plates containing 100 µg ml⁻¹ Ampicillin, 0.004% X-Gal and 2.0 mM IPTG for blue white screening and incubated overnight at 37 °C then stored at 4 °C. White colonies were picked from each library for analysis in 100 µl liquid LB medium containing 100 µg ml⁻¹ Ampicillin using 96-well plates in duplicates. Backup plates were prepared for long-term storage. The liquid cultures were incubated overnight at 37 °C on a rotary shaker then stored at 4°C, while 20% glycerol was added to the backup plates which were stored at -80°C. The clones were screened for the presence of the inserts which were amplified by PCR using vector primers M13F (5`-GTAAAACGACGGCCAG-3`) and M13R (5'-CAGGAAACAGCTATGAC-3'). A fresh overnight culture was prepared from 500 of those clones containing inserts, from each library, stabbed in LB 96-well plates containing 100 µg ml⁻¹ Ampicillin and sent to GATC Biotech Company for full sequencing, applying Sanger sequencing technology. Forward and their complementary reverse sequences obtained from the company were automatically assembled with a minimum overlap of 25 bases and 80% identity in a batch assembly, using the software DNA baser sequence assembly software (Heracle BioSoft S.R.L., Pitesti, Romania). The ambiguities in the resulting contigs were automatically corrected. Low quality regions as well as the vector sequences were automatically detected and trimmed by the same software. The ends were trimmed until there are more than 75% good bases in a 18-bases window. The contigs encompassed sequences with around 1400 nucleotides.

Phylogenetic analysis was performed using the ARB software package [version 5.2] (71) using SILVA database release 102 [SSU Ref 102]. All the approximately full-length 16S rRNA gene sequences were aligned in ARB using the SINA (SILVA incremental aligner) (92) and the alignment was subsequently improved manually considering the secondary structure of the rRNA molecule. Aligned sequences were further refined manually by comparison of closest

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relatives and were added to the tree by ARB Parsimony method using (positional_variability_Bacteria_102) filter without allowing changes in the overall tree topology. Phylogenetic trees were reconstructed based on the maximum likelihood algorithm RaxML (115).

Operational taxonomic units (OTUs) and the richness estimator indexes (Shannon-Weaver Index (70), Simpson Index (59) and boot richness estimator (112) were calculated with DOTUR [Distance-Based OUT and Richness] (108) and MOTHUR and the newer more expandable software (version 1.2.0.3) [<http://www.mothur.org/wiki>]. The furthest neighbour algorithm was used for each distance level from a PHYLIP distance matrix generated in ARB software. Then, 97% and 93% sequences identity were selected to generate the rarefaction curves.

2.3.3 Catalyzed reporter deposition-Fluorescence In Situ Hybridization (CARD-FISH) and cell counting

For CARD-FISH, 10 and 100 ml aliquots were collected twice weekly and fixed with paraformaldehyde or formaldehyde for 1 h at 4 °C (1% final concentration). The fixed samples were directly filtered onto 0.2 µm pore size polycarbonate membrane filters (type GTTP, 47 mm diameter; Millipore, Eschborn, Germany) and stored at - 20 °C.

Prior to CARD-FISH, filters were cut in sections and fixed cells were immobilized by embedding in 0.2% (w/v) agarose (gel strength ≥ 300 g cm⁻², Biozym, Oldendorf, Germany) (85) and they were subsequently permeabilized by treatment with 10 mg ml⁻¹ lysozyme (Fluka, Steinheim, Germany) in 50 mM EDTA, 100 mM Tris/HCl for 1 h at 37 °C. Endogenous peroxidases were inactivated by using 0.01 M HCl for 20 min at RT. Filter sections were hybridized with horseradish peroxidase (HRP)-labelled oligonucleotide probes (Biomers, Ulm, Germany), listed in **Table 1**, and tyramide signal amplification was carried out according to the protocol by Pernthaler and colleagues (86). The probe-delivered horseradish peroxidase was detected with tyramides labeled with fluorescein (86). The general probe mixtrue targeting almost all members of Bacteria (EUB338 I-III, 35% FA, [24]) was used as positive control and the antisense probe NON3338 (127) as negative control.

Accordingly, filter sections were hybridized with the previously mentioned probes (50 ng DNA µl⁻¹ for 2 h at 46°C, where each probe was mixed with the hybridization buffer (see appendix) containing the corresponding % FA in a ratio 1:300. Filter sections were then removed from the hybridization mixture and incubated in 50 ml of prewarmed washing buffer (15 mM EDTA, 20 mM Tris-HCl, x M NaCl [depending on probe, see appendix], 0.01%

w/v SDS) for 10 min at 48°C. In order to equilibrate the probe-delivered HRP, filter sections were placed in 1x PBS for 15 min at RT, then dabbed onto blotting paper in order to remove excess buffer. Then they were immediately transferred to the amplification mixture which consisted of amplification buffer, 100x H₂O₂ and tyramide-fluorescein in a ratio 1000:10:1, respectively and incubated for 45 min at 46°C in the dark.

Probe	Target organism	Probe sequence 5'- 3'	FA [%]	Reference
SAR92-627	SAR92 clade	CAGACAGTTCTAACTGCAGTTCC	20	(117)
SAR86-1245	SAR86 clade	TTAGCGTCCGTCTGTAT	35	(138)
ALT1413	<i>Alteromonas/Glaciecola</i> clade	TTTGCATCCCCTCCCAT	40	(46)
PSA184	<i>Pseudoalteromonas,</i> <i>Colwellia</i>	CCCCTTTGGTCCGTAGAC	30	(46)
NOR5-730	NOR5/OM60 clade	TCGAGCCAGGAGGCCGCC	50	(47), (134)
GV841	family Vibrionaceae	AGGCCACAACCTCCAAGTAG	30	(56)

Table 1 List of probes used in this study and the formamide concentration (v/v) used in CARD-FISH hybridization buffer

After amplification, filter sections were then briefly placed on blotting paper to remove excess tyramide-fluorescein then washed once in 1x PBS for 10 min, once in water and twice in 96% ethanol for 1 min each to decrease background fluorescence before drying. Then they were counterstained with 1 µg ml⁻¹ DAPI [4', 6-diamidino-2-phenylindole] for 3-5 min, washed with Milli-Q water and 96% EtOH for 1 min respectively, then air dried and mounted in a 4:1 mixture of Citifluor (Citifluor Ltd, London, U.K) and the antifading reagent Vecta Shield (Vector Laboratories, Inc., Burlingame, CA,USA). Slides were stored at -20 °C until microscopic analysis. Cells were quantified with an Axioplan II Imaging epifluorescence microscope (Zeiss, Jena, Germany), fitted with 10x eyepieces and 100x (oil immersion) objectives. To determine the relative abundance, about 1000 DAPI cells were counted in variable areas of each filter section using 2 rows of the counting grid and CARD FISH signals of the corresponding grid was counted afterwards. If the cell density was higher than 100 cells per row, only one row per grid was counted till reaching 1000 DAPI cells, such decreasing the counting error.

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To determine total cell count per one ml of filtered volume, the average cell numbers counted per grid was multiplied by the dilution factor [1.11111] and the microscope factor [74506], which represents the ratio of the effective filter tower area [1164156428 μm^2] to the grid area [15625 mm^2], and divided by the filtered water volume according to **Equation 1**. Total cell counts were obtained by multiplying the percentage of relative abundance of target cells with the total cells counts of the corresponding date.

$$\text{TCC} = [\text{number of cells per grid} \times 1,11111 \times 74506 / \text{water volume}]$$

Equation 1 Calculation of Total cell numbers

2.3.4 Probe design and optimization of hybridization conditions

Oligonucleotide probes were designed based on 16S rRNA database release 102 (92) using the probe design tool of the ARB software. Probe specificities were checked *in silico* against the same database containing the full sequences of both winter and spring clone libraries using the probe match tool of the same software. The criteria were high coverage of the target group, and as few hits to organisms outside the target group as possible (see appendix). To optimize the stringency conditions, a series of hybridizations at increasing formamide concentrations (10–50%) were evaluated. For those probes showing signals, the optimal formamide concentration is the highest concentration before signal intensity sharply decreases (Table 2). Melting curves were constructed using ImageJ software [<http://rsbweb.nih.gov/ij/>].

Probe	Target organism	Probe sequence 5'- 3'	FA [%]	Reference
Rei731	<i>Reinekea</i>	TATCAGCCCAGCAAGTCG	20	this study
Bal731	<i>Balneatrix</i> - related clade	TATCAAGCCAGGGCGTCG	25	this study
OM182/707	OM182 clade	CACCGGTATTCCTCAGAA	15	this study
Glac227	<i>Glaciecola</i>	AATCTCGCTTAGGCCACT	30	this study

Table 2 List of probes designed in this study and the optimal FA concentration (v/v) used in CARD-FISH hybridization buffer.

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3.1 Physico-chemical and biological parameters

The winter period in year 2009 was characterized by temperatures around 4°C and low concentrations of chlorophyll, below 2 µg l⁻¹. This scenario changed drastically by beginning of the spring in mid March when the temperature started gradually to increase up to 12°C by end of May and the chlorophyll content increased drastically showing a peak on the 23rd March [28 µg l⁻¹]. It decreased then steadily down to 0.4 µg l⁻¹ by end of April (**Figure 3**). **This tightly followed** a sudden water salinity decrease from 33 to 29 PSU on 20th March. Two further salinity decreases were observed on 22nd April and 11th May dropping from 32 down to 30.5 and 29.5 PSU respectively.

Figure 4 shows the nutrient trends for phosphate, silicate, nitrite and nitrate concentrations. Generally, they decreased substantially after the chlorophyll peak. While phosphate ranged between 0.14 µmol l⁻¹ and 0.6 µmol l⁻¹ in the winter; it was not detectable in the first week after the algal peak then it started to increase to not more than 0.2 µmol l⁻¹. Silicate concentrations were between 2.5 µmol l⁻¹ and 6 µmol l⁻¹ in the winter. A sudden peak of 15 µmol l⁻¹ was detected a couple of days just before the blooming peak. Then no silicate was detected in the first week after the bloom and thereafter values not more than 2.6 µmol l⁻¹ were recorded. Nitrite concentrations were around 1 µmol l⁻¹ in the winter, decreased steadily down to 0.4 µmol l⁻¹ till mid March. They increased then shortly to 0.7 µmol l⁻¹ just before the algal peak then decreased again steadily down to 0.2 µmol l⁻¹ with some exceptional fluctuations. Concentrations of nitrate were below 11 µmol l⁻¹ till the beginning of the bloom. A pronounced peak at 44 µmol l⁻¹ was observed before the chlorophyll peak. Thereafter, concentrations decreased steadily down to 5 µmol l⁻¹ in the following 3 weeks. From end of April until May nitrate had two further small peaks reaching a maximum of 18 µmol l⁻¹ and tightly coupled to the salinity declining peaks (**Figure 4**).

Total bacterioplankton abundances, as determined by DAPI counting, were around 4 x 10⁵ cells ml⁻¹ in the winter, but increased about 6 fold after the development of the phytoplankton bloom within one month to reach a peak of 2.6 x 10⁶ cells ml⁻¹ on the 28th April (**Figure 5**).

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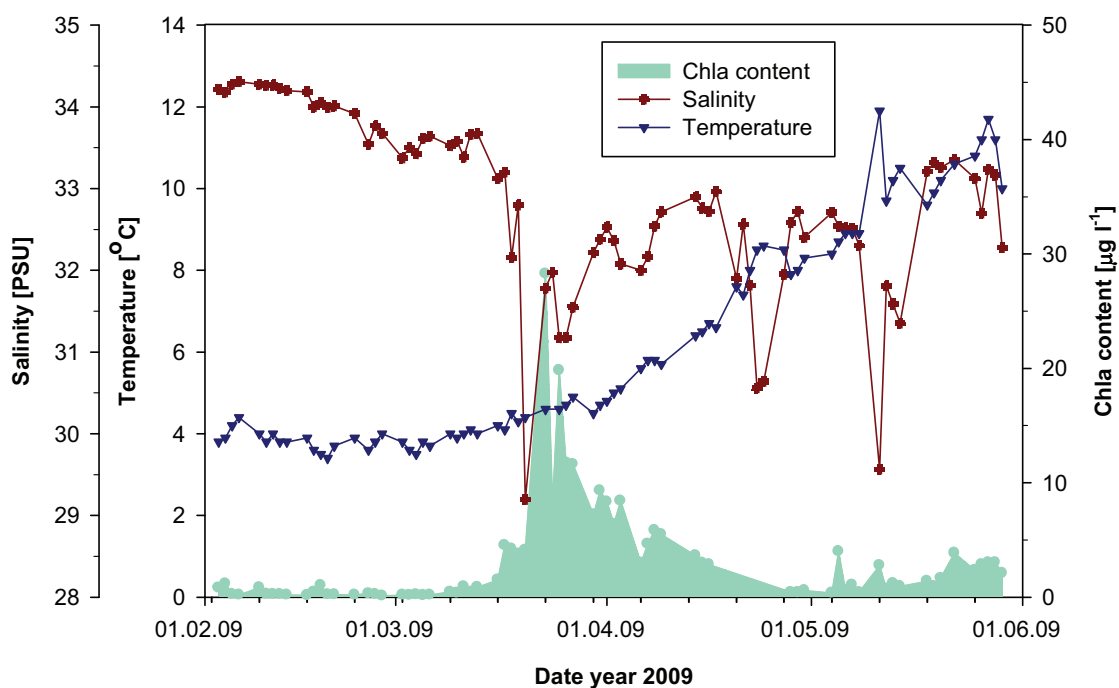


Figure 3 Distribution of temperature, salinity and chlorophyll a (Chla) during the winter-spring transition in year 2009 (daily physico-chemical measurements done by the Biological Station Helgoland, <http://www.pangaea.de/>).

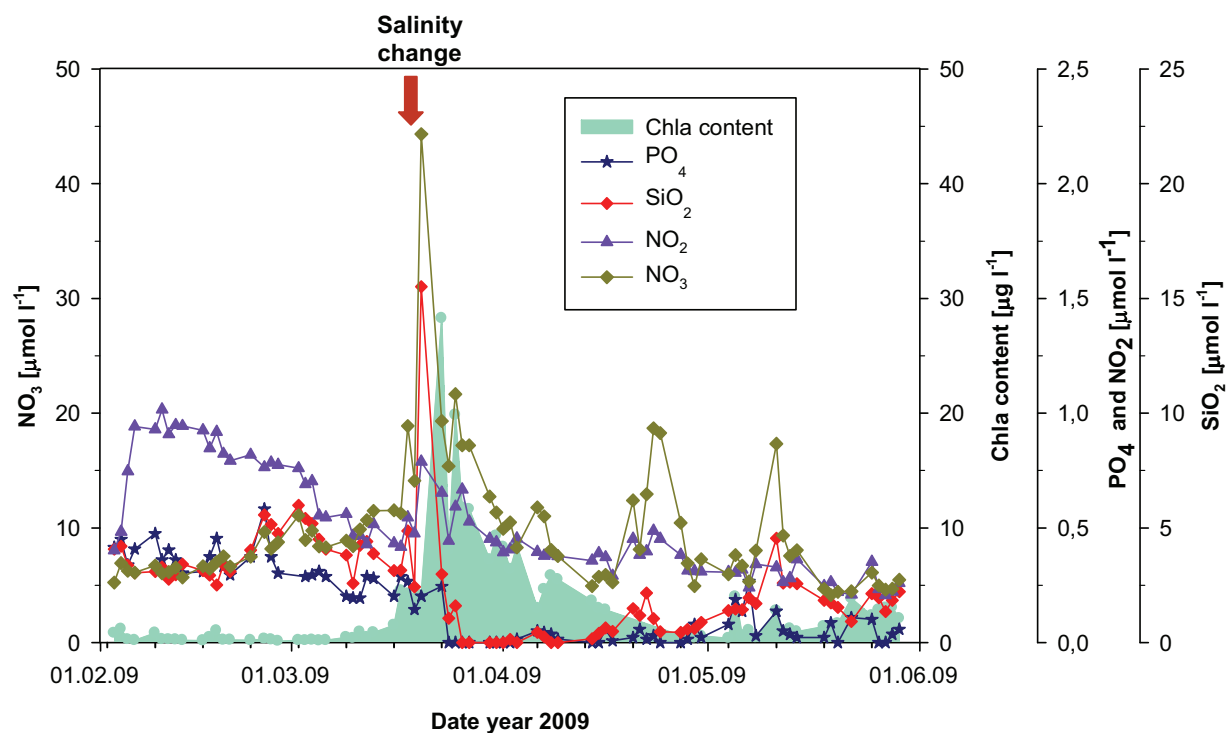


Figure 4 Distribution of phosphate (PO_4), silicate (SiO_2), nitrite (NO_2) and nitrate (NO_3) versus salinity during the winter-spring transition in year 2009 (daily physico-chemical measurements done by the Biological Station Helgoland, <http://www.pangaea.de/>).

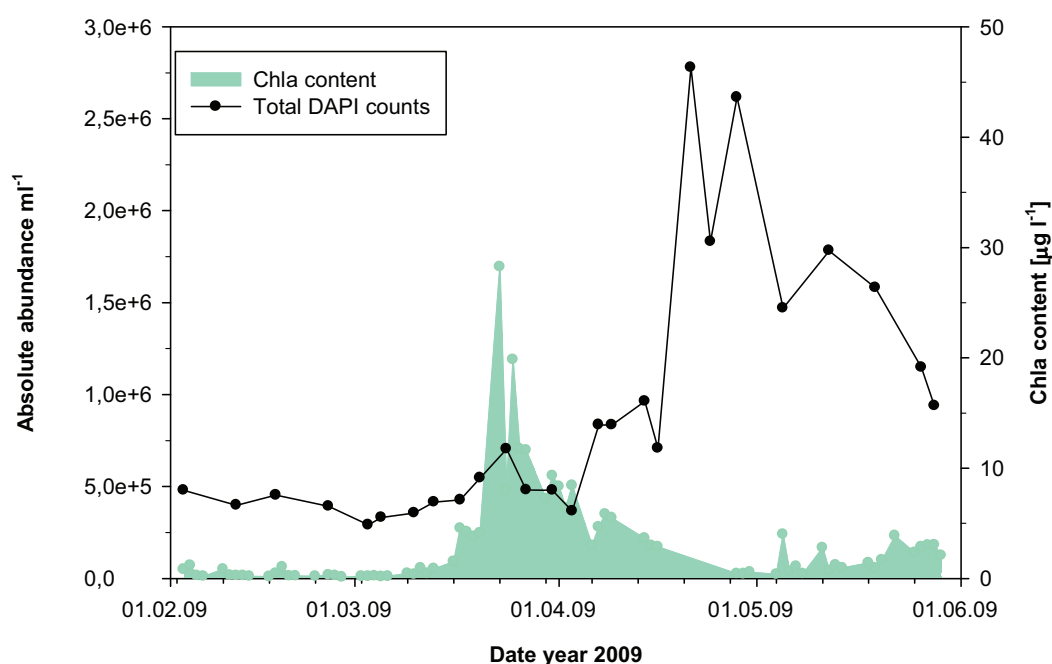


Figure 5 Total DAPI counts in the North Sea at Helgoland Kabeltonne in year 2009.

Winter and spring sampling dates for DNA analysis were chosen according to temperature, salinity and chlorophyll content profiles. The winter sample was taken on 11th February 2009, when temperature was around 4°C, and chlorophyll content was below 2 µg l⁻¹. The spring sample was taken on 14th April, about two weeks after the chlorophyll peak to catch the spring diatom bloom. Temperature was 6.4 °C and the chlorophyll content was 3.6 µg l⁻¹ on that day.

In the year 2010 the water temperature ranged between 1°C and 3°C in the winter (**Figure 6**). By mid of March it increased constantly up to 10 °C. The salinity values ranged from 34 to 32 PSU from February till mid April interrupted by a sudden decline from 33 to 31 PSU on 9th March. Lower values varying between 32 and 30 PSU were recorded from mid of April till end of May. In the meantime, another sudden decline from 31.4 to 28.5 PSU was detected from 4th to 6th of May. The chlorophyll content was below 1 µg l⁻¹ until mid March, and then increased ranging between 1 and 11µg l⁻¹ till 22nd of April when a sudden peak with 45 µg l⁻¹ was detected. It decreased drastically afterwards, but then another smaller peak of 17 µg l⁻¹ was shown on 5th May.

Phosphate concentrations ranged between 0.5 and 1 µmol l⁻¹ from February until mid March (**Figure 7**). It showed a further increasing trend after the first salinity decline and a pronounced peak of 2.4 µmol l⁻¹ was observed at the beginning of April, three weeks before the chlorophyll peak. Then the concentrations drastically decreased reaching zero values with some increasing fluctuations not exceeding 0.7 µmol l⁻¹. The concentration of silicate varied between 8 and 13 µmol l⁻¹. A peak of 21 µmol l⁻¹ coupled the first salinity decline. Then

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values decreased down to zero and hardly increased above $2 \mu\text{mol l}^{-1}$. Nitrite showed generally a decreasing trend from around 1.5 down to $0.9 \mu\text{mol l}^{-1}$ at the beginning of March. This was altered into an increase up to $1.3 \mu\text{mol l}^{-1}$, coupling the first salinity decline. Afterwards it generally continued decreasing down to $0.2 \mu\text{mol l}^{-1}$ till end of May. Nitrate concentrations were mostly between 10 and $17 \mu\text{mol l}^{-1}$ until the first salinity decrease. This is when they increased to a maximum of $30 \mu\text{mol l}^{-1}$ before they dropped down to $8 \mu\text{mol l}^{-1}$ by mid April when another increase was observed but it did not go above $25 \mu\text{mol l}^{-1}$. Afterwards the values just steadily decreased down to $8 \mu\text{mol l}^{-1}$.

Bacterial cell counts ranged from 2 to 4×10^5 cells ml^{-1} in the winter. It increased up to 5×10^5 cells ml^{-1} before the algal peak. Then the increase continued reaching up to 2×10^6 by end of May (**Figure 8**).

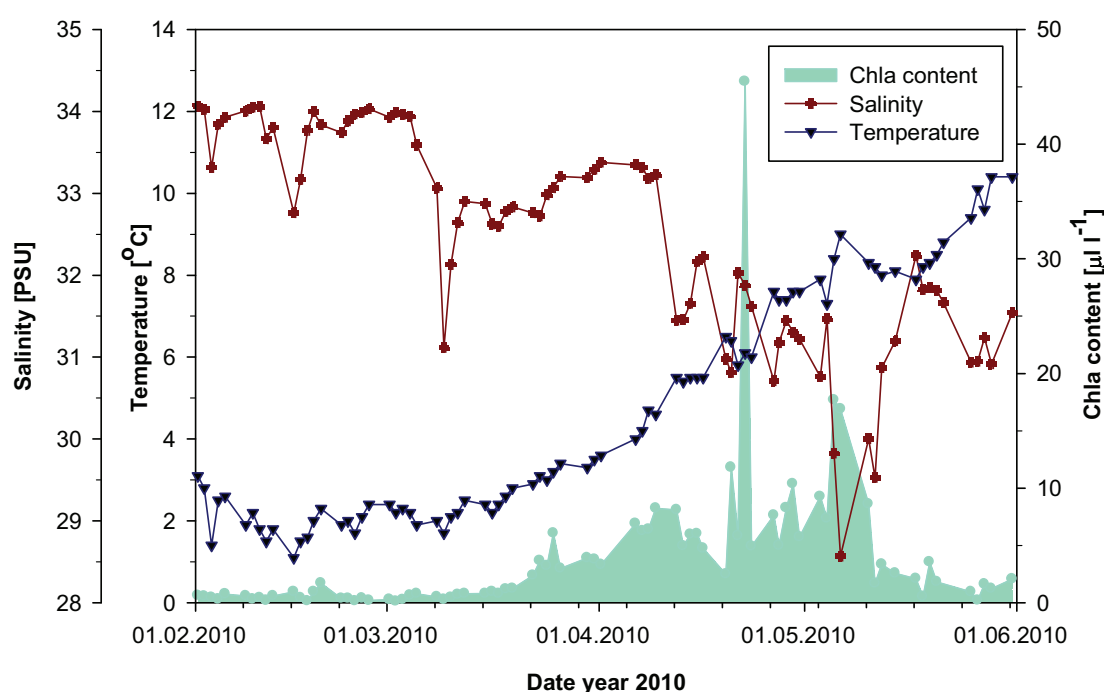


Figure 6 Distribution of temperature, salinity and chlorophyll a (Chla) during the winter-spring transition in year 2010 (daily physico-chemical measurements done by the Biological Station Helgoland, <http://www.pangaea.de/>).

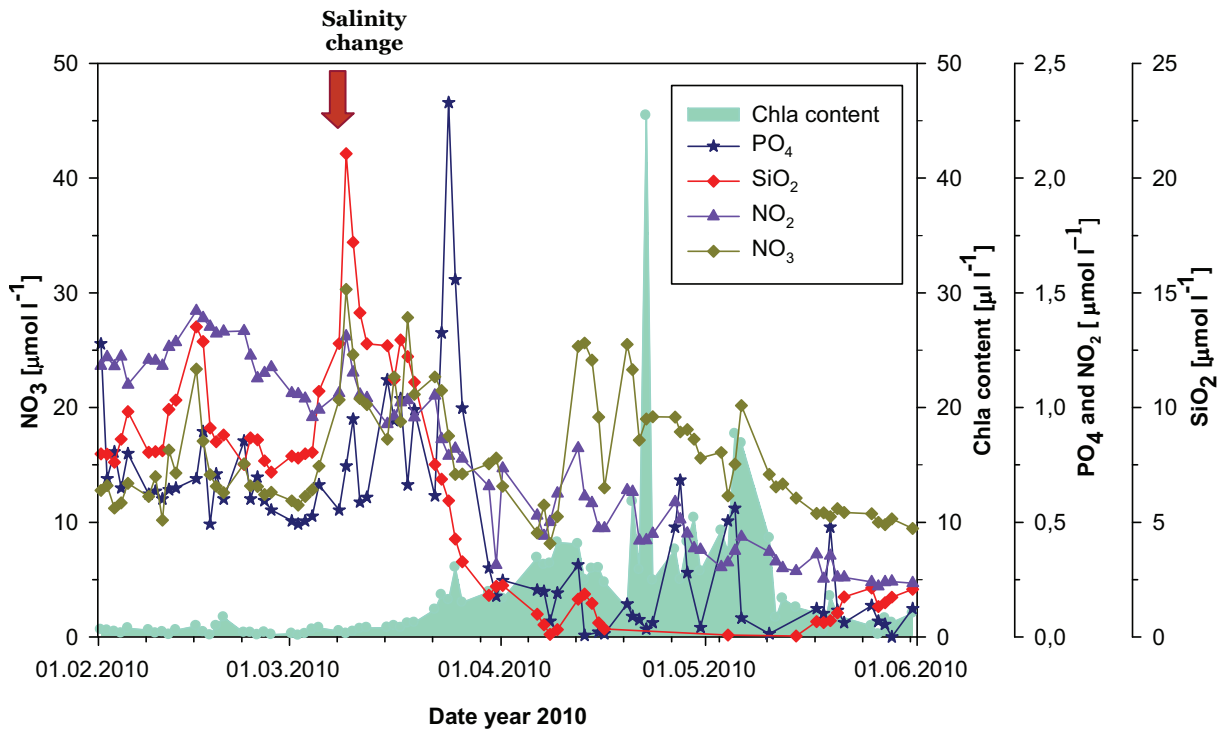


Figure 7 Distribution of phosphate (PO_4), silicate (SiO_2), nitrite (NO_2) and nitrate (NO_3) versus salinity during the winter-spring transition in year 2010 (daily physico-chemical measurements done by the Biological Station Helgoland, <http://www.pangaea.de/>).

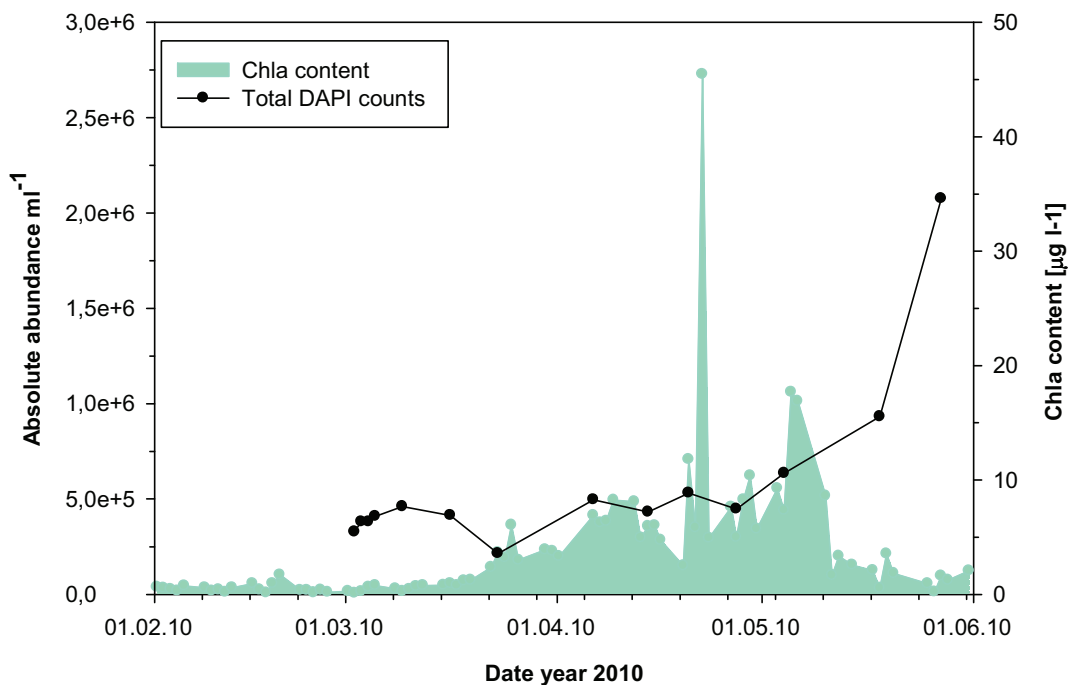


Figure 8 Total DAPI counts in the North Sea at Helgoland Kabeltonne in year 2010.

3.2 Bacterioplankton diversity

3.2.1 Comparison of GM3 and GM3m primer – 16S gene clone library

With the general bacterial primer set GM3 and GM4, one 16S rRNA gene library was constructed from water samples taken at the Kabeltonne station in Helgoland on 11th February 2009 (GM3 library). In parallel, another library (GM3m library) was generated using a different forward primer (GM3 modified) to compare the ability of both forward primers to reflect the richness and the community distribution from the same sample.

From GM3 and GM3m libraries 445 and 454 clones were obtained, respectively, and were fully sequenced. Overall, the two libraries showed minor differences in bacterial richness and composition. Both libraries had a similar number of OTUs at a 16S rRNA similarity level of 97% (Table 3). The richness indexes predicted similar total number of OTUs in both libraries, with 106 for Boot richness estimator, 339 for Shannon-Weaver index (95% CI 325–353) and 0.071 for Simpson index for GM3 library, whereas for the GM3m library, the predicted richness was 104 for Boot richness estimator, 353 for Shannon-Weaver index (95% CI 339–366) and 0.071 for Simpson index. The number of clones used in each library screening did not fully cover the diversity of 16S rRNA genes as indicated by the rarefaction curves which, based on sequence similarity of 93% and 97%, did not yet approach the saturation plateau (**Figure 9**).

	GM3	GM3m
Number of sequences	445	454
Number of OTUs at 97% similarity	86	87
Boot richness estimator	106	104
Shannon-Weaver Index	339	353
Simpson Index	0,071	0,065

Table 3 Comparison of the community richness for GM3 and GM3m clone libraries using standard algorithmic richness estimator and diversity indices.

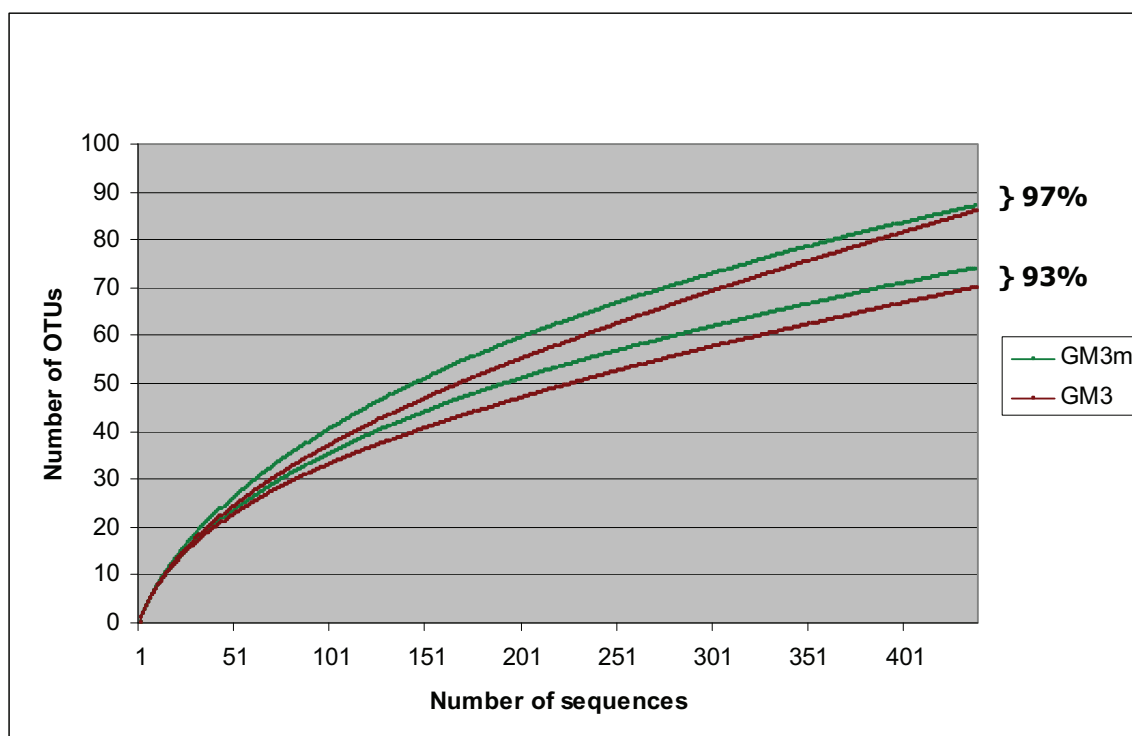


Figure 9 Rarefaction curves generated for 16S rRNA genes in the clone libraries from the winter water sample collected from the North Sea (GM3 and GM3m libraries). Clones were grouped into phylotypes based on sequence similarity of 97% and 93 %. The rarefaction curve, plotting the number of observed phylotypes (S_{obs}) as a function of the number of clones, was computed with the DOTUR software.

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The diversity distribution was almost the same on the phylum level using both primers. About half of the sequences were classified as Alphaproteobacteria (178 and 186 sequences out of 439 and 438 total sequences respectively); 75 and 64 sequences were assigned to Gammaproteobacteria, 39 and 31 sequences as Bacteroidetes and 40 and 36 sequences as Betaproteobacteria, such representing about one tenth of the examined clones (**Figure 10**)

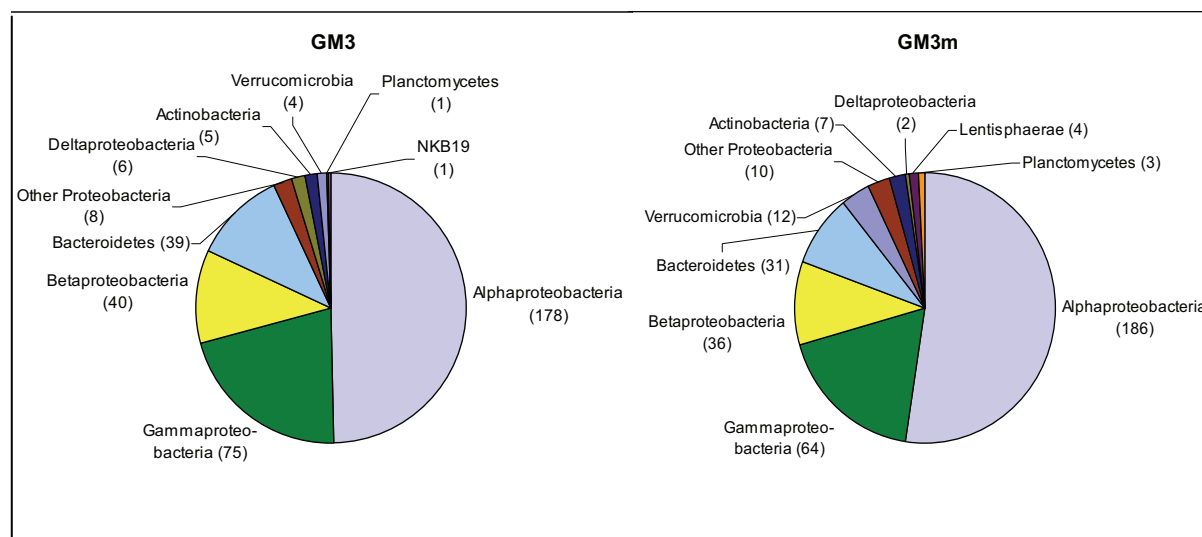


Figure 10 Diversity distributions of the phylogenetic groups in both GM3 and GM3m libraries, generated for the winter sample (11.02.2009) at the phylum/subphylum level.

On a more highly resolved taxonomic level similar numbers of sequences were affiliated to the same subgroups in both libraries, with again minor differences (**Table 4**). In both libraries, the majority of the alphaproteobacterial sequences were affiliated to the SAR11 clade, followed by members belonging to NAC11-lineage; most of the betaproteobacterial community belonged to members of the OM43 clade. There were some differences for the gammaproteobacterial subgroups. In the GM3 library, most of the gammaproteobacterial sequences retrieved were affiliated to members belonging to the SAR86 clade and SAR92 clade with almost equal fractions; a smaller fraction of the gene sequences were assigned to members belonging to the OM182 and NOR5/OM60 clusters. In contrast, the largest fraction of the gammaproteobacterial sequences was affiliated to members belonging to the SAR86 clade in the GM3m library and smaller equal fractions to members belonging to SAR92, OM182 and NOR5/OM60 clusters. Most of the bacteroidetal sequences retrieved in both libraries were members of the NS9 and NS4 marine groups.

Since both GM3 and GM3m libraries of the winter sample showed very similar richness and diversity distribution, the sequences of both libraries were merged together and treated as one, designated as February clone library, with 897 clones in total.

GM3	GM3m	Total	Subgroups	Groups
144	140	284	SAR11 clade	Alphaproteobacteria
35	33	68	OM43 clade	Betaproteobacteria
15	22	37	SAR86 clade	Gammaproteobacteria
15	18	33	NAC11-7 lineage	Alphaproteobacteria
17	7	24	SAR92 clade	Gammaproteobacteria
10	8	18	SAR116	Alphaproteobacteria
11	6	17	NS9 marine group	Bacteroidetes
9	7	16	OM182 clade	Gammaproteobacteria
8	5	13	NS4 marine group	Bacteroidetes
1	6	7	NOR5/OM60 clade	Gammaproteobacteria

Table 4 Number of sequences affiliated to the ten most abundant phylotypes in both GM3 and GM3m clone libraries.

3.2.2 Comparison of winter and spring 16S rRNA gene clone libraries - February - April 2009

Based on the finding for the Feb 2009 sample, the 16S rRNA gene clone library of the spring sample, collected on 14th April 2009, was constructed using only the general bacterial primer set GM3 and GM4 for initial DNA amplification. Eight hundred twenty-two clones were obtained. At the species level (97% similarity) the winter library had 129 total number of OTUs while the spring one 105. Shannon-weaver index was 3603 (95% CI 3499–3707) for the winter library, 3393 (95% CI 3493–3551) for the spring one, while values of Simpson index were 0.067 and 0.065 respectively (**Figure 4**). In contrast to the winter library, the number of clones in the spring library could well cover the diversity of 16S rRNA genes as indicated by the corresponding refraction curves tending to approach saturation on both 93% and 97% sequence similarities (**Figure 11**).

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	Winter library	Spring library
Number of sequences	900	822
Number of OTUs at 97% similarity	129	105
Shannon-Weaver Index	3603	3393
Simpson Index	0.067	0.065

Table 5 Comparison of the community richness for winter and spring clone libraries using standard algorithmic richness estimator and diversity indices.

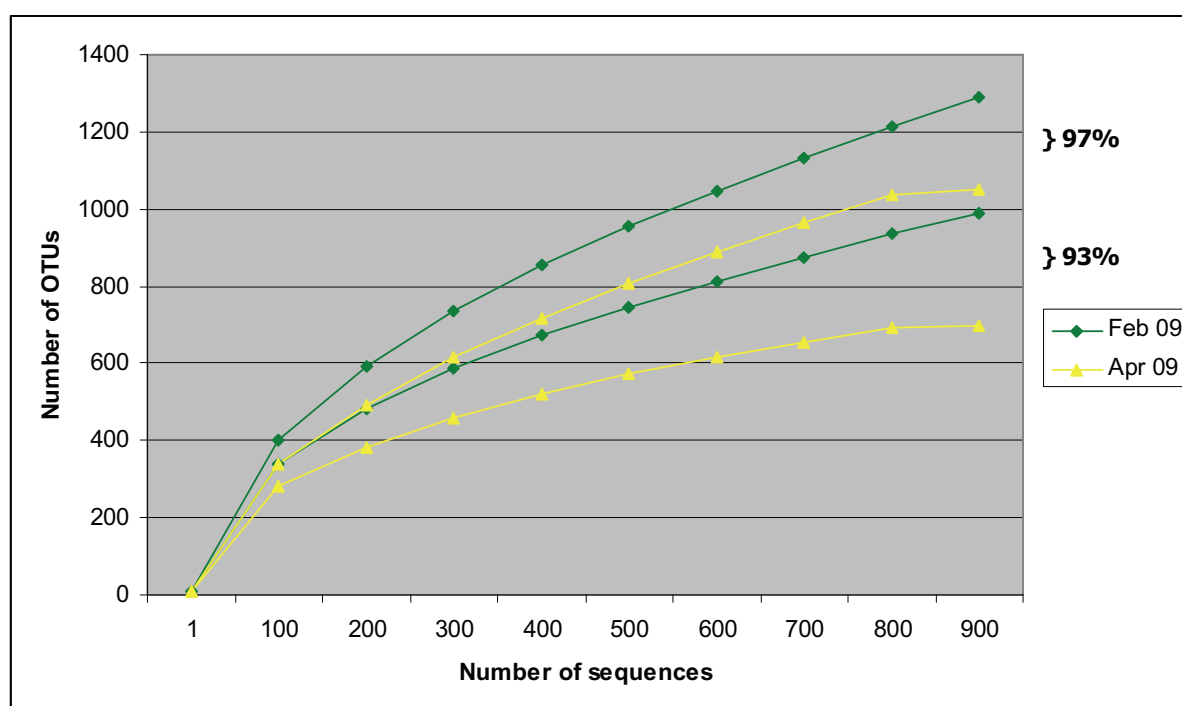


Figure 11 Rarefaction curves generated for the 16S rRNA genes in the clone libraries from the winter water sample collected from the North Sea (GM3 and GM3m libraries). Clones were grouped into phylotypes based on sequence similarity of 97% and 93 %. The rarefaction curve, plotting the number of observed phylotypes (S_{obs}) as a function of the number of clones, was computed with the Mothur software.

A substantial community change has been observed within the four major bacterial groups Alpha-, Gamma-, Betaproteobacteria and Bacteroidetes from the winter to the spring according to the libraries. Comparing the winter and spring sample, some phylogenetic groups, which were prominent in the winter, disappeared and new ones, which were not

found in the winter library, appeared in the community occupying a large fraction of the community. For example, the the alphaproteobacterial SAR11 cluster dominated in the winter with around 32% of the clones which dropped to 19% in the spring library (**Table 6, Figure 12**). The OM43 clade was the second most dominant group following the SAR11 clade in the winter library and the single betaproteobacterial occurring phylotype in both libraries. However, it nearly disappeared in the spring library, where the percentage of the corresponding sequences decreased from 8% to 0.5%.

The diversity distribution within the phylum of Bacteroidetes changed drastically in the winter-spring transition phase. Groups, not previously found in the winter library were abundant in the April 14th 2009 library, such as *Polaribacter* (11%), NS3a marine group (6%), *Formosa* (4%) and *Ulvibacter* (4%). The fraction of sequences affiliated to NS9 and NS4 marine groups remained nearly the same in both libraries.

Also within the Gammaproteobacteria, sequences affiliation in the spring library was shifted towards the genera *Reinekea*, *Balneatrix*-related clade and *Glaciacola* (**Figure 13**). The SAR86 clade was the most dominant gammaproteobacterial group in the winter comprising 4% of the total clones, but completely disappeared from the spring library. The SAR92 showed higher proportion in the spring, where fraction of the affiliated sequences increased from 3% in the winter library to 6% in the spring one. The fraction of the sequences falling within the OM182 and NOR5/OM60 clusters remained nearly constant in both libraries (**Table 6, Figure 12**).

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Phylogenetic groups	February 2009	%	April 2009	%
SAR11 clade	284	32	157	19
<i>Polaribacter</i>	-		94	11
<i>Reinekea</i>	-		76	9
OM43 clade	68	8	4	0,5
SAR92 clade	24	3	48	6
<i>Balneatrix</i> -related clade	-		44	5
SAR86 clade	37	4	-	
<i>Glaciecola</i>	-		32	4
NAC11-7 lineage	33	4	47	6
<i>Formosa</i>	-		36	4
NS3a marine group	-		49	6
<i>Ulvibacter</i>	-		32	4
SAR116	18	2	1	0,1
NS9 marine group	17	2	12	1
OM182 clade	16	2	22	3
NS4 marine group	13	1	1	0,1
NOR5/ OM60 clade	7	1	1	0,1
Total number of clones	899		822	

Table 6 Number of sequences affiliated to the phylogenetic groups found in the winter (February) and spring (April) clone libraries generated from the surface water sample collected from the North Sea in year 2009. For clarity, only the most ten dominant groups on at least one of the two libraries are listed.

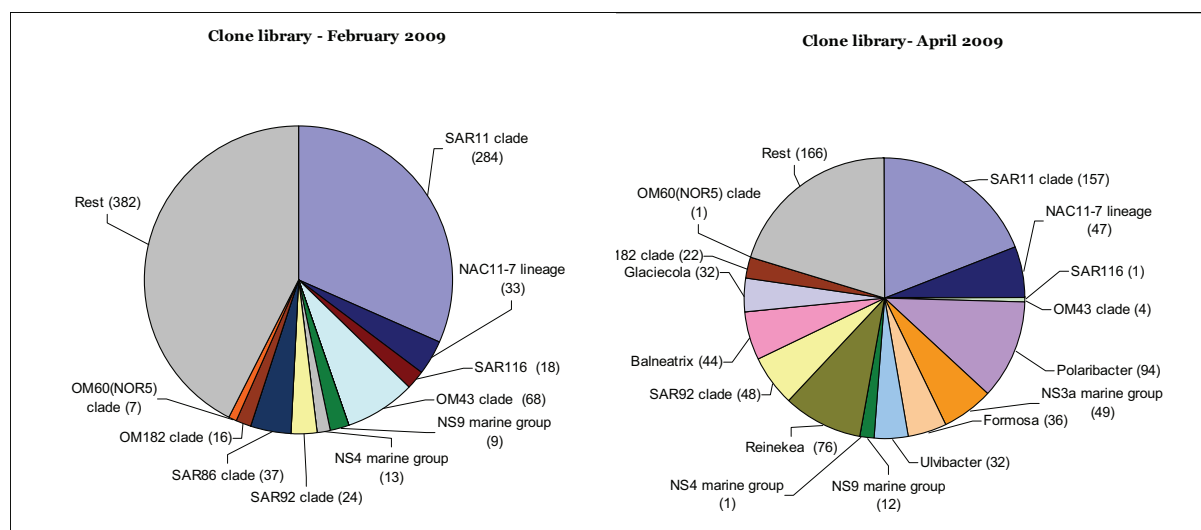


Figure 12 Diversity distributions of the phylogenetic groups of both winter (February) and spring (April) clone libraries - year 2009. For clarity, only the most ten dominant groups in at least one of the two libraries are shown.

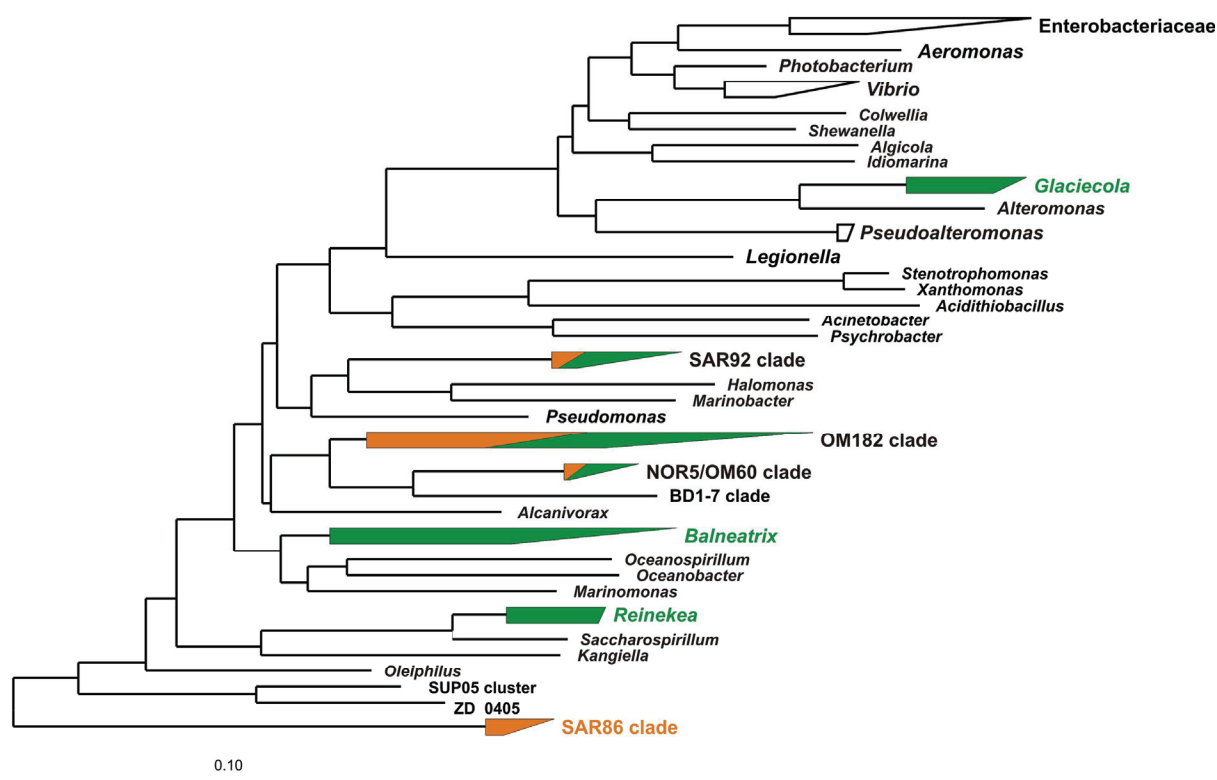


Figure 13 Phylogenetic RAXML tree based on comparative sequence analysis of 16S rRNA gene showing the major gammaproteobacterial groups of the winter and spring clone libraries. Each group is represented by few or one species for simplification. Groups are highlighted in orange, green and in both colours according to the corresponding sequences retrieved only in the winter clone library, only in the spring library and in both libraries respectively. The bar indicates 10% sequence divergence.

3.3 Gammaproteobacterial community composition and abundance

According to the 16S gene tags provided by Anna Klindworth (Microbial Genomics Department, Max Planck Institute for marine Microbiology, Bremen, Germany) for year 2009, important groups within the Gammaproteobacteria were affiliated to *Reinekea*, SAR92 clade, SAR86 clade, OM182 clade, *Balneatrix*-related clade, and *Glaciecola*. In order to quantify these groups before, during and after the algal bloom, new oligonucleotide probes were developed and evaluated for *Reinekea*, *Balneatrix*-related clade, OM182 clade and *Glaciecola*. These were used together with already published probes for SAR92, SAR86, NOR5/OM60 clades, the family Vibrionaceae and the genus of *Pseudoalteromonas*. The probe sequences and properties are listed in materials and methods (**Table 2**).

3.3.1 *Reinekea*

Reinekea was one of the gammaproteobacterial groups firstly occurring in the spring library, where 75 clones were classified as *Reinekea*. They comprised 9% of the library. Seventy four of the sequences were >99% similar and showed about 94% to 95% similarity to the previously isolated strains *Reinekea blandensis* and *Reinekea marinisedimentorum*. Only clone 709-M1 was more distant from the remaining clones by 3% (**Figure 14**).

One probe, probe Rei731 was designed and targeted all *Reinekea* species isolated so far. The probe match tool in the ARB software also indicated that the probe targeted all *Reinekea* clones present in the library except for one clone. Hybridization conditions were optimal using 35% FA concentration. This was tested on a pure culture of *Reinekea marinisedimentorum* (DSM 15388) (**Figure 15**). However, when tested on environmental sample using CARD-FISH, signals were lost above 20% FA, and consequently the protocol was changed accordingly. The probe had up to 0.9 weighted mismatches with non-target sequences which did not belong to marine groups.

During the winter *Reinekea* counts were below the detection limit. In comparison to the SAR92 clade, the absolute abundance of *Reinekea* increased drastically from 4×10^3 cells ml⁻¹ on 9th April to 1.2×10^5 cells ml⁻¹ on 14th April. According to CARD-FISH counts, by beginning of May the absolute abundance of that group was again below the detection limit (**Figure 16**). The total cell counts for *Reinekea* was around 1×10^3 cells ml⁻¹ in March 2010, increased up to 6.5×10^3 cells ml⁻¹ during the algal bloom and reached its peak with 7.3×10^4 cells ml⁻¹ on 18th May, about one month after the bloom. No data was available for the month of June since sampling had not taken place during that month and no cells were detected for *Reinekea* in July 2010 (**Figure 17**).

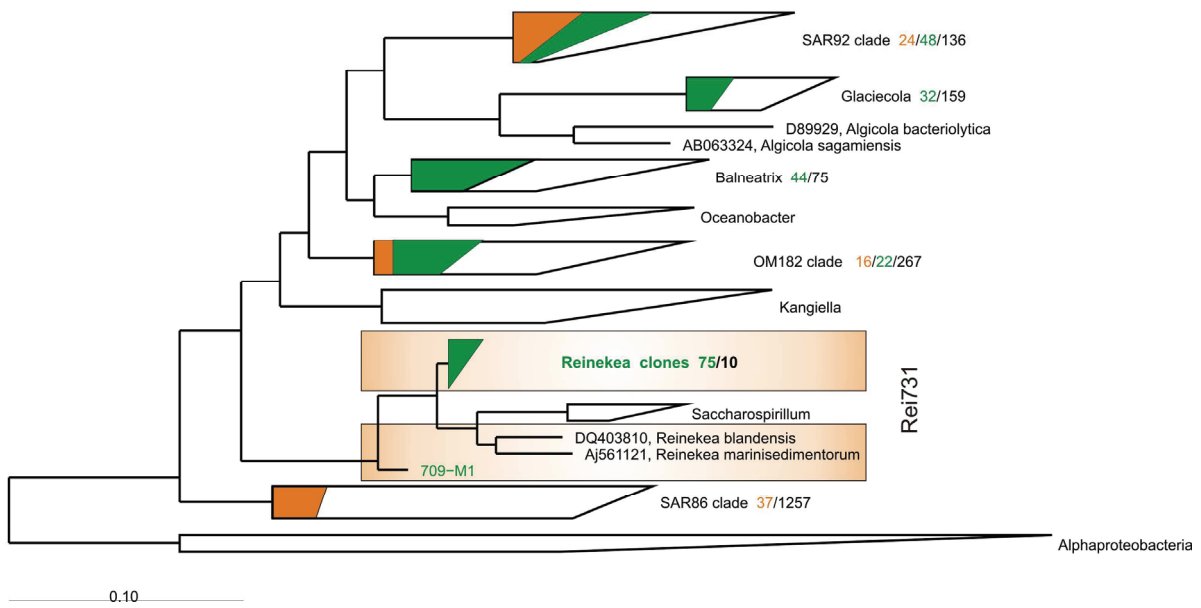


Figure 14 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences classified as *Reinekea*. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers on the right indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same groups already present in the database. Sequences targeted by the newly designed probe Rei731 at zero weighted mismatches are highlighted in boxes. The scale bar represents 10% estimated sequence divergence.

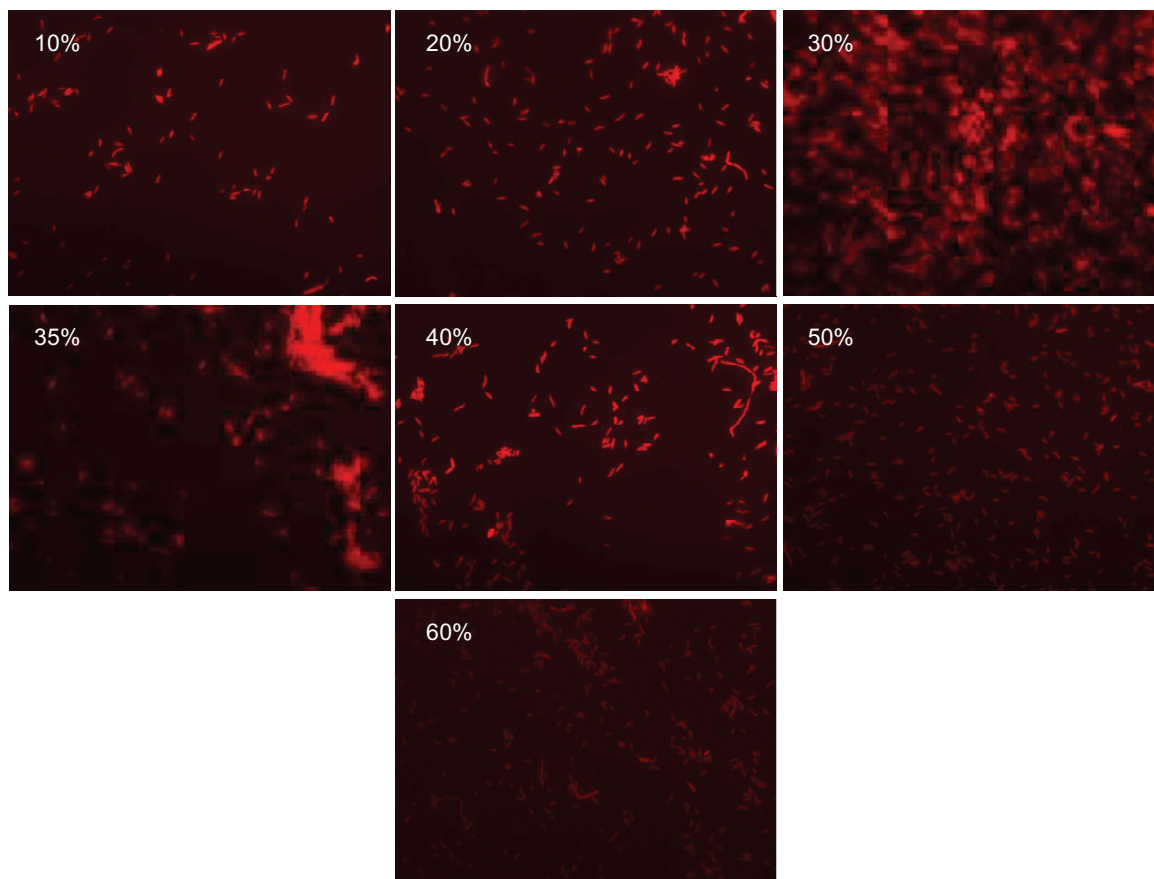


Figure 15 Picture gallery of epifluorescence micrographs showing the signal intensity obtained by probe Rei731 at different FA% concentrations when tested on a pure culture of *Reinekea marinisedimentorum* (DSM 15388), using standard – FISH with Cy3-monolabelled probe.

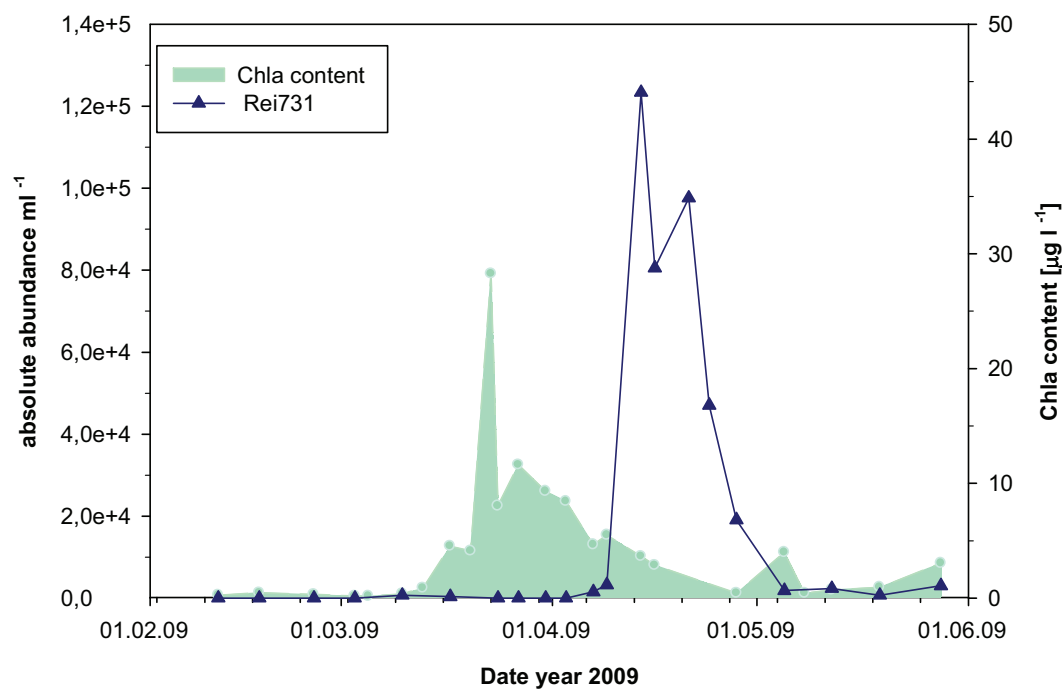


Figure 16 Absolute abundances of the phylogenetic group *Reinekea* in year 2009.

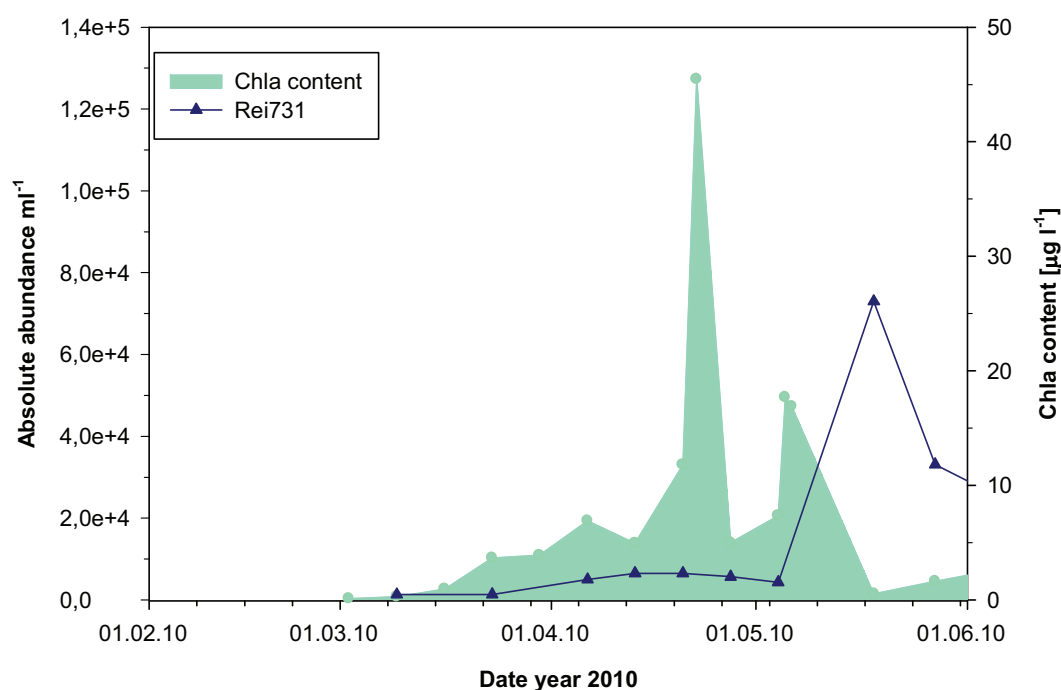


Figure 17 Absolute abundances of the phylogenetic group *Reinekea* in year 2010.

3.3.2 SAR92 clade

The SAR92 clade occurred in both libraries, with 24 (3%) and 48 clones (6% relative clone frequency) in the winter and spring libraries, respectively. The winter clones formed one cluster within the clade and were 96% to 99% similar. In addition, the spring clones formed another cluster having the same sequence similarity. Both clusters were about 4% distant from each other at maximum (**Figure 18**). Using the probe match tool of ARB software, the previously designed probe SAR92-627 (117) targeted all clones, except for two sequences, clones 334-M1 and 403-M1, and it was used to determine the absolute counts for that group in winter and spring 2009. The SAR92 clade showed low abundances in the winter of year 2009 ranging between 6×10^3 cells ml^{-1} and 2×10^4 cells ml^{-1} till the beginning of the bloom. In the first two weeks of the bloom they increased up to 1×10^5 cells ml^{-1} . Then they further increased culminating in a peak of 3×10^5 cells ml^{-1} on 24th April, about one month after the blooming peak. Thus, the SAR92 clade was the most abundant gammaproteobacterial group after the spring algal bloom in year 2009, as detected by CARD-FISH. The absolute abundance of SAR92 clade decreased by beginning of May but had another peak of 1.8×10^5 cells ml^{-1} on 12th May (**Figure 19**). In the winter of year 2010 the total cell counts of SAR92 was around 5×10^3 cells ml^{-1} . They started to increase at the beginning of the bloom, reached

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9×10^4 cells ml⁻¹ during the algal peak, and reached their peak with 1.6×10^5 cells ml⁻¹ on 18th May, about one month after the blooming peak (**Figure 20**).

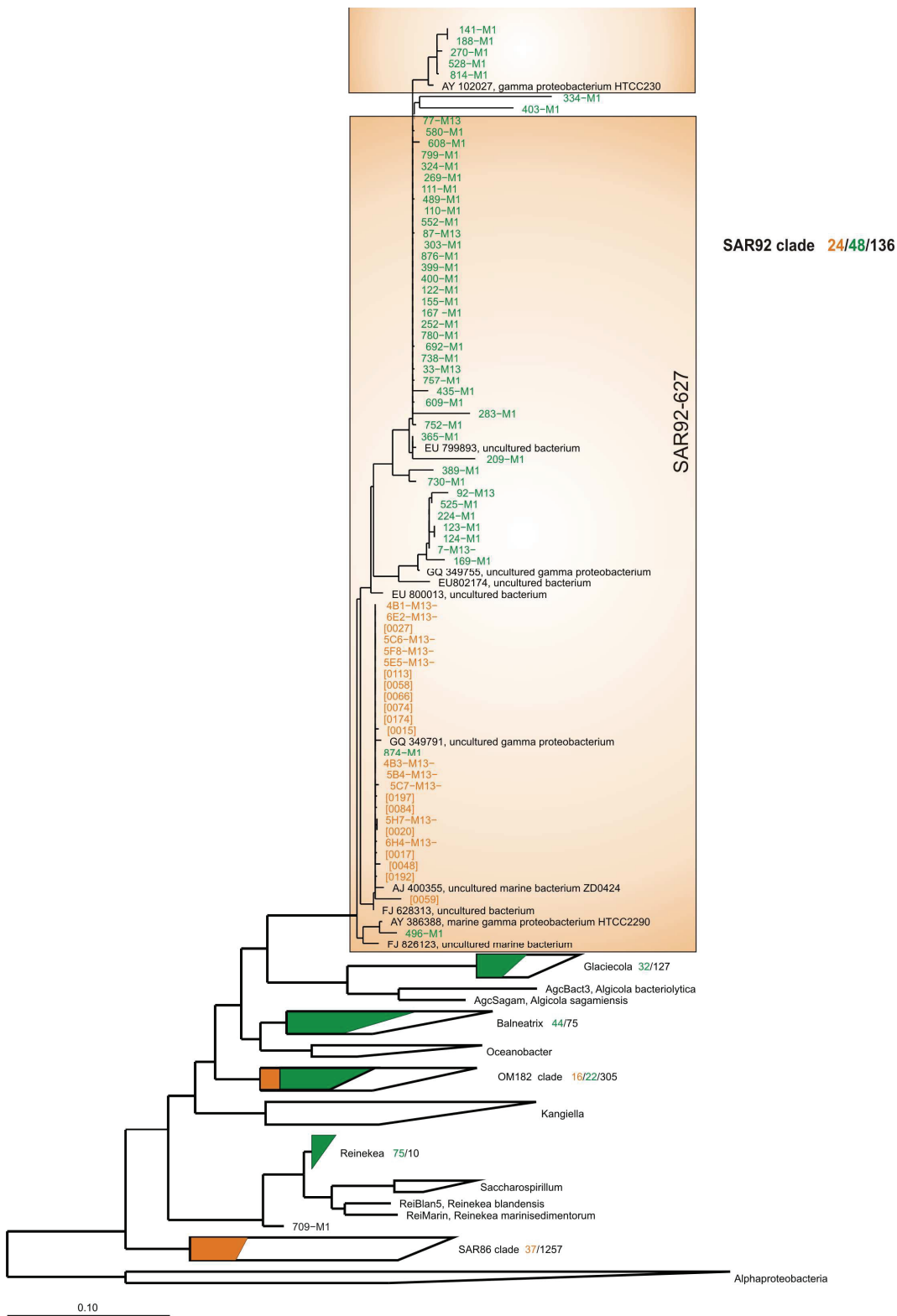


Figure 18 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences classified as SAR92 clade. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers on the right indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same group already present in the database. Sequences targeted by the previously designed probe SAR92-627 at zero weighted mismatches are highlighted in boxes. The scale bar represents 10% estimated sequence divergence.

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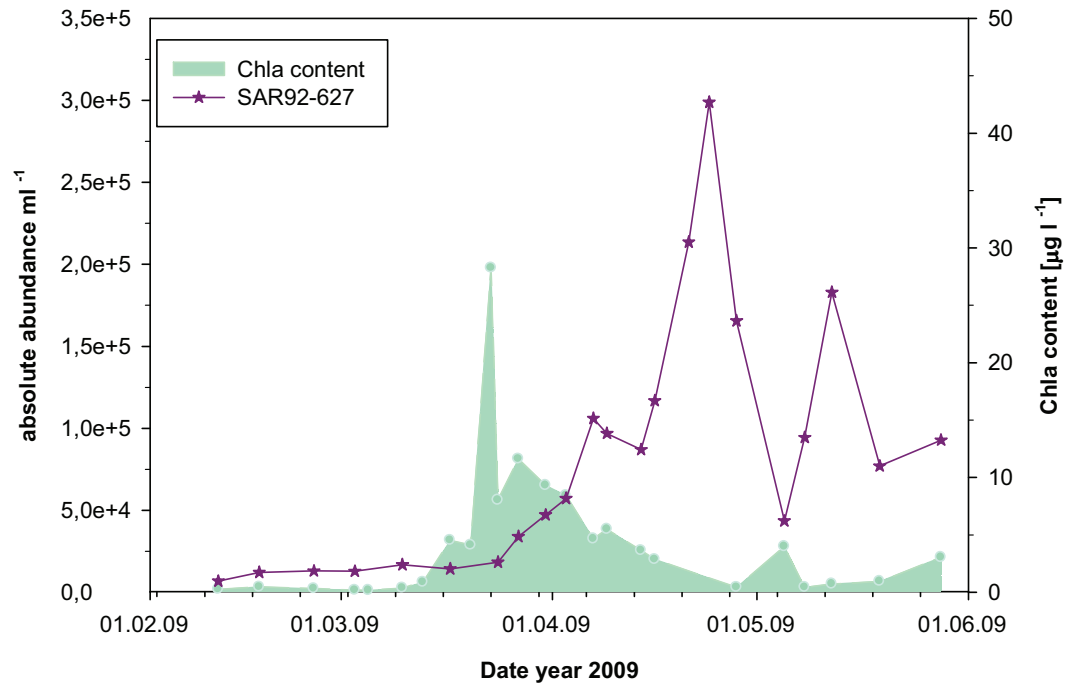


Figure 19 Absolute abundances of SAR92 clade in year 2009.

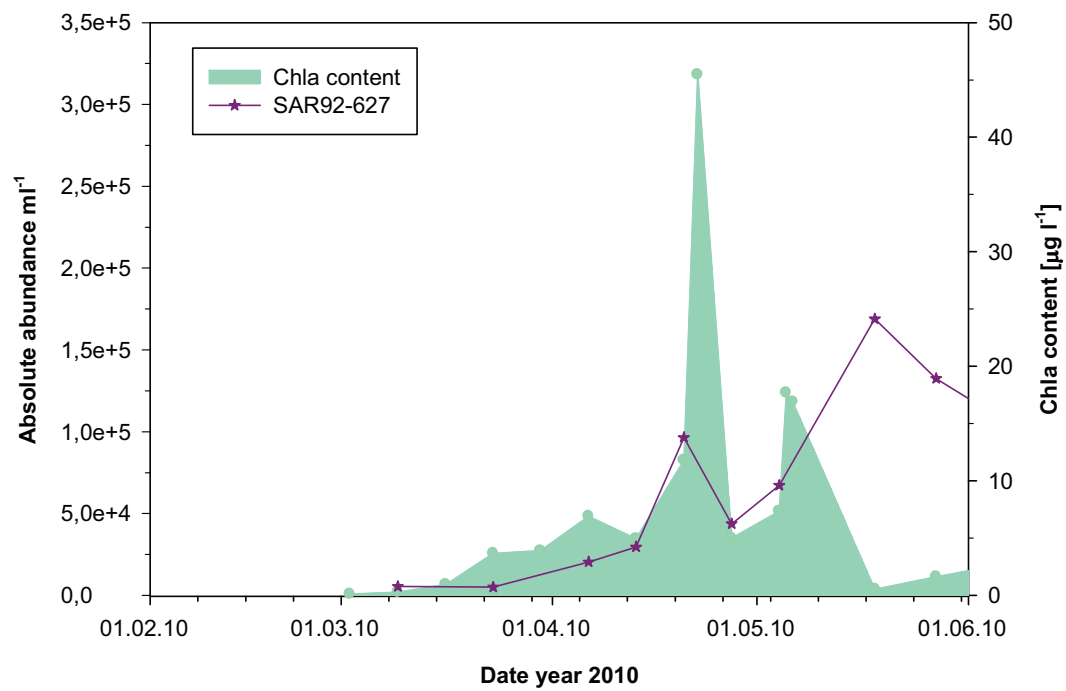


Figure 20 Absolute abundances of SAR92 clade in year 2010.

3.3.3 SAR86 clade

Thirty seven clones were retrieved within the SAR86 cluster in the winter clone library but no sequences were found for that group in the spring library. Although this clade is highly diverse, most of the clones formed three main clusters within the group, which were about 1% to 2% distant from each other. Few clones were about 3% to 6% distant from those clusters, except for one clone, 5H10-M13, which shared only 84% to 85% similarity with those clusters (**Figure 21**). The previously designed SAR867-1245 probe targeted all clones in situ except for two sequences; one of them was clone 5H10, which was the most distant one.

The absolute cell numbers counted with SAR86-1245 probe ranged between 1×10^4 cells ml⁻¹ and 3×10^4 cells ml⁻¹ during the winter. The counts decreased during the bloom and reached down to 2×10^3 cells ml⁻¹ till end of May. Then they started to increase culminating in a peak of 2×10^5 cells ml⁻¹ on 12th May. However, they dropped again but did not go below the level of 3×10^4 cells ml⁻¹ not reaching the low level that had been registered during the bloom (**Figure 22**). Same trend was observed for the absolute abundance of that group in year 2010 where it was around 1×10^4 cells ml⁻¹ in the winter, decreased down to 2×10^3 cells ml⁻¹ during the algal bloom but increased up to 8×10^4 cells ml⁻¹ about one month after the bloom (**Figure 23**). It was not known if this was the peak for the absolute abundance of this group, since no sample was collected in the month of June 2010.

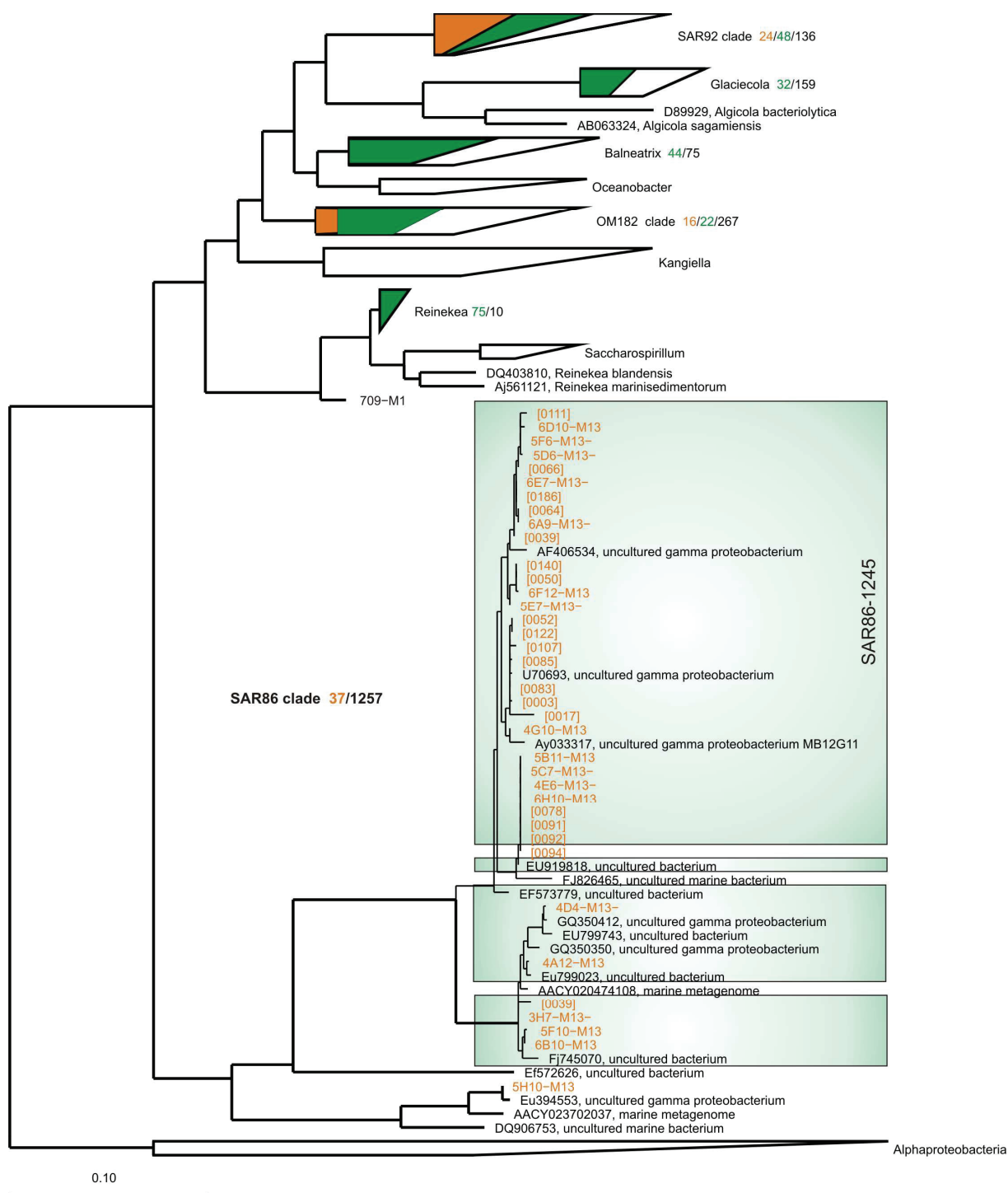


Figure 21 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences classified as SAR86 clade. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers in brackets indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same group already present in the database. Sequences targeted by the previously designed probe SAR86-1245 at zero weighted mismatches are highlighted in boxes. The scale bar represents 10% estimated sequence divergence.

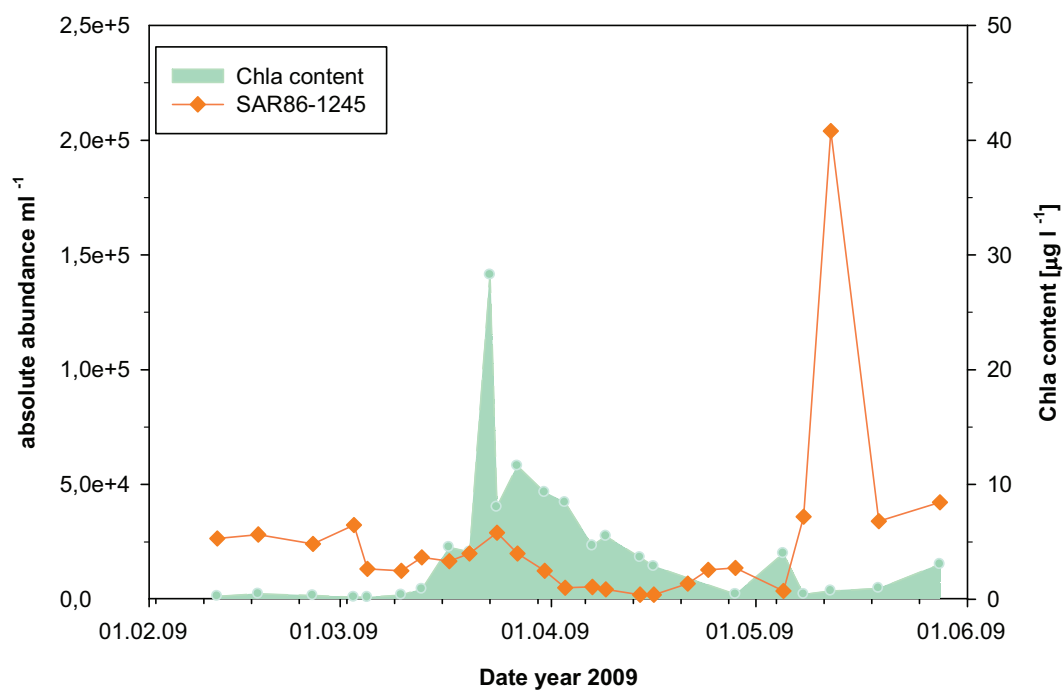


Figure 22 Absolute abundances of SAR86 clade in year 2009.

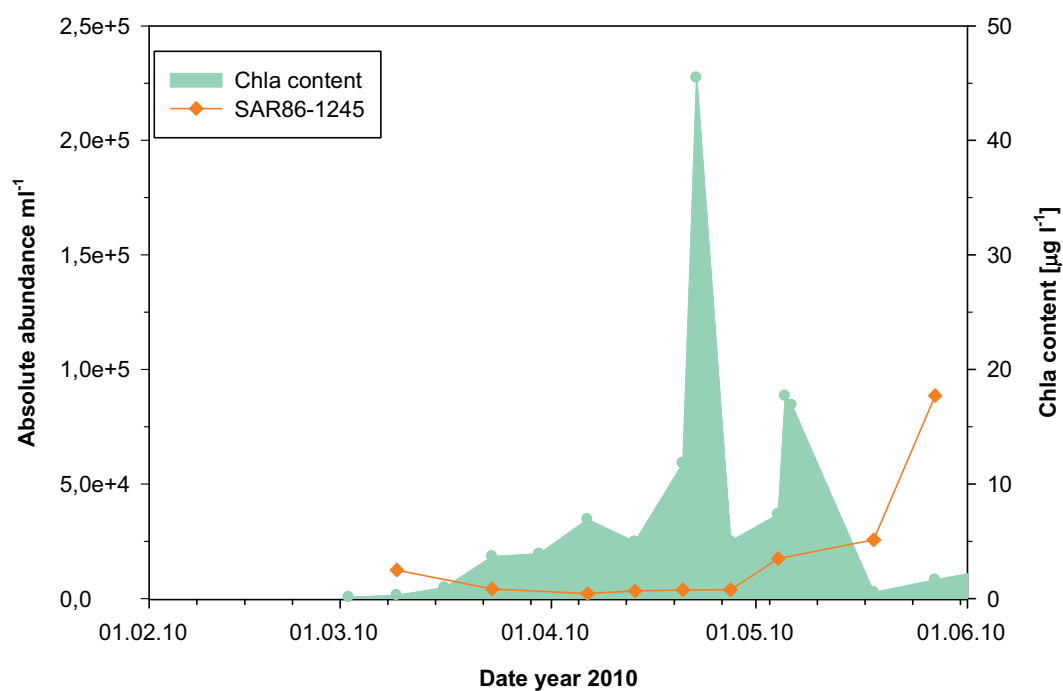


Figure 23 Absolute abundances of SAR86 clade in year 2010.

3.3.4 NOR5/OM60 and OM182 clades

Seven and two clones were obtained within the NOR5/OM60 cluster in winter and spring clone libraries, respectively, while 16 and 22 clones were affiliated within the OM182 cluster. For the OM182 clade, the clones of each library were clustered in about three groups and were distributed within the clade (**Figure 24**).

Since the OM182 clade formed two main clusters within the dataset, which were about 10% to 11% distant from each other, three probes were designed to cover both clusters of the clade. Probe OM182/707 targeted one cluster while probes OM182/838 and OM182/838-a targeted the other one. Probe OM182/838-a differed from the previous one by one mismatch. However, only probe OM182/707 could hybridize to the rRNA showing optimal signals using 15% FA (**Figure 25**) and had at least one strong peripheral mismatch (0.3 weighted mismatches), with Enterobacteriaceae (**Table 7**). The probe covered all of the clones present in the targeted cluster (**Figure 24**).

Probe NOR5-730, previously designed by Yan Shi (134), was used to determine the absolute counts for the NOR5/OM60 cluster in winter and spring 2009 at the station Kabeltonne, Helgoland. It targeted all of the clones found in both clone libraries (**Figure 26**).

Both NOR5/OM60 and OM182 clades showed similar succession pattern within the same time frame. During the winter their absolute abundances were below 7×10^3 cells ml⁻¹ and 1.3×10^4 cells ml⁻¹, respectively as counted by probes NOR5-730 and OM182/707 respectively. During the algal bloom they increased and reached peaks of 9×10^4 cells ml⁻¹ and 1.2×10^5 cells ml⁻¹, respectively, by end of April (about one month after the algal peak) (**Figure 27**). In year 2010 the absolute cell numbers counted with probe OM182/707 were around 1×10^4 cells ml⁻¹ at the beginning of the algal bloom. They steadily increased to 2.2×10^4 cells ml⁻¹ in two weeks after the algal peak and reached a maximum of 1×10^5 cells ml⁻¹ two weeks later (one month after the algal peak) (**Figure 28**). However, it remains unclear if this was the absolute abundance peak due to the unavailability of samples from June 2010.

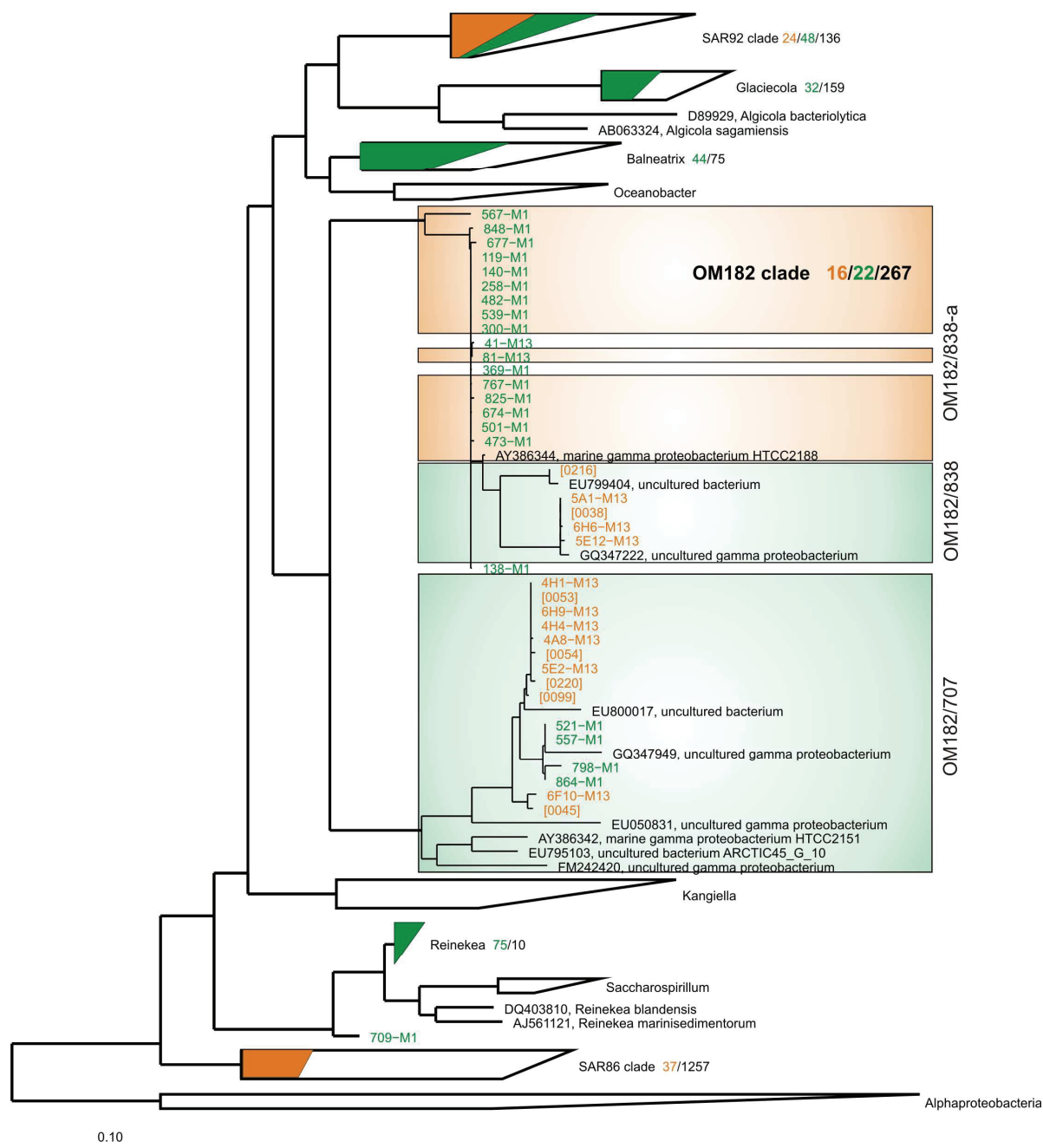


Figure 24 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences classified as OM182 clade. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers on the right indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same group already present in the database. Sequences targeted by the newly designed probes OM182/707, OM182/838 and OM182/838-a at zero weighted mismatches are highlighted in boxes. The scale bar represents 10% estimated sequence divergence.

Results

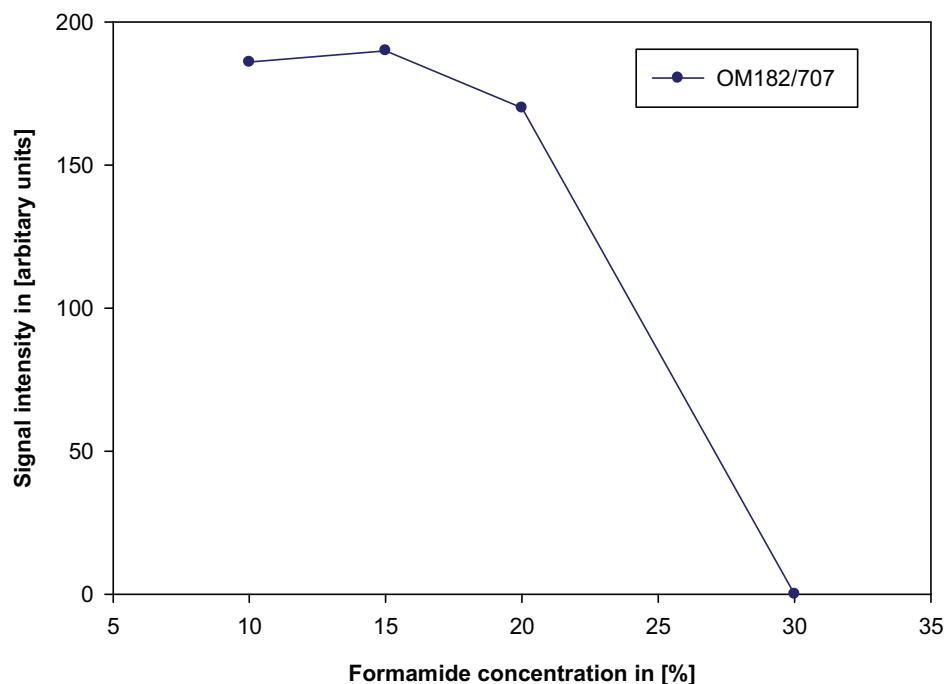


Figure 25 Melting curve for probe OM182 clade when tested on environmental sample using CARD-FISH.

Probe	No. of weighted mismatches	Binding site
OM182/707		3' AAG ACT CCT TAT GGC CAC 5'
target		5' UUC UGA GGA AUA CCG GUG 3'
OM182 clade uncultured, strains HTCC2180, HTCC2151, HTCC2178	0.0	*** **
Enterobacteriaceae	0.3	A ** **
Candidate division OP10	0.5	**G** **
SUPO5 cluster	0.6	A ** *G** **
Thiotricales	0.7	**A** **
Deltaproteobacteria	0.9	A ** G** **
<i>Shewanella</i>	0.9	A ** G** **
Deltaproteobacteria	1.0	**U G** **
Betaproteobacteria	1.0	**U G** **
OM182/838		3' CCC AUU AGG GAA AUC ACC 5'
target		5' GGG UAA UCC CUU UAG UGG 3'
OM182 clade uncultured		*** **
<i>Geobacillus</i> , Firmicutes	1.5	**U** ** *C
<i>Geobacillus</i> , Firmicutes	1.6	*** *U** ** *C
S24-7, Bacteroidetes	1.6	*** **
OM182 clade uncultured, strain HTCC2188	0.8	*** ** U** **
OM182/838-a		5' GGG UAA UCC CUU UAG UGG 3'

Table 7 Fullmatch and mismatch reference 16S rRNA sequences showing probe-binding sites for the three probes designed for the OM182 clade according to the ARB v5.2 Probe Match Tool. Normal letters stand for weak mismatches and bold letters for strong ones.

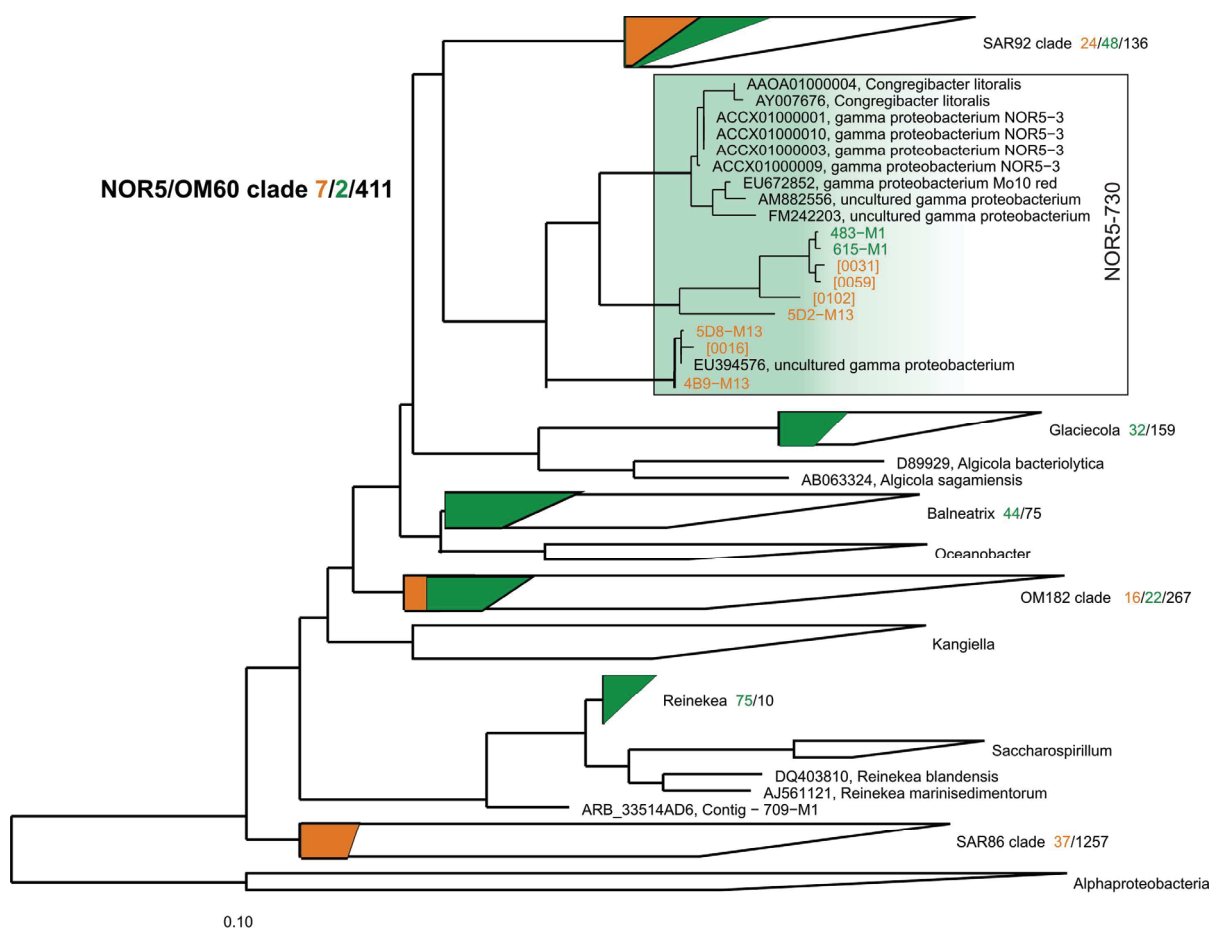


Figure 26 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences classified as NOR5/OM60 clade. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers on the right indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same group already present in the database. Sequences targeted by the previously designed probe NOR5-730 at zero weighted mismatches are highlighted in a box. The scale bar represents 10% estimated sequence divergence.

Results

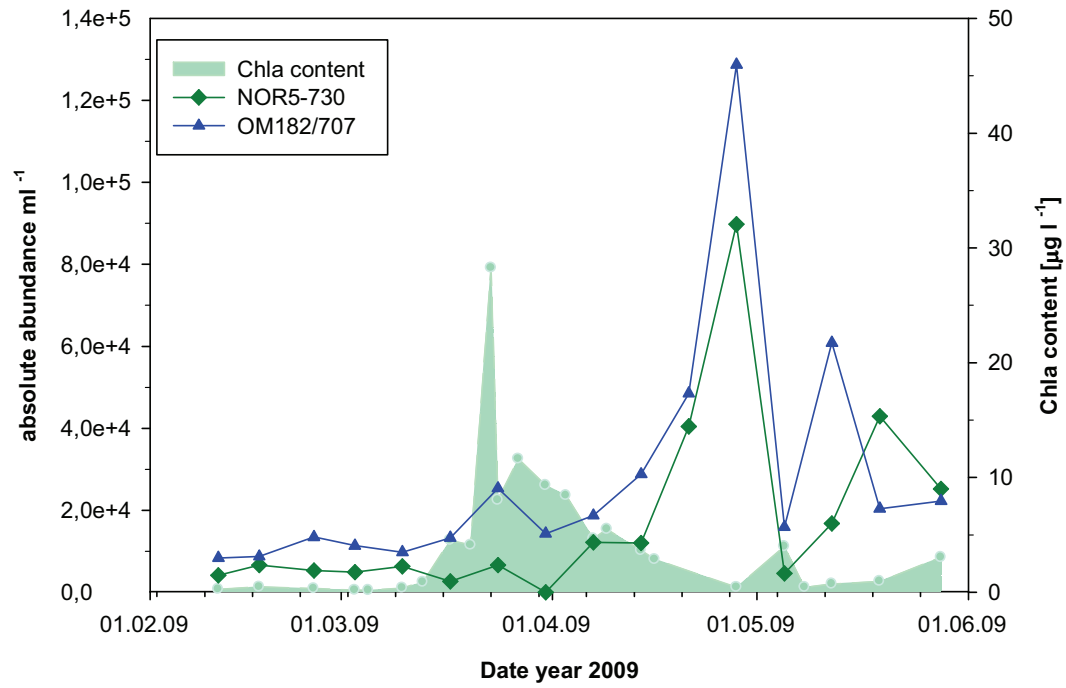


Figure 27 Absolute abundances of NOR5/OM60 and OM182 clades in year 2009.

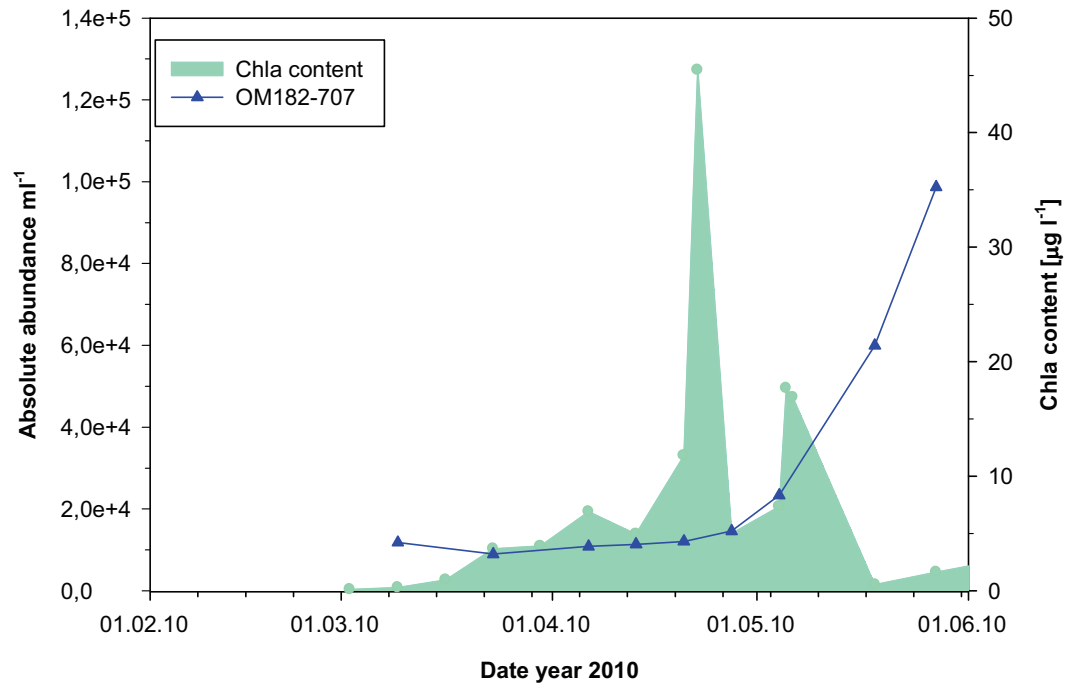


Figure 28 Absolute abundances of the OM182 clade in year 2010.

3.3.5 *Balneatrix*-related clade and *Vibrio*

Clones affiliated to the *Balneatrix* group were only found in the spring clone library, where 44 sequences were classified as *Balneatrix*-related clade such comprising 5% of the total clones. Most clones formed one cluster sharing 96% to 99% similarity. The most distant clone, 941-M1, shared only 92% to 95% similarity with the rest of the clones. Except for that clone, most of the clones shared about 90% to 91% similarity with the reference strain *Balneatrix alpica* and 94% to 99% with the selected uncultured reference sequences (*Balneatrix*-related clade) (**Figure 29**). Probe Bal731 did not cover the reference strain *Balneatrix alpica* but targeted all of the clones and 80% of the uncultured sequences belonging to that group. The probe showed optimal hybridization conditions using 25% FA (**Figure 30**). The probe had at least two mismatches (one strong and one weak) with the OM182 clade (1.7 weighted mismatches) (**Table 8**).

The *Balneatrix*-related clade counts were below the detection limit during the winter. By the beginning of the bloom, the absolute counts of *Balneatrix*-related clade started gradually to increase and reached their peak with 3.6×10^4 cells ml⁻¹ by beginning of May (about one month after the algal peak). Similar pattern was observed for absolute cell numbers of *Vibrio*, as counted with the previously designed GV841 probe showing an increase up to 3×10^4 cells ml⁻¹ (**Figure 31**). In year 2010 *Balneatrix* -related clade counts started to increase during the first weeks of the bloom, reached 1.7×10^4 cells ml⁻¹ by the blooming peak and reached their maximum of 2.2×10^4 cells ml⁻¹ by one month after the algal peak (**Figure 32**).

Results

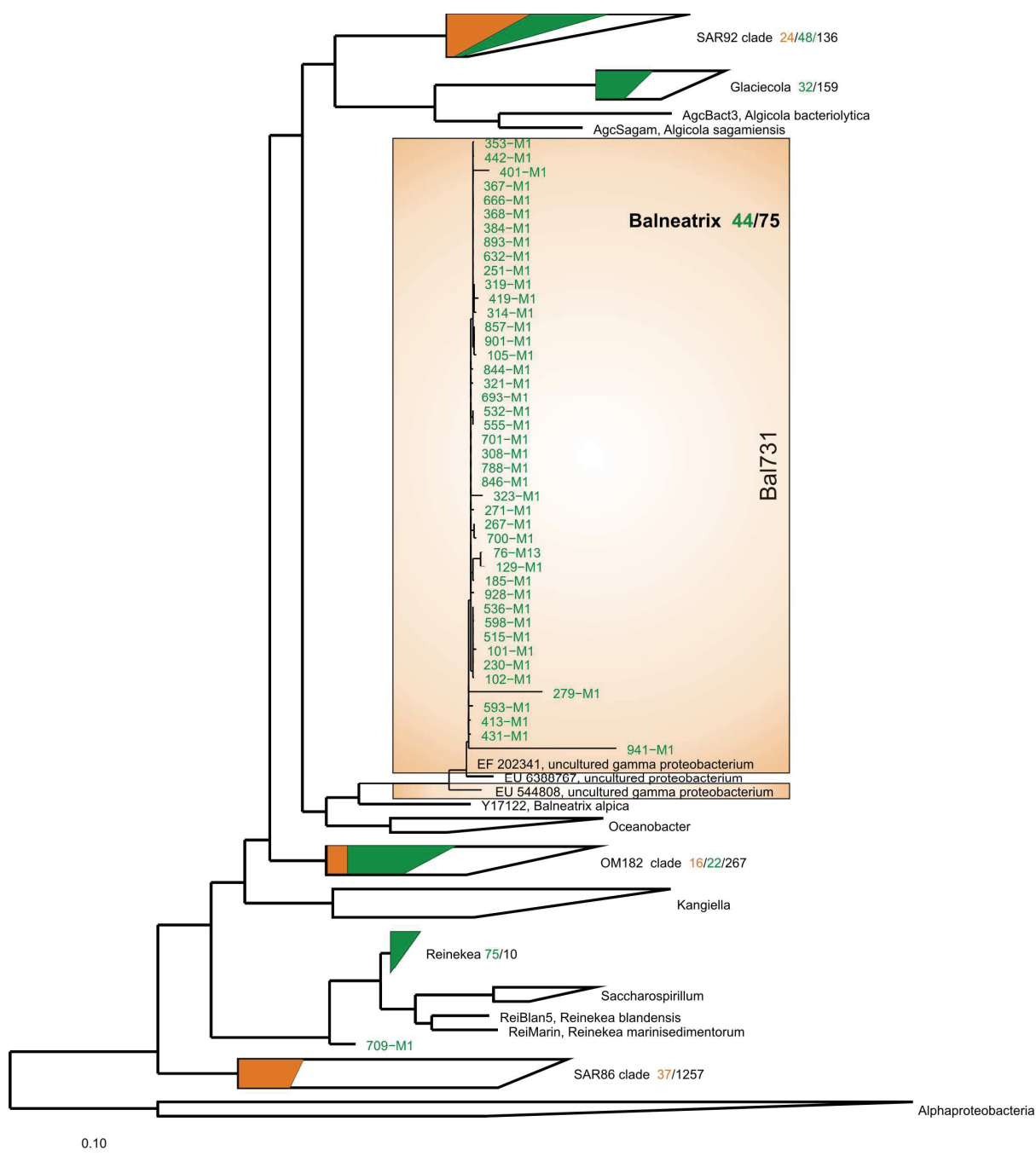


Figure 29 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences, classified as *Balneatrix*-related clade. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers in brackets indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same group already present in the database. Sequences targeted by the newly designed probe Bal731 at zero weighted mismatches are highlighted in boxes. The scale bar represents 10% estimated sequence divergence.

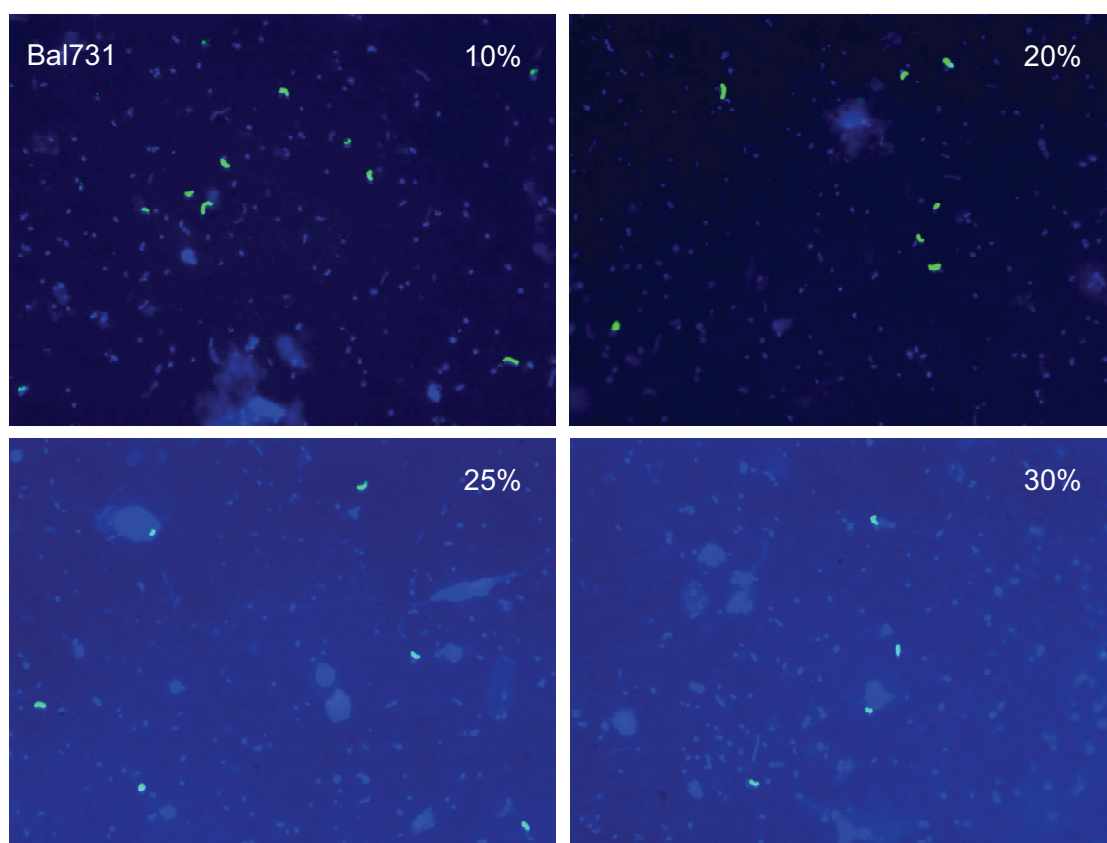


Figure 30 Picture gallery of epifluorescence micrographs showing the signal intensity obtained by probe Bal731 at different FA% concentrations when tested on environmental sample using CARD-FISH.

Probe	No. of weighted mismatches	Binding site
Bal731		
target		3' CGT GCG GGA CCG AAC TAT 5'
		5' CGA CGC CCU GGC UUG AUA 3'
<i>Balneatrix</i> uncultured bacteria	0.0	*** **
<i>Balneatrix</i> uncultured bacteria	1.1	*** ** A *** **
Eukarya	1.6	GGG * G * *** G ** *** ** U
OM182 clade	1.7	** G * C * *** **

Table 8 Fullmatch and mismatch reference 16S rRNA sequences showing probe-binding sites for the probe designed for *Balneatrix*-related clade according to the ARB v5.2 Probe Match Tool. Normal letters stand for weak mismatches and bold letters for strong ones.

Results

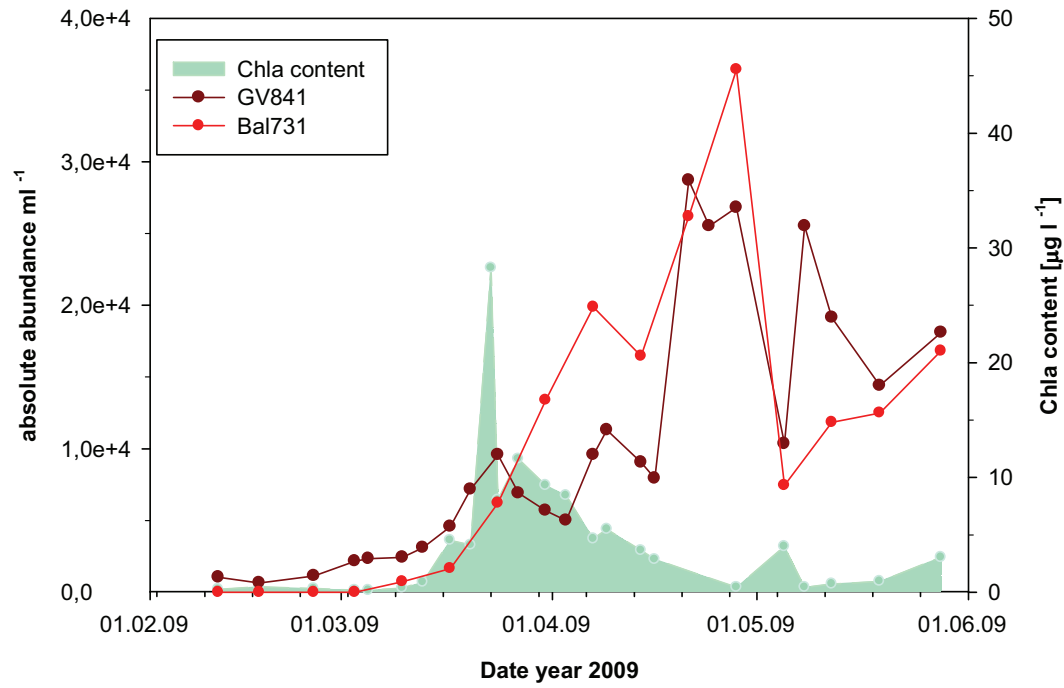


Figure 31 Absolute abundances of the phylogenetic groups of *Balneatrix*-related clade and *Vibrio* in year 2009.

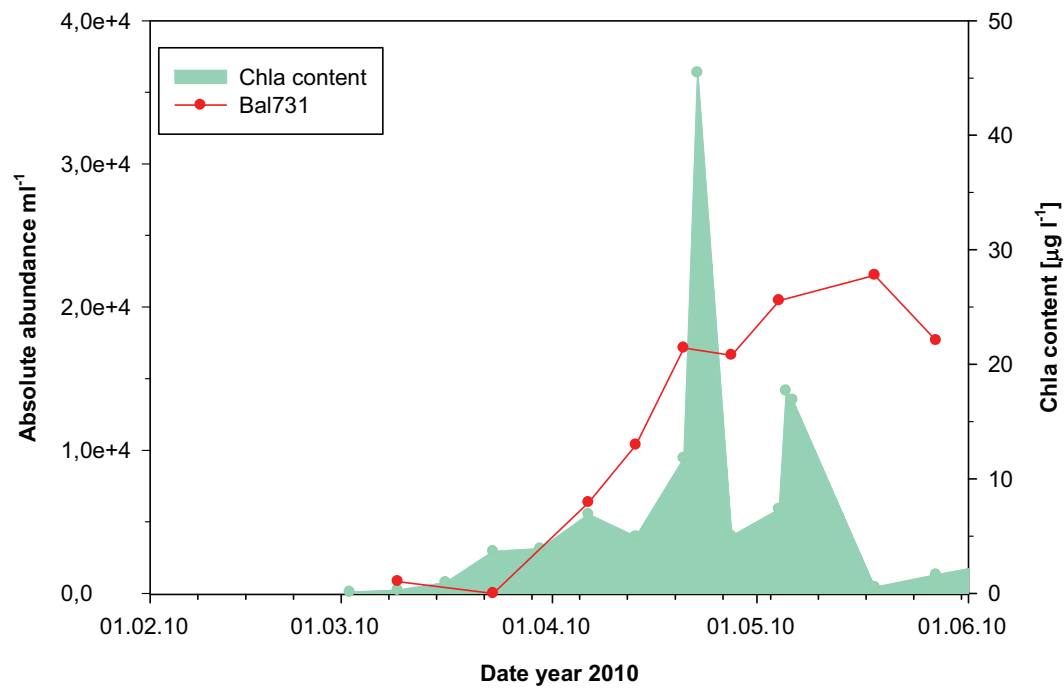


Figure 32 Absolute abundance of the phylogenetic group of *Balneatrix*-related clade in year 2010.

3.3.6 *Glaciecola*

In the spring library, 32 sequences were affiliated as *Glaciecola* such comprising 4% of the clones. The clones formed one cluster with 96% to 99% similarity and their similarity to the various previously isolated strains of *Glaciecola* ranged from 91% to 98%. Just clone 444-M1, was 5% to 7% distant from this cluster, but was 98% similar to the previously isolated *Glaciecola nitratreducens* (**Figure 33**). Two probes targeted the group of *Glaciecola*, probe Glac1276 and probe Glac227. However, only the second one could show signals *in situ* and the optimal hybridization conditions were obtained using 30% FA. The probe targeted all of the clones except the most distant one. It targeted the reference strains *Glaciecola polaris*, *G. psychrophila*, *G. mesophila* and *G. punicea*. It had at least two mismatches (one strong and one weak; 1.5 weighted mismatches) with non-target sequences. The details of the probe mismatches are given in (**Table 9**).

Also *Glaciecola* absolute counts were below the detection limit during the winter but increased up to 1×10^4 cells ml⁻¹ just after the algal blooming peak. The counts started to decline end of April and was barely detectable during May (**Figure 34**). In year 2010 *Glaciecola* counts were firstly detected by the beginning of the bloom and ranged around 2×10^3 cells ml⁻¹ till mid April. They increased to 4.7×10^3 cells ml⁻¹ during the algal peak and reached their maximum of 5.5×10^3 cells ml⁻¹ one week later then drastically declined down to 5×10^2 cells ml⁻¹ (**Figure 35**).

Results

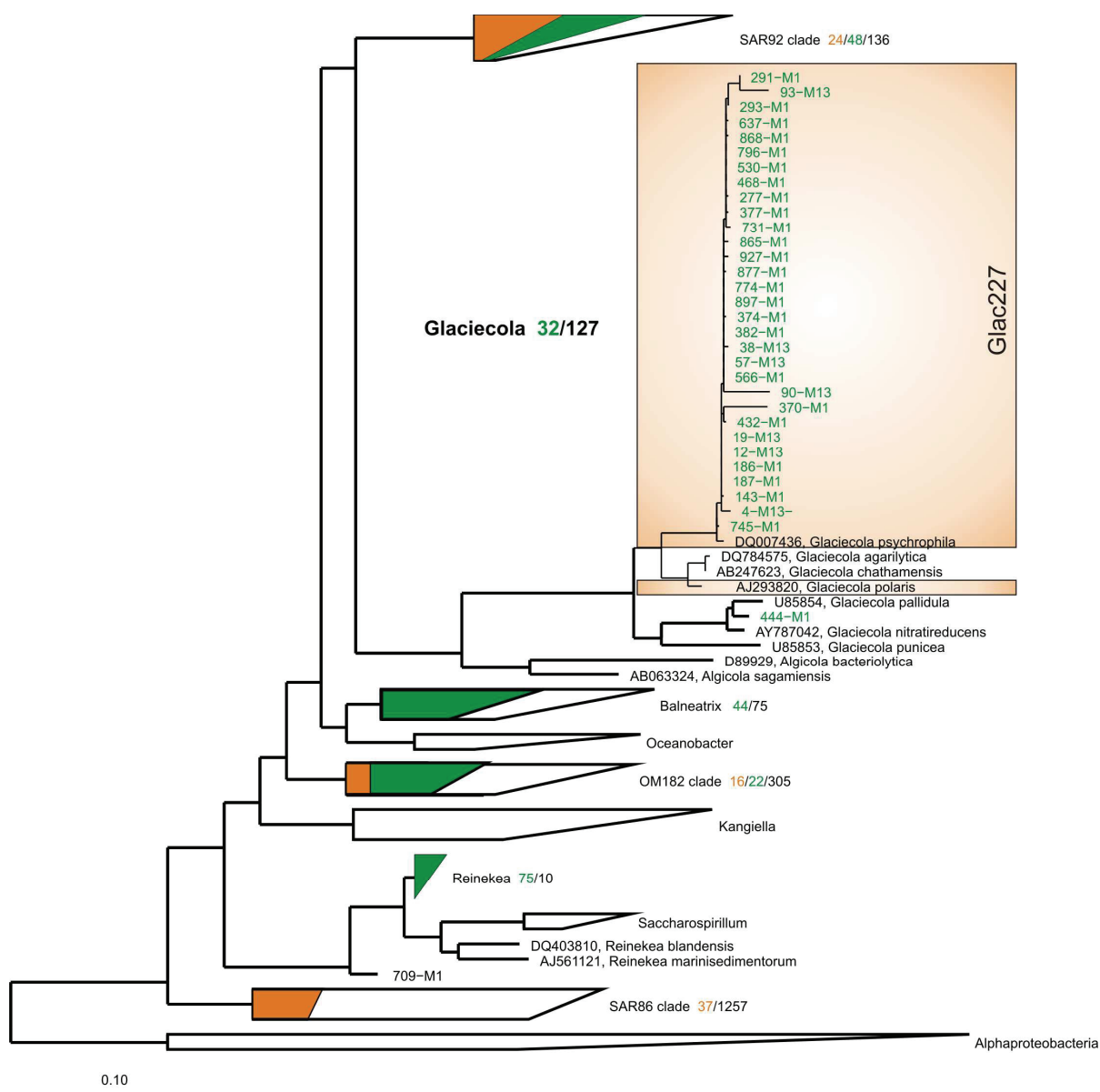


Figure 33 Phylogenetic RAxML tree reconstructed based on the 16S rRNA of members of the Gammaproteobacteria showing the relationship of the 16S rRNA clone sequences classified as *Glaciecola*. Sequences retrieved in this study are pointed out by a color code (winter clone library: orange; spring library: green). The numbers in brackets indicate the numbers of sequences retrieved in the corresponding library versus the number of sequences of the same group already present in the database. Sequences targeted by the newly designed probe Glac227 at zero weighted mismatches are highlighted in boxes. The scale bar represents 10% estimated sequence divergence.

Probe	No. of weighted mismatches	Binding site
Glac1276		
target		3' GCC TAG GGA ATT GCT AGC 5'
		5' CGG AUC CCU UAA AGC UCG 3'
<i>Glaciecola polaris</i> , <i>G. chathamensis</i> , <i>G. mesophila</i>	0.0	*** **
<i>Pseudoalteromonas tunicata</i>	1.1	** A *** **
<i>Glaciecola punicea</i>	1.1	*** * C * **
Glac227		
target		3' TCA CCG GAT GCT CTC TAA 5'
		5' AGU GGC CUA AGC GAG AUU 3'
<i>Glaciecola polaris</i> , <i>G. psychrophila</i> , <i>G. mesophila</i> , <i>G. punicea</i>	0.0	*** **
<i>Glaciecola lipolytica</i>	0.7	*** ** ** * U ***
<i>Glaciecola agarilytica</i> , <i>G. chathamensis</i>	1.3	*** U ** ** ** **
<i>Bowmanella denitrificans</i>	1.5	** A *** ** * U *** **
uncultured Bacteroidetes (2)	1.6	*** ** ** ** * A * G *
<i>Marinobacter</i> (1)	1.6	G ** * U U ** ** ** * G
uncultured Planctomycetae bacterium	1.6	*** ** G ** ** ** * G *

Table 9 Fullmatch and mismatch reference 16S rRNA sequences showing probe-binding sites for the probe designed for *Glaciecola* according to the ARB v5.2 Probe Match Tool. Normal letters symbolize weak mismatches and bold letters symbolize strong ones.

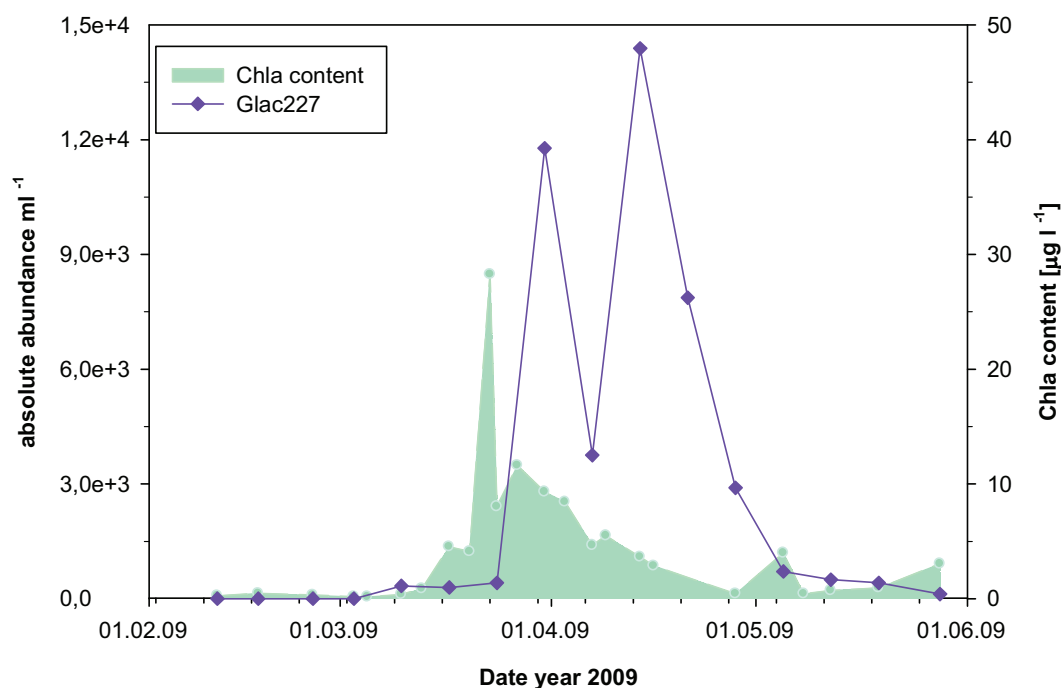


Figure 34 Absolute abundances of the genus *Glaciecola* in year 2009.

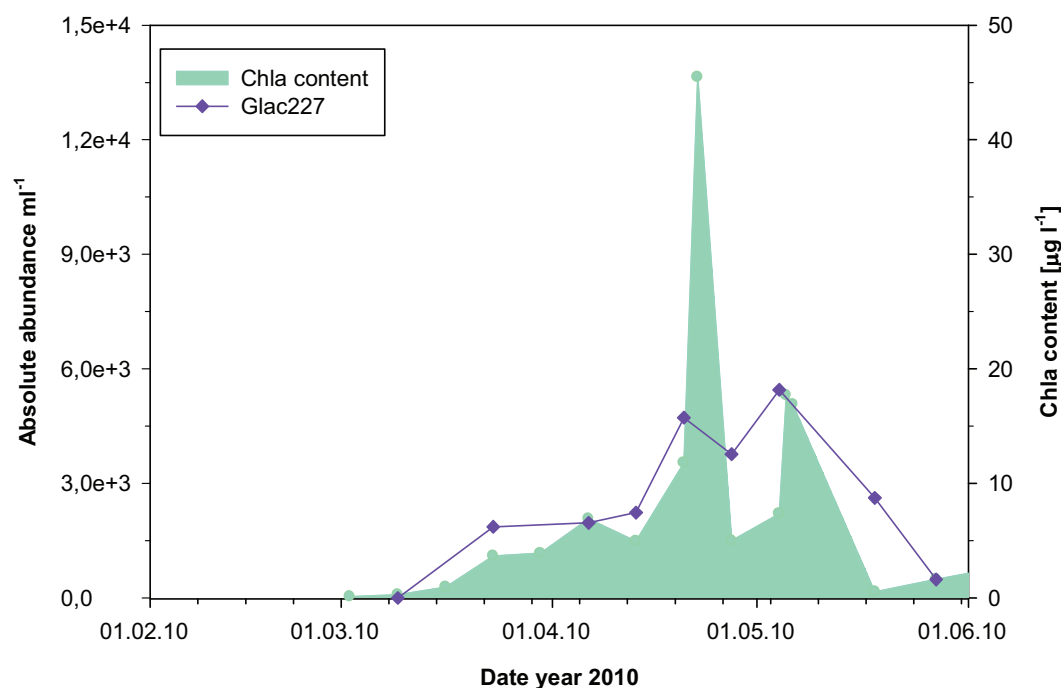


Figure 35 Absolute abundances of the genus *Glaciecola* in year 2010.

3.3.7 *Pseudoalteromonas* and *Colwellia*

No clones were found for *Pseudoalteromonas* in the winter clone library, but 8 clones were retrieved in the spring clone library. For the genus *Colwellia* 8 clones were retrieved, all in the spring library. Probe PSA184 perfectly targeted 4 *Pseudoalteromonas* clones and 4 *Colwellia* clones. In addition, it covered 50% of the *Glaciecola* sequences present in the database and targeted all of the *Glaciecola* clones. The PSA184 probe has at least one strong mismatch (0.8 weighted mismatches) with *Psychromonas* species. The probe was used to determine the absolute counts for the genus *Pseudoalteromonas* and *Colwellia*. The counts ranged between 4×10^2 cells ml^{-1} and 4×10^3 cells ml^{-1} in the winter. Just after the bloom within one week, the counts increased up to 8×10^3 cells ml^{-1} and further increased to 9×10^3 cells ml^{-1} by mid April (14th April). However, by beginning of May the counts were already dropped to 1.6×10^3 cells ml^{-1} (Figure 36).

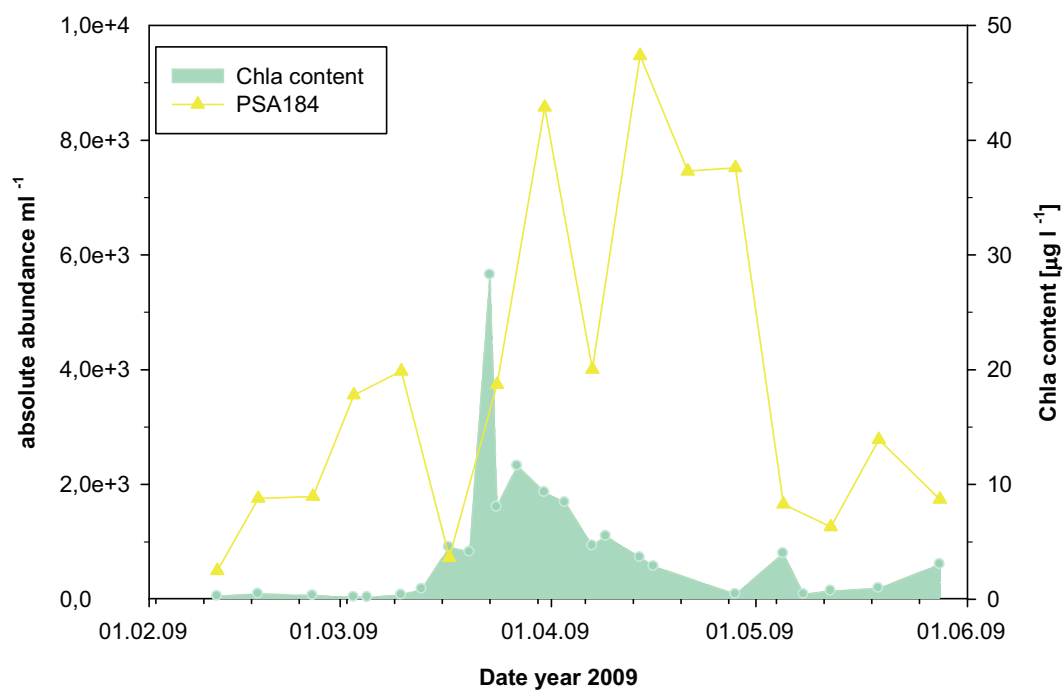


Figure 36 Absolute abundances of the genera *Pseudoalteromonas/Colwellia* in year 2009.

Discussion

4 Discussion

Seasonal changes in bacterial diversity have been previously reported (2), especially those linked to a spring algal bloom (1), (25), (101). It has been assumed that there are specific factors and effects controlling specific bacterial populations (103). Temperature was previously found to be the most important factor influencing the bacterioplankton composition (64), and the blooming of phytoplankton was found to display strong effects on the bacterial community, as previously reported for the bacterioplankton community of Helgoland Roads (103).

The physico-chemical and biological parameters measured during the winter–spring transition at the Kabeltonne Station, Helgoland provided a descriptive overview to the environmental changes that had been taking place in the North Sea during that time. Such, the increase of the chlorophyll content by end of March 2009 was attributed to an algal bloom- mostly diatoms, as indicated by the peak on the 23rd March (**Figure 3**). This phytoplankton bloom might have been enhanced by the increase of temperature. In the same time, the decrease of water salinity revealed an accumulation of freshwater near the coasts and a following concentrated transport of this freshwater body, most likely from river Elbe to the sea, and thereby, an inflow of various inorganic nutrients. This was strongly indicated by the sudden increase of various nutrients concentrations such that of silicate, nitrite and nitrate coinciding with the freshwater intrusion on 20th March (**Figure 4**). The availability of such nutrients seems to have enhanced the diatom blooming. However, the inorganic nutrient concentrations decreased drastically just after the blooming peak indicating their rapid consumption by the algae. As primary producers, the blooming phytoplanktons are known to produce a high amount of dissolved organic carbon, which then triggers the succession of bacterioplankton communities (10). The steady decrease of the chlorophyll content after the blooming peak indicated the death of the previously dominating diatoms. The decay of phytoplankton resulted in the release of other organic mainly polymer compounds which seemed to further enhance the succession of certain bacterioplankton communities. This succession was also indicated by the increase of total bacterial cell counts after the bloom (**Figure 5**). In this study, the provided 16S rRNA gene tag sequences (Anna Klindworth, Microbial Genomics Department, Max Planck Institute for marine Microbiology, Bremen, Germany) in combination with 16S rRNA gene clone libraries gave insight into the diversity and community structure of the North Sea bacterioplankton at the winter – spring transition period in year 2009. CARD-FISH allowed a close monitoring of the changes occurring within the gammaproteobacterial community composition.

4.1 Overall bacterioplankton diversity and composition

4.1.1 Comparison of GM3 and GM3m primers

The discrimination of PCR primers for and against certain sequences has been previously investigated (119), (61). Therefore, it was expected that the application of multiple PCR primer sets would minimize the biases and recover substantially more species. Since the GM3m primer is longer than GM3 primer and has one more variable position, there was a higher possibility of annealing to sequences, not covered by the original primer and such to recover more species. Therefore, both primers were used to initially amplify the DNA extracted from the collected water sample from the North Sea for the winter clone library generation, to assess the necessity of combining both primers. However, both primers showed to have the same ability to cover the community richness and distribution on both phyla and genus level with minor differences in the number of sequences affiliated to the various subgroups (**Figure 10, Table 4**). Only few sequences were detected by one primer and not the other, such as *Lentisphaerae* and *NKB19*, which represented only a low fraction of the libraries community. This finding is supported by the quite high similarity of the various richness estimators values (**Table 3**). However, rarefaction analysis showed that more sequences are required to fully cover the diversity as indicated by the rarefaction curves not yet reaching saturation (**Figure 9**).

4.1.2 Comparison of 16S rRNA gene winter and spring clone libraries – 2009

The winter and spring clone libraries had similar numbers of OTUs at the species level and showed similar values of community richness as indicated by Shannon-Weaver and Simpson indices (**Table 5**). Rarefaction analysis revealed that the number of clones sequenced in the spring library was sufficient to obtain detailed insights into the diversity of the bacterial assemblage as indicated by the saturation tendency of the corresponding rarefaction curves at both species and genus level. Yet the coverage of diversity is incomplete and more sequences are still required, especially for the winter library, to improve the coverage of the bacterial diversity (**Figure 11**).

Both winter and spring clone libraries gave first insights in the microbial community of the North Sea at the long-term ecological research station Helgoland Roads in the winter and the spring of year 2009. However, it is important to mention that the fraction of sequences affiliated to a certain group within the clone library is not necessarily reflecting the relative abundance of the group within the community since the number of sequences generated are

influenced by the PCR bias and the variable *rrn* operon copy number of each organism. Thus PCR primer may be discriminated against certain templates and the amplification of the more abundant templates may be inhibited by self-annealing during the PCR process (61). Genomic properties such as genome size, copy number of 16S rRNA genes, and G+C content would also influence the PCR product ratios (55). The heterogeneity of *rrn* operon copy number may lead to an over- or underestimation diversity of a certain group according to the number of sequences amplified. This is why diversity would be only qualitatively analyzed by clone libraries.

Overall, the community was dominated by four bacterial groups in both seasons: Alphaproteobacteria, followed by Gammaproteobacteria, Betaproteobacteria and Bacteroidetes in the winter (**Figure 12**). These groups ought to be expected in marine surface water. Alphaproteobacteria, especially members of the SAR11 clade, are among the most common prokaryotes in the marine plankton (78). SAR11 bacteria are the most abundant bacterial group in the Atlantic Ocean (107). They may comprise between 24% and 55% of prokaryotic cells at the Bermuda Atlantic Time Series (BATS) station (78), and similar proportions were found in the Atlantic Ocean (72), (74), the Mediterranean Sea (2) and the coastal North Sea (5). Members of the NAC11-7 lineage which are related to the *Roseobacter* spp., are another common component of coastal and offshore microbial assemblages, and they may constitute up to 25% of the marine bacterioplankton. Also Betaproteobacteria are found predominantly in freshwater and coastal areas. The water mass displacement at the sampling station could be a reason why the betaproteobacterial OM43 clade was detected in the winter but not in the spring. The Elbe river has a strong impact on the tidal cycles when there are eastern winds and this might lead to the appearance or disappearance of certain groups. However, further studies are needed within this context to determine whether such impacts can be correlated with detection or disappearance of specific bacterial species.

A substantial community change has been observed within the winter-spring transition, where the community was overtaken by members related to Bacteroidetes and Gammaproteobacteria in the post-blooming phase, according to the spring clone library. The drastic change of the diversity distribution within these two major groups seems to be strongly related to the algal blooming event.

Considering Bacteroidetes, members of this phylum are among the most abundant groups of bacteria in coastal marine waters (65). Previous studies confirmed that members belonging to this phylum are remarkably responsive to phytoplankton blooms (90). Various lines of evidence suggested that some members of this phylum play an important part in the degradation of complex and polymeric organic matter (65). This is why these bacteria could be particularly important in the processing of organic matter during such events, since they

Discussion

also have been found not only free living but also attached to organic aggregates (90). Moreover, Bacteroidetes, particularly *Flavobacteria*, have been previously found in high abundances during natural and induced phytoplankton blooms and as primary colonizers of marine phytoplankton, suggesting a potential role as consumers of algae-derived metabolites (6). Thus the appearance of certain flavobacterial groups in the post-blooming phase could be explained by recent findings about several flavobacterial groups. For instance, *Polaribacter* was found to contain a substantial number of genes for attachment to surfaces or particles, gliding motility and polymer degradation (57); this, in addition to membrane transporters which play important roles in making use of available nutrients. Thus, proteins and polysaccharides provided by the algae, represented important carbon and nitrogen sources for this flavobacterial group. In addition, the whole genome and functional analysis of the predicted proteome of '*Gramella forsetii*' KTO803 revealed a substantial suite of genes encoding hydrolytic enzymes, a predicted preference for polymeric carbon sources and a distinct capability for surface adhesion which suggest a clear adaptation of this marine *Bacteroidetes* representative to the degradation of high molecular weight organic matter (13). Furthermore, *Ulvibacter* was identified as a member of a microbial community whose metagenome was enriched with carbohydrate metabolism genes (42). This supports the assumption that this group could be also involved in this high-molecular weight dissolved organic matter consumption. Although, these findings agree well with the currently life strategy of marine Bacteroidetes, further investigations need to be conducted to find out more about these groups, for instance about their free living and attached fraction.

Bacteria belonging to the Gammaproteobacteria were abundant throughout the winter and spring of year 2009. Together with the phylum of Bacteroidetes, they dominated the peak in prokaryote abundance following the spring bloom (**Figure 37**). Generally, the Gammaproteobacteria are very diverse and abundant in coastal and temperate surface waters. Previous studies have shown that members of the Gammaproteobacteria respond to phytoplankton blooms with an increase in abundance. Members of this group are thought to colonize and contribute to the mineralization of organic aggregates released during the wane of phytoplankton blooms. Many groups known to be able to degrade carbohydrates are assigned to this subphylum as well as they are well known for their role in marine polysaccharide degradation (42). Furthermore, they showed to increase in abundance during the decay of algal blooms (90). Although the ecology of marine representatives of these bacteria remains largely unknown, it can be expected that they contain substantial physiological and metabolic variability considering the ample phylogenetic diversity within this subphylum.

The libraries showed that there are some gammaproteobacterial groups which are present as part of the bacterioplankton community despite of the season such as the SAR92, OM182

clades and the NOR5/OM60 cluster. Genera strongly increasing in the spring such as *Reinekea*, *Balneatrix*-related clade and *Glaciecola* seem to respond to the substrate availability caused by the bloom (bottom-up effect). On the contrary, other gammaproteobacterial groups, which are usually present in the community but decreased in spring, like members of the SAR86 clade are apparently negatively influenced by the phytoplankton. This clade was the most dominant gammaproteobacterial group in winter but seem to be overgrown at the time of the bloom.

Thus, strictly monitoring the succession of such defined gammaproteobacterial groups will increase the understanding of their role in the microbial community especially at that time of the year. Furthermore, investigating the possibility of such process to annually reoccur will further explain a lot about factors that contribute to the succession of these groups. This was achieved by using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH).

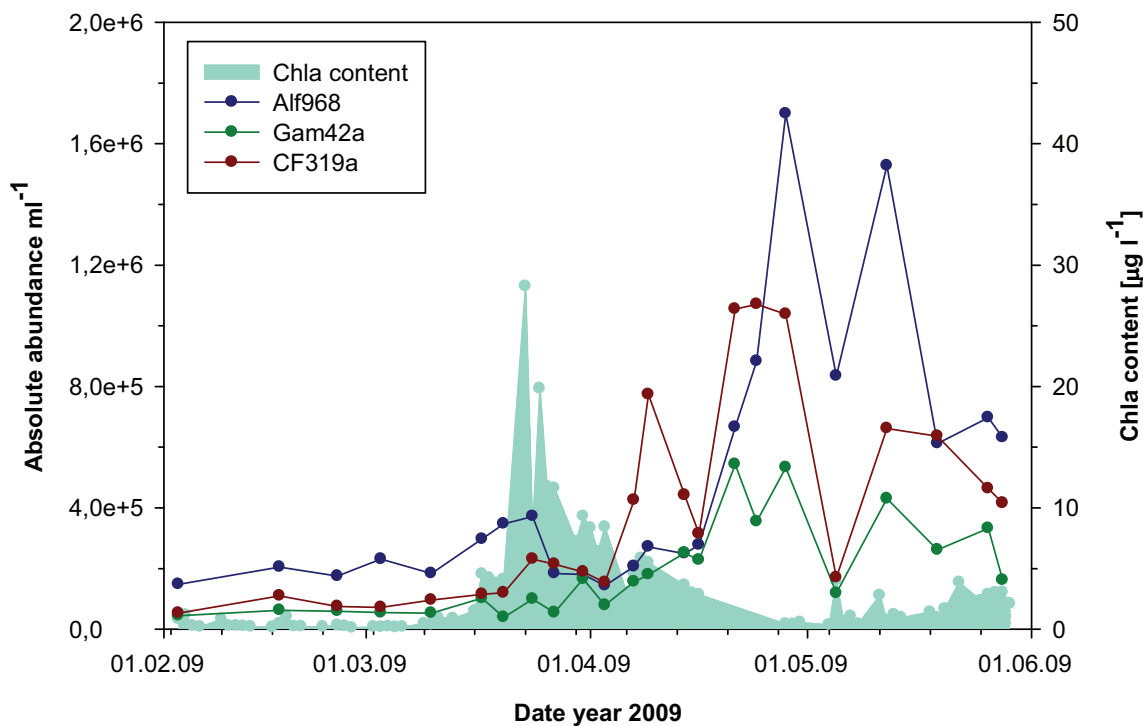


Figure 37 Absolute abundances of major phylogenetic groups in the North Sea from February to May 2009.

4.2 CARD-FISH counts for Gammaproteobacteria – 2009

Bacterial succession would not be only triggered by phytoplankton bloom, since several other biotic and abiotic factors contribute to such succession as well. However, it is often the combination of several factors or the absence of certain factors at a given time that result in the succession of certain groups in a certain time frame (28). Bottom-up effects such as substrate supply (i.e., dissolved and particulate organic and inorganic nutrients) (30) as well as top-down effects, comprising protistan grazing (97) and viral lysis (18), represent other strong factors that tandemly shape the bacterial community structure. Furthermore, tidal cycles and constant change of water masses might interfere with the monitoring of such a succession or the rapid decline of a certain bacterial bloom. As an open coastal area, Helgoland Roads are characterized by strong tidal currents and strong flushing rates (109) combined with constant freshwater inflow from rivers Elbe and Weser, which might contribute to the sudden appearance or disappearance of a certain group within the community.

In this study, CARD FISH counts with group-specific probes showed a community shift within the Gammaproteobacteria subphylum in spring 2009. Groups such as *Reinekea*, SAR92, SAR86, OM182 clades and NOR5/OM60-730 played an important role into this shift and dominated in a tight successive pattern after the algal bloom. Although the absolute abundances of other groups such as *Balneatrix-related clade*, *Vibrio*, *Glaciecola* and *Pseudoalteromonas* remained relatively low within the community after the bloom they also participated into this shift. Thus, these groups responded differently and with a different onset of action to the bloom. Except for the SAR86 clade, the rest of the gammaproteobacterial groups seemed to respond to the substrate availability caused by the bloom (bottom up-effect). However, the onset of succession differed between the groups.

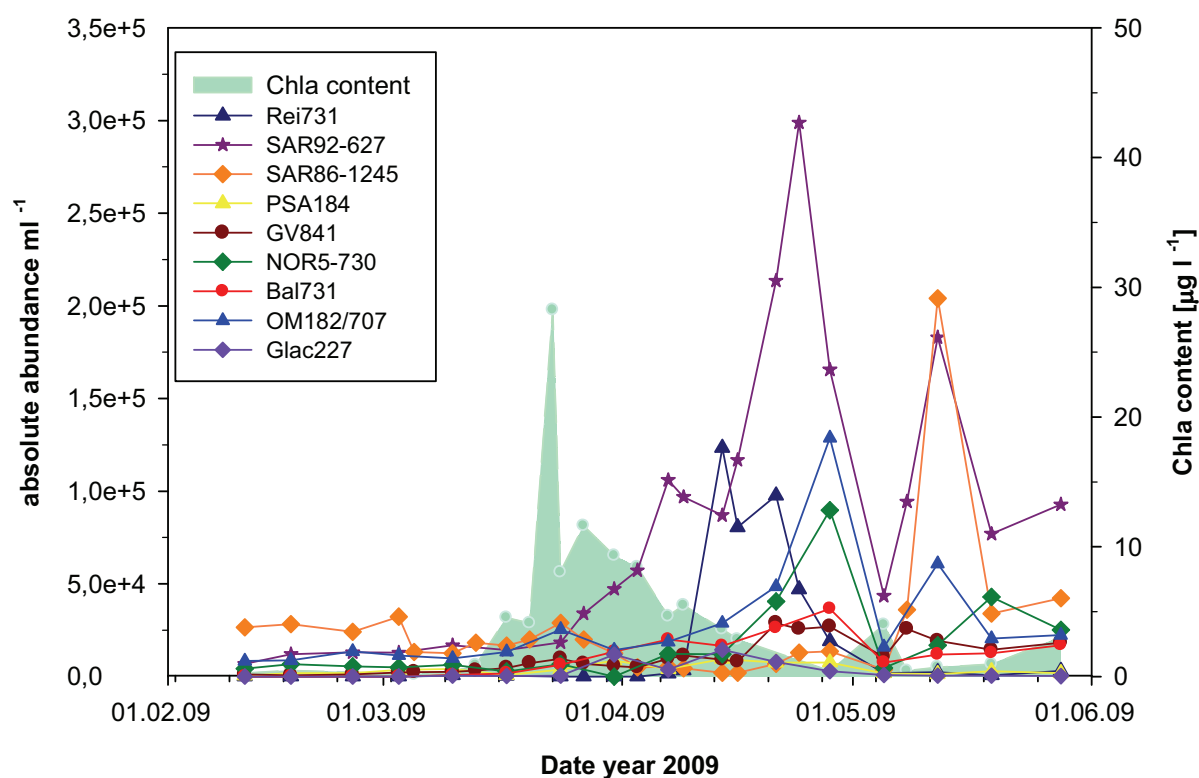


Figure 38 Absolute abundances of the main gammaproteobacterial groups as determined by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) in the North Sea from February to May 2009. The target of each probe is detailed in ‘Materials and Methods’. The figure is only to show the proportionality of the abundances between the various groups.

SAR92 clade

According to the CARD-FISH counts, the absolute abundance of SAR92 started gradually to increase just after the algal bloom peak on the 23rd March, increased to 1.0×10^5 cells ml^{-1} in two weeks and continued to reach up to 3.0×10^5 cells ml^{-1} on the, one month after the bloom (**Figure 38**). This is in good agreement with the fact that the growth rate of SAR92 clade is directly affected by the increase of nutrient concentrations, despite of being oligotrophic (117). The immediate succession of that group seems to be directly promoted by the bloom derived organic substrate. One good reason might be the possession of PR proteins by this clade which might be leading to an effective use of the produced carbon. Recent metaproteomic findings annotating TonB-dependent transporters for that clade further suggest the involvement of that group in oligomer/polymer uptake and degradation (Teeling, H., Gardebrecht, A., personal communication). Although most of the SAR92 clade cells were found usually unattached during microscopical examination, a large fraction of the cells were found in the vicinity of phytoplankton cells or attached to macroscopic organic matter particles. This was observed during the succession of the SAR92 clade, especially at the peak

Discussion

of their bloom (around the 24th April) (**Figure 39**). This further suggests a clear adaptation of this clade to the degradation of oligomers and, possibly, higher molecular weight organic matter. The dominance of the SAR92 clade over other gammaproteobacterial groups during the wane of the phytoplankton bloom makes it evident that this clade optimally benefits from the algal bloom as a carbohydrate degrading bacteria. In addition, the retrieval of two different clusters for SAR92 clade in winter and spring clone libraries respectively (**Figure 18**) indicates different substrate preference and microbial activity within the clade which might be mainly attributing to its rapid growth and its consequent dominance during the spring. However, further genomic information might illustrate more about the microbial activity of this clade and its growth pattern.

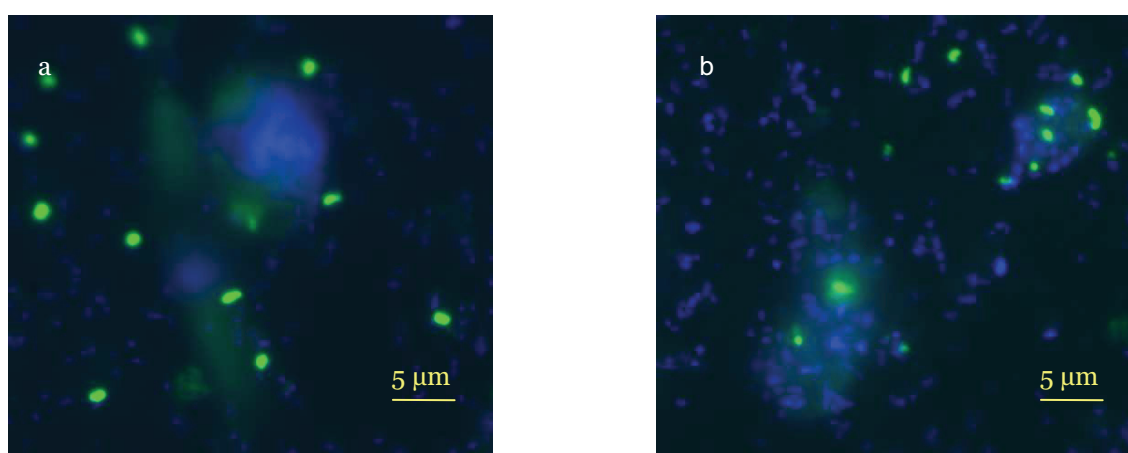


Figure 39 Epifluorescence micrographs showing cells of the SAR92 clade in the vicinity of a phycosphere of a phytoplankton cell (12.05.09) (a) and attached to particles (28.04.09) (b).

Reinekea

The succession of the SAR92 clade was accompanied by a tight succession of the genus *Reinekea* reaching up to 12×10^4 cells ml^{-1} (**Figure 38**). The *Reinekea* bloom seems to be also enhanced by the availability of the organic substrate. Polysaccharide degrading enzymes have been previously annotated for that group (27), and the genome sequenced for *R. marinsedimentarium* revealed the presence of genes involved in attachment and that of hydrolytic enzymes including a considerable fraction of ABC transporters (<http://www.ncbi.nlm.nih.gov/>). In addition, preliminary metaproteomic data recently revealed that carbohydrate-active enzymes, prominently expressed during the bacterial bloom, were mostly annotated to the genus of *Reinekea* (still unpublished). Accordingly, these proteins are involved in binding to polymers, such as chitin and peptidoglycan, such proposing that *Reinekea* might be potentially specialized in using proteins and other polymers for growth. However, the abundance of genes involved in attachment and the range

of hydrolytic enzymes is still not well known about that genus and needs to be further investigated. The large cell size of *Reinekea* makes it a potential candidate for grazing by protists (top-down effect) which might further explain the rapid decline for its bloom (14), (39).

In addition, this genus seems not to be originally present in the water column but firstly brought to the surface layer of the sea by the strong tidal currents of the southern North Sea (109). This explains its sudden appearance after the bloom and its complete disappearance complete disappearance from the water column after the depletion of the algal derived organic matter by beginning of May. This hypothesis could be further supported by the fact that strains belonging to this genus were originally isolated from sediment samples such as *R. marinsedimentarium* (99) and *R. aestuarii* (33). Nevertheless, this is not to exclude the possibility of natural existence of this genus in a very low abundance in the water column in the North Sea.

NOR5/OM60 and OM182 clades

A further succession of members of the OM182 clade was detected by end of April, when the absolute abundance of that group increased from 2×10^4 cells ml⁻¹ to 12×10^4 cells ml⁻¹ coupled by an increase of the absolute abundances of the NOR5/OM60 clade from 1×10^4 to 9×10^4 cells ml⁻¹. The presence of these two groups throughout the year is common since they are oligotrophic (31). In addition, members of the cultured NOR5/OM60 clade are known to form substantial populations in the German Bight during all seasons (14). However, this is the first study to give an indication about the abundance of the OM182 clade in the North Sea. Accordingly, it seems that this clade also forms a substantial part of the bacterioplankton community in the German Bight. The similar succession patterns for these two clusters might be an indication of similar activities. However, their blooms seem to be strongly influenced by the substrate availability of the algal bloom. Although no much is known about the OM182 clade genome, the diverse metabolic potential among members of the NOR5/OM60 clade makes this clade a potential utilizer for substrates derived from the algal bloom. This clade primarily encompasses AAnP bacteria that participate in the marine carbon cycling to a great extent.

Phytoplankton are known not only to release large amount of carbohydrates (1) but also a substantial amount of lipids and fatty acids during spring blooms (104), which contributes to the overall organic carbon production (76). As parts of the phytoplankton cellular content (17) and structural and functional component of the cell membrane (135), triglycerides and phospholipids must be also released into the environment during the wane of the bloom. Degradation of such complex lipids often requires specific extracellular enzymes. Genomic

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analyses of the reference strain KT71 revealed the presence of several genes coding for putative lipase/esterases and proteases/peptidases that might be involved in the breakdown of lipids and peptides. Furthermore, the lipase/esterase activity was confirmed for this strain. Although this strain is not able to degrade complex proteins or polysaccharides, it might prefer to utilize oligopeptides or partly degraded proteins, released by the algae. This is supported by the fact that this strain harbors two transporters for oligopeptides with up to five amino acids, *oppABC* (KT71_06839–06854) and *oppF* (KT71_00435) (49). Also ABC transporters for amino acids were found in the IMCC3088 genome (63). Furthermore, members of the NOR5/OM60 clade seem to indirectly benefit from the algal bloom since there is microscopic evidence that they are able to attach to macroscopic particles. By attaching to the surface of organic particles, they may directly use oligomeric substrates or they may commensally benefit from the exoenzymatic activities of the blooming polymer-degrading bacteria (49).

Three studies on North Sea surface water had reported NOR5/OM60 blooms up to 13% co-occurring with algal bloom (14), and similar observations were recently reported for other coastal areas (2), which strongly suggests that the photoheterotrophic members of the NOR5/OM60 clade benefit from algal photosynthesis (134).

Other members of this clade also harbor a wide variety of hydrolytic enzymes and enzymes involved in various metabolic pathways, where glucose is required as initial substrate, such indicating that these bacteria are using a wider spectrum of substrates as carbon and energy source (63).

SAR86 clade

By beginning of May, the absolute abundances of the groups *Reinekea*, SAR92, OM182 and NOR5/OM60 clades significantly decreased, which could be caused by the grazing effect of protists and viruses to a large extent (top-down effect). However, the smaller cells of the SAR86 clade (45) seemed to be able to escape such grazing effect as indicated by its sudden increase. This was observed when the absolute abundance of this group increased from counts around 2.0×10^3 cells ml⁻¹ culminating in a peak of 2.0×10^5 cells ml⁻¹ by 12th May (**Figure 38**). The common presence of the SAR86 clade as part of the bacterial community in the North Sea has been previously reported (85). The dominance of this clade over other gammaproteobacterial groups during the winter could be due its possession of the light-dependent proton pump PR so that it could effectively use the concentration-limited organic carbon (26). In addition, earlier findings showed that SAR86 population constituted 8.1% of the total bacterial population but also approximately 50% of all DNA-synthesizing bacteria in coastal North Sea during early autumn, a season in which there is generally declining bacterial activity (87). This indicated that this clade might be an important contributor to

total bacterioplankton activity in coastal North Sea water during periods of low phytoplankton primary production and furthermore, that the small size and low ribosome content of SAR86 cells are probably not indications of inactivity or dormancy. This might further explain its dominance in the winter among the Gammaproteobacteria. However, this group seemed to be overgrown and outcompeted, due to its small size (46), by larger bacteria, such as *Reinekea* and *Glaciecola* firstly occurring after the algal bloom (**Figure 40**). In addition, the decreased SAR86 counts during the spring are consistent with previous studies reporting about their more rapid growth in late summer than during spring in the German Bight (84).

Although SAR86 and SAR92 clades share common features such as being oligotrophic (123) (31), harboring PR proteins (102), (117), and having small cell sizes (45), (34)), it seems that the winter growth conditions are more favored by SAR86 clade while that of the post-blooming phase by SAR92 clade, according to their abundances. This might be due to different abilities of substrate utilization and further suggests that SAR86 is negatively or not affected by the algal bloom.

On one hand it is not yet known if members of this lineage are able to degrade polymers so, but they are well known of being free living (3). Thus one can speculate their limitation to attach to particles or contribute to the mineralization of organic aggregates, as it is the case for SAR92 clade. This is further supported by previous indications that the activity of SAR86 may not be primarily governed by even the availability of algal derived monomeric substrates, since members of this clade showed similar leucine incorporation patterns during prebloom and bloom situations (3). However, more genetic information might reveal more about the different growth patterns and activity of those two clades.

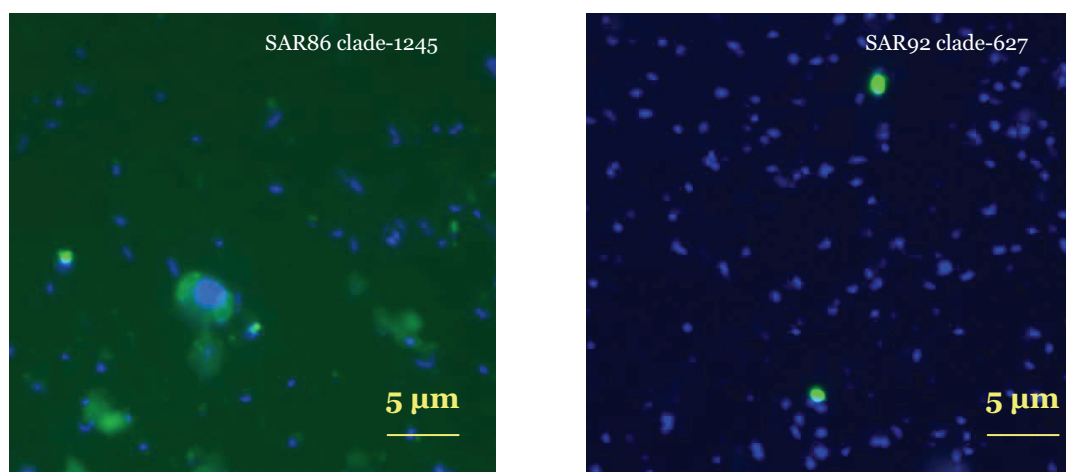


Figure 40 Epifluorescence micrographs showing the size of SAR86 clade cell (20.04.10) in comparison to the size of SAR92 clade cells (05.05.10).

Vibrio and *Pseudoalteromonas*

Despite the low abundances of the genera *Vibrio* and *Pseudoalteromonas*, relatively to the other gammaproteobacterial groups, both groups were clearly positively influenced by the algal bloom due to their abundance increase in April (**Figure 31, Figure 36**). This is supported by the fact that they belong to the attached fraction of the marine bacterial community (46) and their surface-associated life style is commonly known (110). Recent studies have showed that *Vibrio* species are positively affected by the occurrence of algae, because they can utilize the bioavailable dissolved organic substrate released by the algae (79), (44). Thus they can use algal released substrates very efficiently as they attach to algal cells by the formation of biofilms (113). Furthermore, the combination of high temperatures with events of low salinity would promote the growth of *Vibrio* bacteria (73).

Members of the genus *Pseudoalteromonas* are generally associated with higher organisms including a wide range of algae (60). Thus a niche adaptation for the acquisition of substrates for growth from the extracellular digestion of surface-associated polymeric substances and uptake and utilization of their respective monomers was previously indicated by the *P. tunicata* genome analysis (121). The characterization of the largest group of characterized proteins in the *P. tunicata* secretome as hydrolytic enzymes further suggests that the bacterium is an efficient degrader of complex organic matter in the marine environment and thus may play a similar role as the Bacteroidetes group on surfaces. Moreover, chitinolytic activity has been previously displayed in both *Pseudoalteromonas* (121) and *Vibrio* species (62) as surface associated bacteria. However, *Pseudoalteromonas* species seem to play a different role in the vicinity of the algal cells. Their algicidal activity has been previously displayed (60). Several species which produce antifouling metabolites have been isolated from the surface of marine red algae such as *P. tunicata* (121) and *P. ulvae* (43), where they prevent growth by other algae. Thus they might be strongly exerting an algicidal effect on the bloom as has been previously displayed for species of this genus (60). Eventhough not all *Pseudoalteromonas* species have negative effect on algal host surfaces (121). This in addition to the large size and high ribosome content of their cells could be an indication of recent metabolic activity.

The detection of low population densities of these two groups, despite their frequent isolation from the North Sea, have been commonly reported (14). Despite of being copiotrophic and their rapid growth, they seem to be highly grazed by selective predators due to their large size (top-down effect), as previously suggested (14). This further explains the rapid decline of their abundances by May (**Figure 31, Figure 36**). Marine *Vibrio* and *Pseudoalteromonas* bacteriophages were found in the North Sea. They seem to contribute to the rapid death of species belonging to these two genera such as the *Vibrio* phages which are

members of the virus family *Myoviridae* (77), (128) and the *Pseudoalteromonas* phage species H103/1 belonging to the virus family *Siphoviridae* (129).

***Balneatrix*-related clade**

This study is the first to investigate the *Balneatrix*-related clade counts in the North Sea. The occurrence of this clade in marine environment was rarely reported, however one marine strain has been previously assigned to this genus (116). Furthermore, this genus seems not to be originally part of the water column community of the North Sea since it was absent or below the detection limit during the winter as indicated by clone libraries and CARD-FISH, respectively. In contrast to *Reinekea*, the succession of *Balneatrix*-related clade followed a gradual increase of its abundance which indicates that it was brought with the transported freshwater to the sea, especially that it started to appear the same time the water salinity began to decrease in **(Figure 31)**. In addition, GM. Garrity described it previously as a freshwater bacterium in Bergey's Manual for Systematic Bacteriology (52). In contrast to *Reinekea*, *Balneatrix*-related clade did not disappear from the water column in May. Although the cells are large enough, they seem to escape the grazing effect exerted by higher protists, which seem to favor larger cells such as these of the *Reinekea* and *Glaciecola* species.

The affiliation of about 5% of the clones to *Balneatrix*-related clade in the spring clone library supports the CARD FISH counts and further suggests that these sequences, which are 96% to 99% similar, are forming a new marine cluster within the genus. Although *Balneatrix* species are not primarily of marine origin (40), the clones are closely related to the marine uncultured sequences so far found for this genus in the SINA database, sharing 94% to 99% similarity, while they have only 90% to 91% similarity to the pathogenic *Balneatrix alpica*. Since the newly designed probe Bal731 had at least two mismatches with OM182 clade, it is excluded that the probe targeted sequences other than members of the *Balneatrix*-related clade group.

Glaciecola

Although the genus *Glaciecola* was the least abundant group among the Gammaproteobacteria in spring 2009, it also participated in the bacterial community shift and it responded immediately to the bloom as indicated by the CARD-FISH counts **(Figure 34)**. Same as *Reinekea*, this group was not detected in the winter but firstly after the algal bloom. Since this genus is well adapted to diverse marine environments (29), it might have been brought from the North Sea sediments to the water column by the strong tidal cycles or

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it might have been released from the surface of the algae to the surrounding seawater with the senescence of the algae as previously reported(66). *Glaciecola* succession (2) and its association with algal bloom (68) have been previously reported. In depth analysis of the single genome, sequenced so far for this genus, revealed the presence of numerous ORFs encoding carbohydrate active enzymes like glycoside hydrolases, glycosyl transferases and carbohydrate esterases, making this organism a potential polysaccharide degrading bacterium (66). In addition, this genus was identified as a member of a cellulose-degrading microbial community (42) which further suggests that the algal derived carbohydrates were a potential target for degradation by this phylotype. The large cells of *Glaciecola* could have been strongly susceptible to the grazing effect by protists and viruses which further might explains the rapid decline of this phylotype by end of April. CARD-FISH counts detected with probe Glac227 are restricted to the genus of *Glaciecola* since the probe had at least two mismatches with non-target sequences. They are further supported by the affiliation of 32 sequences to this genus in the spring clone library sharing 92% to 98% similarity with the various reference strains. This confirms the occurrence of this genus in the North Sea water column, experiencing a short bloom following the algal bloom.

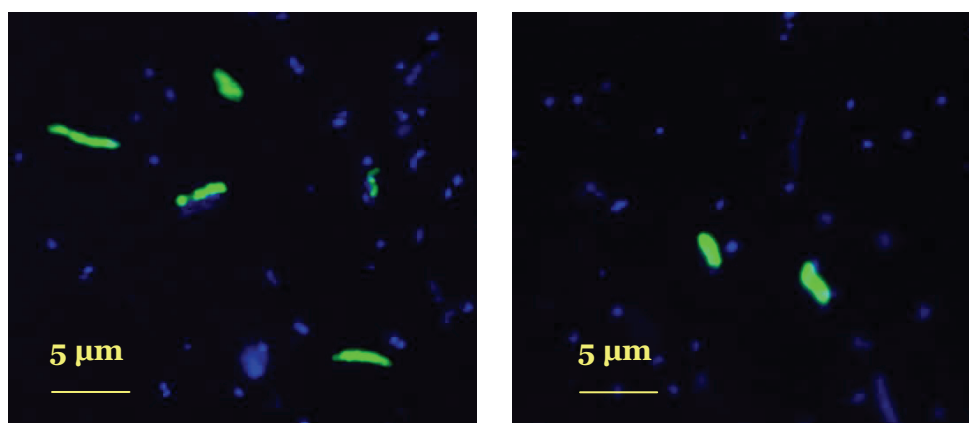


Figure 41 Epifluorescence micrographs showing the size of *Glaciecola* cells (31.03.2009).

4.3 Comparison between 16S rRNA tags and 16S clone libraries

The distribution of 16S rRNA gene tags (provided by Anna Klindworth, Microbial Genomics Department, Max Planck Institute for marine Microbiology, Bremen, Germany) correlated well with sequences obtained in the 16S rRNA clone libraries. Thus the largest fraction of the gammaproteobacterial 16S tags were affiliated to SAR86 clade on 11th February (**Figure 42**), which dominated the winter clone library. Also the largest fraction on 14th April was affiliated to the genus *Reinekea* which comprised about 9% of the total clones in the spring library (**Figure 12, Table 6**). Thus, 16S rRNA tag sequencing presents a promising tool for prescreening of samples since it is quicker and more inexpensive as clone libraries. Although much larger numbers of sequences can be obtained, almost full-length 16S rRNA sequences of high quality are superior for probe design.

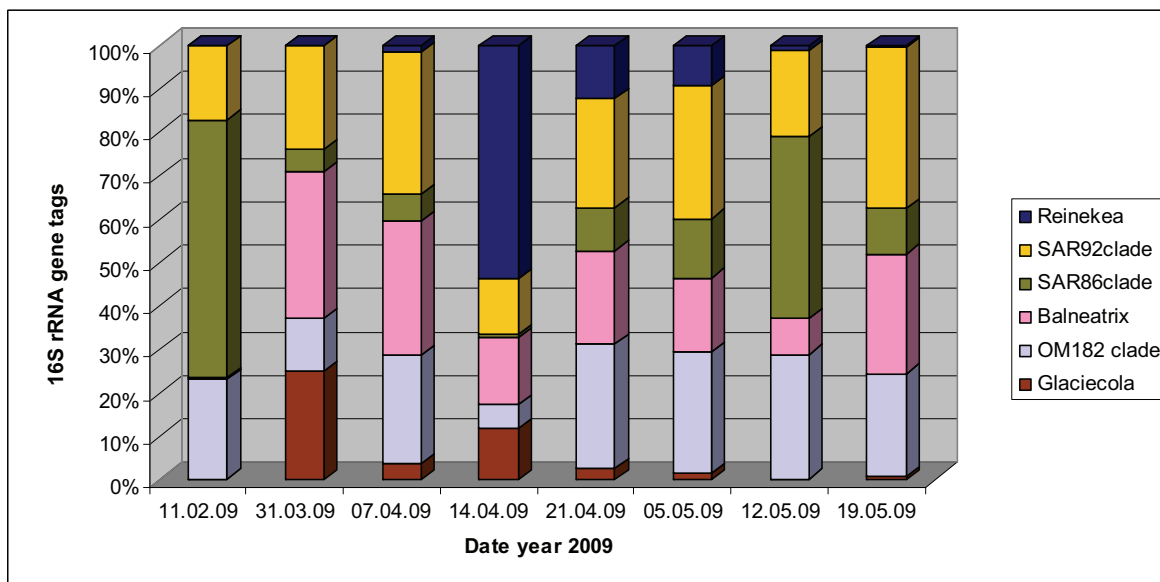


Figure 42 Percentage of 16S rRNA tag reads within the Gammaproteobacteria (Anna Klindworth, Microbial Genomics Department, Max Planck Institute for marine Microbiology, Bremen, Germany).

4.4 Comparison between CARD-FISH counts for Gammaproteobacteria in year 2009 and year 2010

Comparing physico-chemical parameters for the winter-spring transition period in years 2009 and 2010 at Station Kabeltonne, North Sea, a similar picture of phytoplankton succession was observed with only some differences. While the phytoplankton peak was tightly coupled to the first salinity decline in year 2009 a time interval of about 6 weeks separated between both events in year 2010 (**Figure 43**). The pronounced phytoplankton peak was reflected in a sudden increase of the chlorophyll content in 2009. In contrast, a recognizable prebloom phase that lasted for almost a whole month preceded the algal peak in year 2010. In addition, the chlorophyll level was relatively higher in year 2010. In contrast, year 2010 was characterized by lower temperatures of about 2°C in both winter and spring seasons. Salinity values were generally similar in both years as well as the various nutrients values with only some minor differences. In both years, the freshwater discharge of nutrients accompanying the first salinity decline was probably the main trigger for the phytoplankton blooms, in addition to the temperature increase. In year 2009 the two following freshwater intrusions in April/May seemed not to have a significant effect on the chlorophyll level which remained low despite this further inflow of nutrients indicated by the short sharp increase of nitrate concentrations. In contrast, the level of other nutrients remained low suggesting that they might have been limiting for the algae. In contrast, the second salinity decline on 6th May 2010 and the accompanied inflow of nutrients seemed to result into a further smaller chlorophyll peak of 17 µg l⁻¹. This is also supported by higher phosphate, nitrite and nitrate values.

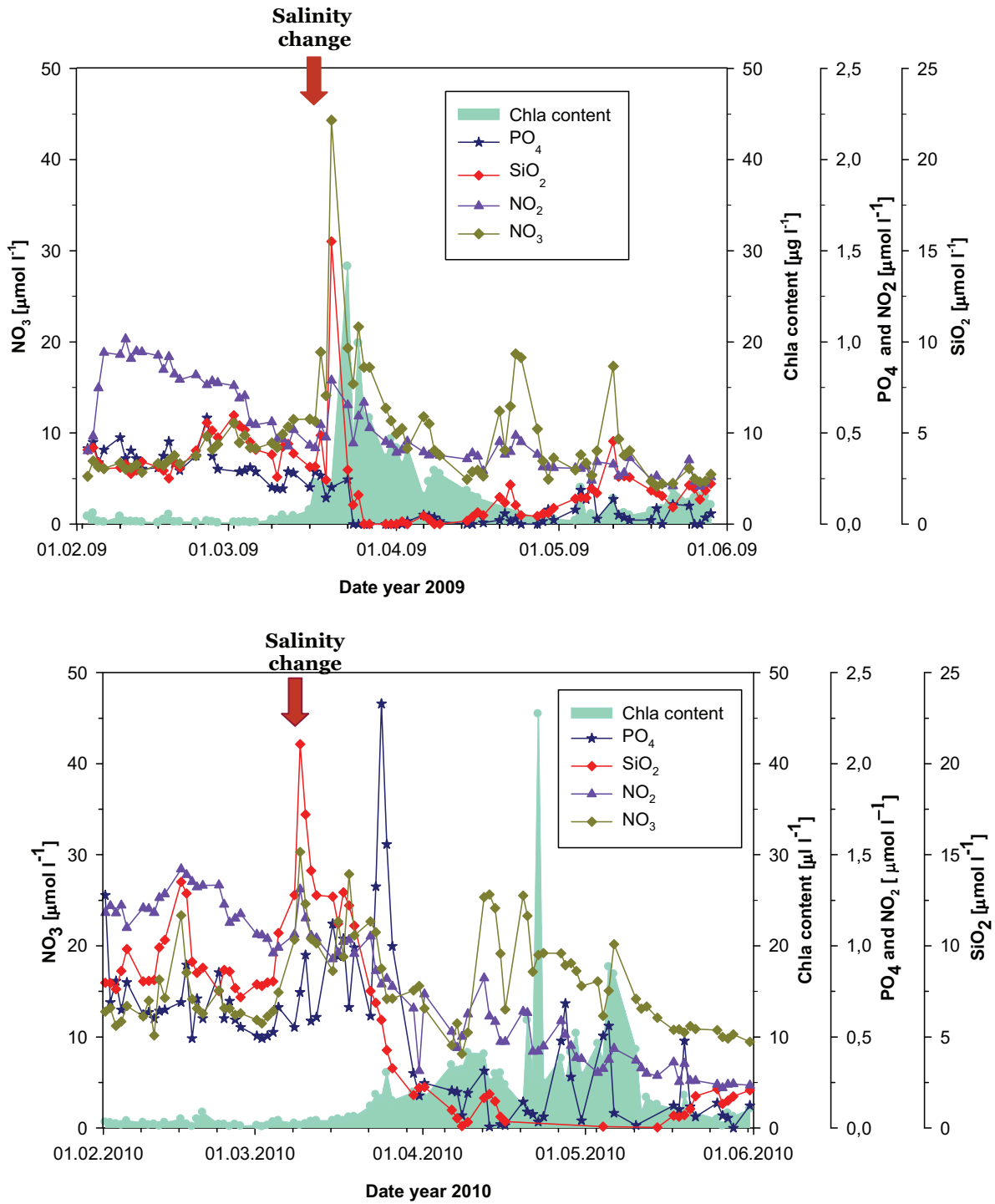


Figure 43 Distribution of phosphate (PO_4), silicate (SiO_2), nitrite (NO_2) and nitrate (NO_3) versus salinity in the winter-spring transition in years 2009 and 2010. The figure is illustrated for comparison.

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Thus, a phytoplankton bloom took place in both years during the winter-spring transition period, but shifted back by one month in the year 2010. Although all of the gammaproteobacterial groups, whose abundances were investigated in year 2010, showed lower absolute abundances by about half of the counts recorded for year 2009, they all had a spring succession during/after the algal bloom and most of them showed similar succession patterns in both years (Figure 44). This meant that the peaks of the individual bacterial successions also occurred about one month later in year 2010 like the peak of the algae spring bloom. The lower absolute counts might be due to lower temperatures in 2010 or to stronger mixing events.

The most dominating gammaproteobacterial group was SAR92 clade in both spring seasons. In both years, it showed a gradual increase having a first peak in the same time of the algal bloom and a second peak one month after the peak of the bloom. However, in year 2010 the first peak occurred earlier, directly accompanying the algal bloom peak. This indicates that this clade might be directly responding to the substrates produced by the algae at the beginning of the bloom where the chlorophyll content was already up to $11 \mu\text{g l}^{-1}$. This is further supported by preliminary metaproteomic data showing the domination of the early bloom by the activity of SAR92 clade. The succession pattern for *Reinekea* and OM182 clade was also similar in both years, where the peak of its succession occurred within similar time frames in regards to the algal peak. The *Balneatrix*-related clade firstly occurred after the bloom and showed relatively similar succession in both years. In year 2009, their succession resulted into a relatively sharp peak 5 weeks after the algal peak while in year 2010 their succession was a result of a gradual increase, which declined again within one month from the algal peak. In addition, *Glaciecola*, which was below the detection limit in both winters, occurred during both spring algal blooms and their abundances remained relatively low. The succession pattern of SAR86 clade was also observed in the following year, but it reoccurred 2 weeks earlier relatively to the phytoplankton peak. Thus it was observed just one week after the succession of SAR92 clade and *Reinekea*, while the time interval ranged between 2 to 3 weeks in year 2009 between their successions. However, the drastic decrease of SAR86 clade absolute abundance below $5 \times 10^3 \text{ cells ml}^{-1}$ was common in both years which further confirm the overgrowth and outcompeting of this lineage during the algal bloom. Thus it seems that the succession of the various gammaproteobacterial groups mainly depends on the onset of the algal bloom. Accordingly, the initial chlorophyll increase (prebloom phase) in year 2010 might have resulted in an earlier onset of the bacterial succession in regards to the phytoplankton peak, as in the case of SAR92 clade and *Balneatrix*-related clade. Furthermore, slight time shifts would be expected since this coastal system is very complex.

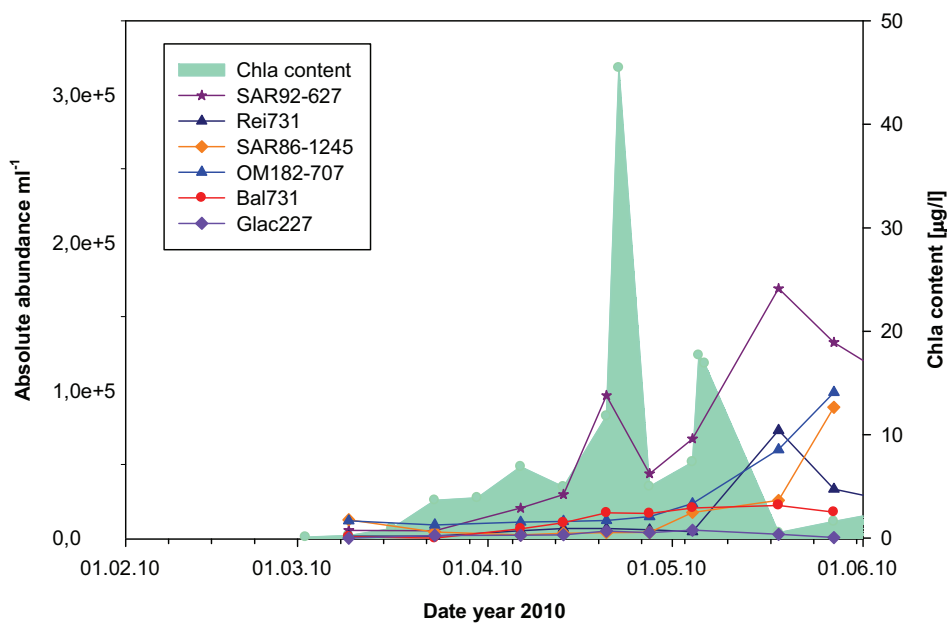
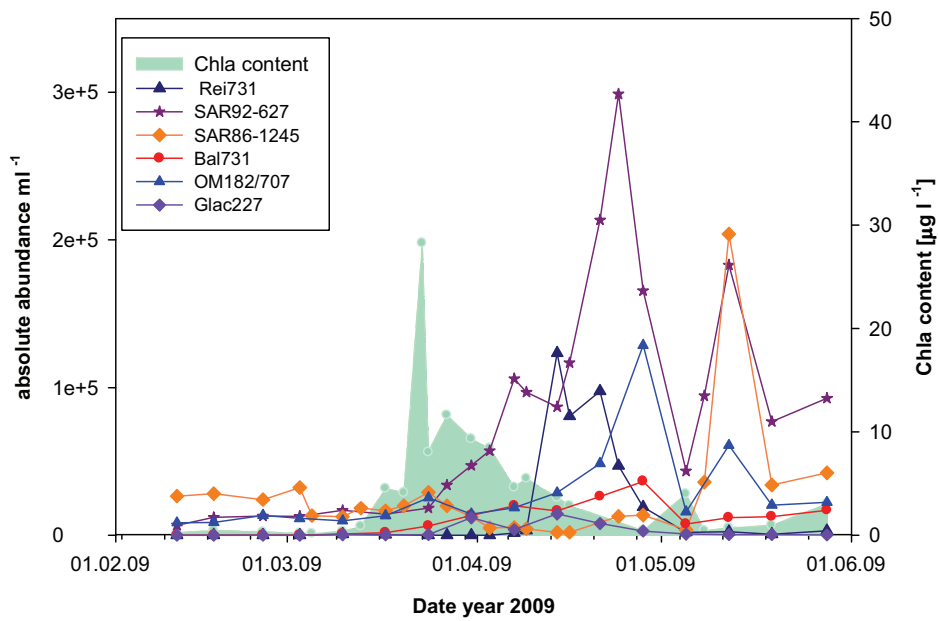


Figure 44 Absolute abundances of the main gamma-proteobacterial groups as determined by catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) in the North Sea from February to May in year 2009 and 2010. The target of each probe is detailed in 'Materials and Methods'. The figure is only to show the proportionality of the abundances between the various groups.

4.5 Probes evaluation and validation

Most of the newly designed probes covered at least one of the reference strains of the targeted subgroups as well as most of the corresponding clones as determined by the probe match tool in the ARB software such as probes Rei731, Bal731 and Glac227. The probability of targeting sequences outside the group was excluded for most of the probes since they either had the lowest number of mismatches with non marine groups such as probes Rei731 and OM182/707 or they targeted sequences within their own group such as probe Gla227. Probe Bal731 had at least two mismatches with the next non-target group, and probe Gla227 had more than one strong mismatch with non-target sequences. Some clones were more distant from the remaining clustered clones most probably due to some sequence errors and were such not targeted by the corresponding probe. This was the case for clones belonging to *Reinekea*, SAR92 clade, and SAR86 clade. Although probe OM182/838 could not cover the whole clade due to a distance of 10-11% between the two clusters, the counts with this probe could reflect the succession of this clade. However, another probe targeting the second cluster is required in order to determine the real abundance of OM182 bacteria.

The sum of FISH counts of all gammaproteobacterial subgroups tested so far correlated well with the count obtained with the gammaproteobacterial probe GAM42a, with only some minor differences (**Figure 45**). This indicates that the previously designed gammaproteobacterial probes in addition to the newly designed probes covered well the Gammaproteobacteria in this coastal system despite of probe GAM42a drawbacks which have to be considered within this evaluation (12). Probe GAM42a mainly targets the 23S rRNA in Gammaproteobacteria at positions 1027-1043. As a broad spectrum group specific FISH probe, it also detects some microorganisms outside the target group (false positives), and misses microorganisms within the subphylum lacking the target sequence (false negatives) (12), (7). Thus, it was previously reported that it can bind to *Competibacter* and *Accumulibacter* due to nucleotide polymorphisms which often leads to inconsistent FISH results (12). This might explain why the sum of counts as determined by the subgroup specific probes exceeded the counts of probe GAM42a on some dates. However, for such a quick comparison and a rapid prescreening of the samples, it showed remarkably consistent results.

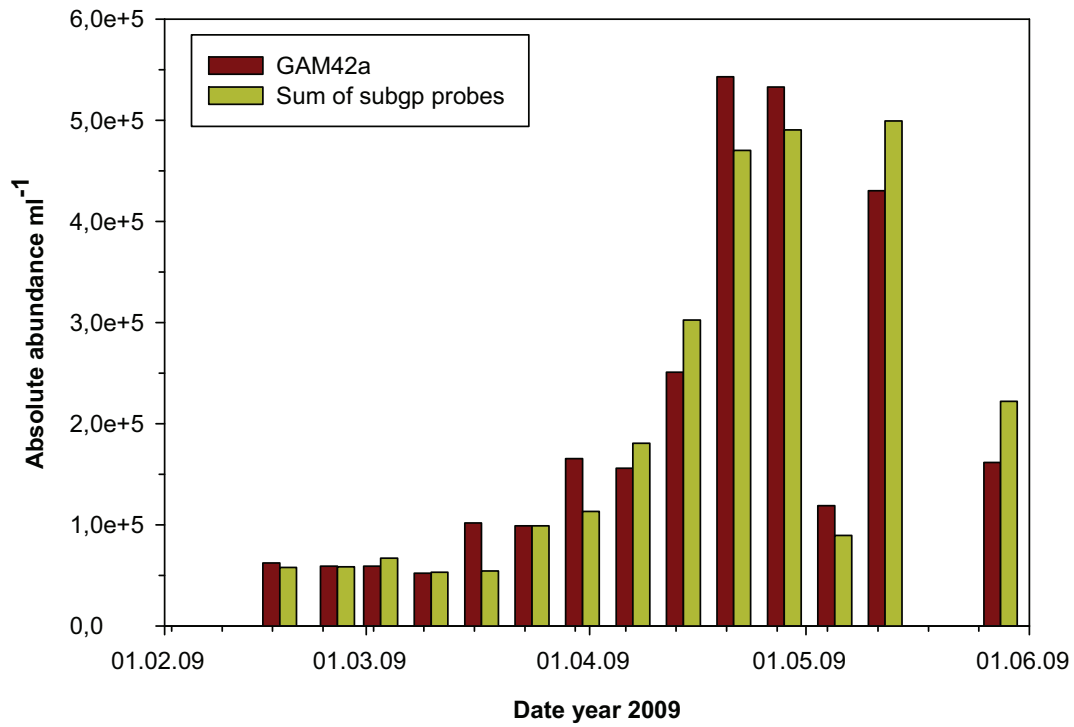


Figure 45 Sum of the absolute counts of the investigated gammaproteobacterial subgroup as detected by their corresponding probes versus the absolute abundance as counted by the general probe GAM42a.

5 General conclusions and Outlook

With regard to the in situ abundance of the marine gammaproteobacterial groups during the winter - spring transition period in year 2009 in the North Sea surface water, several succession patterns were detected: (a) abundant groups present throughout the period with a clear abundance peak after the algal bloom such as SAR92, NOR5/OM60 and OM182 clades, (b) groups present throughout the period slightly increasing in numbers after the algal bloom but remaining relatively low abundant such as *Vibrio* and *Pseudoalteromonas*, (c) groups firstly detected after the bloom with a relatively pronounced succession such as the genera *Reinekea*, *Balneatrix*-related clade and *Glaciecola* and (d) the in winter dominating SAR86 clade which is outcompeted during the bloom and only recovers to higher abundance after the decline of the other gammaproteobacterial groups.

With regard to the onset of succession (a) an immediate response was found for the genera SAR92 clade (first peak), *Glaciecola* and *Pseudoalteromonas*, (b) followed by *Reinekea* three weeks after the chlorophyll peak, (c) followed again by SAR92 (second peak), NOR5/OM60 and OM182 clades, *Balneatrix*-related clade and *Vibrio* one week later and (d) a late increase of the SAR86 clade seven weeks after the algal peak. This suggests different substrate degradation potentials for the individual clades. The SAR92 clade, *Glaciecola* and *Pseudoalteromonas* dominated the early bloom which might be based on utilization of exudates or of readily available substrates. This was followed by the rise of *Reinekea* which might be growing on partly degraded polymers. The late bloom, where polymers derived monomers and lipids released from the dying algae would be rather available, was dominated by NOR5/OM60, OM182, *Balneatrix*-related clades and *Vibrio* further suggesting another substrate preference (**Figure 46**). SAR86 clade was not directly affected by the blooming available substrates but rather members of this clade firstly succeeded after the declining of the other competing bacterial groups. Its dominance in the winter further suggests that it may represent an important component of the active fraction of the bacterioplankton in coastal North Sea surface water during seasons with generally declining bacterial activity as previously indicated (87). Thus the small cell size and low ribosome content do not imply nongrowth or dormancy in this bacterial lineage.

Groups such as *Reinekea*, the *Balneatrix*-related clade and *Glaciecola* which suddenly occurred after the bloom, could be resuspended from sediments and brought by tidal cycles to the water surface if not originally present in the water column. In addition, these groups are not oligotrophic and do not possess such kind of proteorhodopsins which would have enhanced their survival in an environment with low carbon content such as the North Sea surface water before the algal bloom.

The year 2010 was characterized by relatively lower temperatures, relatively lower bacterial cell counts, higher chlorophyll content and longer algal bloom. The reoccurrence of bacterial succession was observed for the gammaproteobacterial groups investigated in this year so far *Reinekea*, SAR92 clade, SAR86 clade, OM182 clade, *Balneatrix*-related clade and *Glaciecola*. Although their individual successions was generally observed one month later than in year 2009, they occurred relatively earlier after the algal blooming peak, which indicates that such bacterial succession might be strongly affected by the onset of the algal bloom. Since Helgoland is characterized by strong tidal currents (109) and shifting of different water bodies besides extreme weather events (73) such as the increase of storm events and wind speed observed in the North Sea (133) minor differences within each succession pattern would be expected such as the lower abundances observed for those gammaproteobacterial subgroups.

General conclusions and Outlook

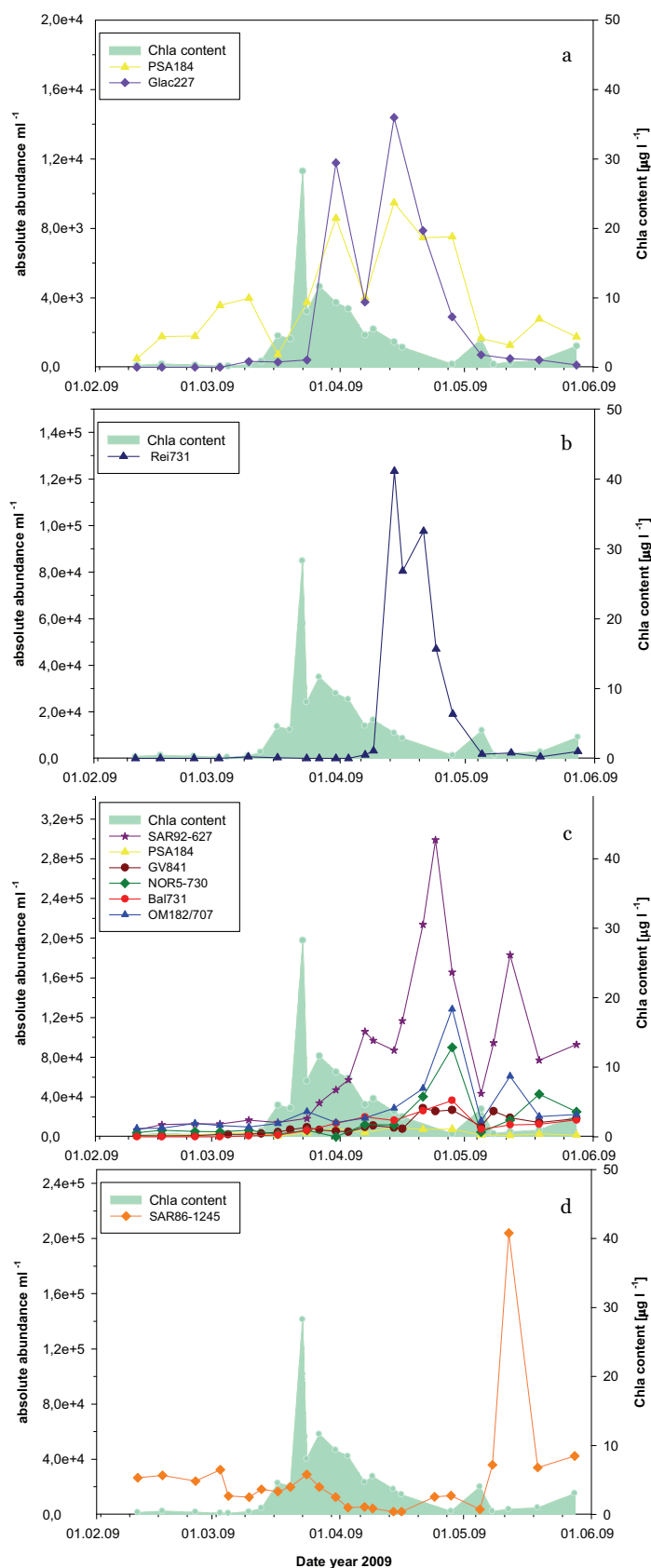


Figure 46 Absolute abundances of the main gammaproteobacterial groups according to their onset of succession in the North Sea in from February to May 2009 as described in the text. The target of each probe is detailed in 'Materials and Methods'. The proportionality of the abundances between the various groups is not considered into this figure.

Future work should focus on additional probe design. For instance, probes specifically targeting the winter and spring clusters of the SAR92 clade would enable the discrimination between the two groups and their separate quantification. This combined with further genomic information and investigations about the microbial activity of this clade might give deeper insights and indications why SAR92 has been dominating spring blooms in the North Sea. An alternative for probe OM182/838 needs to be designed and optimized to cover more members of the OM182 cluster not covered by probe OM182/707. Although all clones retrieved in this study belonging to the NOR5/OM60 cluster were targeted by probe NOR5-730, this probe could be combined with probe NOR5-1238 (CCCTCTGTGCGTTCCATT) (134) for further validation, and a nested probe set for this cluster could be designed. In addition, one or two probes complementary to probe Glac227 could be designed to cover the rest of the *Glaciacola* species.

Observations of this study could be further complemented by a search for additional habitats for the genera *Reinekea* and *Glaciacola* in the North Sea. Thus, North Sea sediments could be examined for their presence.

Abundances of groups such as NOR5/OM60 cluster, *Vibrio*, *Pseudoalteromonas* need to be investigated for year 2010 since they participated in the community shift in year 2009 as members of lower abundance. Thus it would be interesting to investigate their successional patterns in other years. A closer analysis of the algal induced spring succession of Gammaproteobacteria requires future water monitoring, determination of the physico-chemical parameters and further investigation of the abundances of the gammaproteobacterial subgroups of interest using CARD-FISH and the newly designed probes. In parallel, newly occurring gammaproteobacterial groups could be detected by tag sequencing of 16S rRNA genes. This would also require probe evaluation and design of new probes. In this context, it should be mentioned that full-length 16S rRNA gene sequences of high quality are the best starting point for the design of new probes. In addition, this would provide detailed sequence information within each subgroup. Diversity monitoring in combination with comparative genomics of selected pure cultures, metagenomics and metaproteomics might reveal the distinct role of particular clades within the bacterioplankton community of the North Sea.

6 Supplements

Probe	No. of weighted mismatches	Binding site
Rei731		3' GCT GAA CGA CCC GAC TAT 5'
target		5' CGA CUU GCU GGG CUG AUA 3'
<i>Reinekea</i>	0.0	*** *** *** *** *** ***
Firmicutes	0.1	**G *** *** *** *** ***
Acidobacteria	0.6	*** *** *** *** *** *C*
Firmicutes	0.8	*** *** *** *** *** U**
Firmicutes	0.9	**G *** *** *** *** U**
Bal731		3' CGT GCG GGA CCG AAC TAT 5'
target		5' CGA CGC CCU GGC UUG AUA 3'
<i>Balneatrix</i> uncultured bacteria	0.0	*** *** *** *** *** ***
<i>Balneatrix</i> uncultured bacteria	1.1	*** **A *** *** *** ***
Eukarya	1.6	GGG *G* *** G** *** **U
OM182 clade	1.7	**G *C* *** *** *** ***
OM182/707		3' AAG ACT CCT TAT GGC CAC 5'
target		5' UUC UGA GGA AUA CCG GUG 3'
OM182 clade uncultured, strains HTCC2180, HTCC2151, HTCC2178	0.0	*** *** *** *** *** ***
Enterobacteriaceae	0.3	A** *** *** *** *** ***
Candidate division OP10	0.5	*** G** *** *** *** ***
SUPO5 cluster	0.6	A** **G *** *** *** ***
Thiotricales	0.7	**A *** *** *** *** ***
Deltaproteobacteria	0.9	A** G** *** *** *** ***
<i>Shewanella</i>	0.9	A** G** *** *** *** ***
Deltaproteobacteria	1.0	**U G** *** *** *** ***
Betaproteobacteria	1.0	**U G** *** *** *** ***
OM182/838		3' CCC AUU AGG GAA AUC ACC 5'
target		5' GGG UAA UCC CUU UAG UGG 3'
OM182 clade uncultured		*** *** *** *** *** ***
<i>Geobacillus</i> , Firmicutes	1.5	**U *** *** *** *** **C

<i>Geobacillus</i> , Firmicutes	1.6	*** * U * *** *** *** **C
S24-7, Bacteroidetes	1.6	*** *** *** *** *** ***
OM182 clade uncultured, strain HTCC2188	0.8	*** *** *** U ** *** ***
OM182 clade uncultured OM182/838-a	5'	GGG UAA UCC CUU UAG UGG
Glac1276		3' GCC TAG GGA ATT GCT AGC 5'
target	5'	CGG AUC CCU UAA AGC UCG 3'
<i>Glaciecola polaris</i> , <i>G. chathamensis</i> , <i>G. mesophila</i>	0.0	*** *** *** *** *** ***
<i>Pseudoalteromonas tunicata</i>	1.1	**A *** *** *** *** ***
<i>Glaciecola punicea</i>	1.1	*** *C* *** *** *** ***
Glac227		3' TCA CCG GAT GCT CTC TAA 5'
target	5'	AGU GGC CUA AGC GAG AUU 3'
<i>Glaciecola polaris</i> , <i>G. psychrophila</i> , <i>G. mesophila</i> , <i>G. punicea</i>	0.0	*** *** *** *** *** ***
<i>Glaciecola lipolytica</i>	0.7	*** *** *** *** **U ***
<i>Glaciecola agarilytica</i> , <i>G. chathamensis</i>	1.3	*** U ** *** *** *** ***
<i>Bowmanella denitrificans</i>	1.5	**A *** *** **U *** ***
uncultured Bacteroidetes (2)	1.6	*** *** *** *** **A *G*
<i>Marinobacter</i> (1)	1.6	G** **U U** *** *** **G
uncultured Planctomycetae bacterium	1.6	*** *** G ** *** *** *G*

Table 10 Fullmatch and mismatch reference 16S rRNA sequences showing probe-binding sites according to the ARB v5.2 Probe Match Tool. Normal letters stand for weak mismatches and bold letters for strong ones.

7 List of abbreviations

AAnP	aerobic anoxygenic phototrophic
A	adenine
C	cytosine
CTAB	Hexadecyltrimethylammonium-bromide
CARD-FISH	catalyzed reporter deposition fluorescence <i>in situ</i> hybridization
DAPI	4, 6-diamidino-2-phenylindole
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
DOC	dissolved organic carbon
DOTUR	Distance–Based OUT and Richness
EDTA	ethylenediaminetetraacetic acid
FA	formamide
FISH	fluorescence <i>in situ</i> hybridization
G	guanine cytosine
g	gravity
h	hour
HRP	horseradish peroxidase
min	minute
OTU	Operational taxonomic units
PCR	polymerase chain reaction
PHYLIP	PHYLogeny Inference Package
PR	proteorhodopsin
RaxML	Randomized Axelerated Maximum Likelihood
RISA	ribosomal intergenic spacer analysis
rRNA	ribosomal ribonucleic acid
RT	room temperature
SINA	Silva incremental aligner
SDS	sodiumdodecyl sulphate
SSU	small subunit
T	tyrosine

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Erklärung

Hiermit erkläre ich, dass ich die Arbeit mit dem Titel:

Diversity and abundance of Gammaproteobacteria during the winter-spring transition at station Kabeltonne (Helgoland)

selbstständig verfasst und geschrieben habe und ausser den angegebenen Quellen keine weiteren Hilfsmittel verwendet habe.

Ebenfalls erkläre ich hiermit eidesstattlich, dass es sich bei den von mir abgegebenen Arbeiten um 3 identische Exemplare handelt.

.....

(Unterschrift)