# BACTERIAL CHEMOTAXIS IN THE ULTRAOLIGOTROPHIC EASTERN MEDITERRANEAN SEA

## - MOLECULAR AND CULTURE-BASED ANALYSES

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# 1 SUMMARY

The Eastern Mediterranean Sea is one of the most oligotrophic habitats on Earth and contains very low concentrations of phosphate, nitrate and ammonium (Krom *et al*., 2004). In the surface waters, growth of planktonic microorganisms appears to be limited by inorganic phosphate (Krom *et al*., 2004; Thingstad *et al*., 2005). In the following thesis, adaptive strategies of bacterioplankton in response to nutrient-depletion were analyzed.

Growth of heterotrophic bacterioplankton in dialysis cultures was stimulated by organic carbon substrates and inorganic phosphate but not by nitrogen compounds. Copiotrophic bacteria eventually dominated dialysis cultures.

The bacterial community analyzed exhibited high alkaline phosphatase activities, which were shown to correlate with a change in community composition due to the concentration process used in order to gain sufficient amount of organisms for *in situ* measurements.

Using natural bacterioplankton communities, the chemotactic response toward organic carbon compounds, inorganic nitrogen compounds and phosphate was investigated and the bacteria involved were identified based on their 16S rRNA gene sequences. Whereas organic carbon substrates and phosphate elicited a pronounced chemotactic response, no accumulation was observed in capillary assays containing ammonium and nitrate. Different copiotrophic members of the bacterioplankton exhibited distinct responses. Whereas *Pseudoalteromonas* sp. and *Alteromonas* sp. reacted toward organic carbon compounds and phosphate, the *Alphaproteobacterium Thalassospira* sp. was attracted only by inorganic phosphate, and *Vibrio* sp. was exclusively present in capillaries containing organic carbon substrates. Most of the chemotactically active members of these genera represented previously unknown phylotypes. Detailed phylogenetic analyses identified at least 13 novel different lineages of *Alteromonas* and 5 different lineages of *Pseudoalteromonas*. *Thalassospira* sp. accumulated by a relative enrichment factor of up to 60,000 in the chemotaxis capillaries. This previously unrecognized component of marine ultraoligotrophic bacterioplankton thus seems to be specifically adapted to accumulate at point sources of phosphate. The present study provides the first evidence for a chemotactic activity of marine bacterioplankton toward

phosphate, thereby identifying an alternative strategy of motile bacteria to enhance their phosphate acquisition. The pronounced and differential chemotactic response of *Thalassospira*, *Alteromonas, Pseudoalteromonas* and *Vibrio* spp. as determined in the present work has implications for our understanding of the ecological niche of these typical but low abundance marine planktonic genera and their potential role in the marine carbon and phosphorus cycles.

Chemotaxis toward inorganic phosphate constitutes a potential strategy to cope with phosphate limitation, but so far has only been demonstrated for two bacterial pathogens and an archaeon, and not in any free-living planktonic bacterium. In the present study, bacteria affiliated with the genus *Thalassospira* were found to constitute a regular, low abundance, member of the bacterioplankton that can be detected throughout the water column of the Eastern Mediterranean Sea. A representative (strain EM) was isolated in pure culture and exhibited a strong positive chemotaxis toward inorganic phosphate that was induced exclusively in phosphate-starved cultures. Phosphate-depleted cells were twofold larger than phosphate-repleted cells in exponentially growing cultures and 43% of the cells retained their motility even during prolonged starvation over a time period of 10 days. In addition, *Thalassospira* sp. EM was chemotactically attracted by complex substrates (yeast extract and peptone), amino acids and 2-aminoethylphosphonate but not by sugar monomers. Similarly to the isolate from the Eastern Mediterranean, chemotaxis toward phosphate was observed in starved cultures of two motile isolates of this genus, *T. lucentensis* DSM 14000$^T$ and *T. profundimaris* WP0211$^T$. Although *Thalassospira* sp. represents only up to 1.2% of the total bacterioplankton community in the water column of the Eastern Mediterranean Sea, its chemotactic behavior potentially leads to an acceleration of nutrient cycling and may also explain the persistence of marine copiotrophs in this extremely nutrient limited environment.

# 2 INTRODUCTION

## 2.1 THE ROLE OF HETEROTROPHIC BACTERIOPLANKTON IN GLOBAL NUTRIENT CYCLING

According to the concept of the "microbial loop" (Azam *et al.*, 1983; Ducklow *et al.*, 1986), heterotrophic bacteria utilize dissolved organic matter (DOM) produced by phytoplankton as nutrient and energy source. A part of energy and carbon is subsequently conveyed to higher trophic levels as bacteria are consumed by nanoflagellates, which in turn are preyed upon by larger zooplankton. In oligotrophic waters such as open ocean habitats photosynthetic bacteria are the main primary producers and only 1% to 2% of fixed carbon is finally assimilated by fish (Ducklow *et al.*, 1986). A major part of carbon and energy stays within the "microbial loop" and bacteria constitute a large fraction of biomass in the oceans (Figure 2-1).

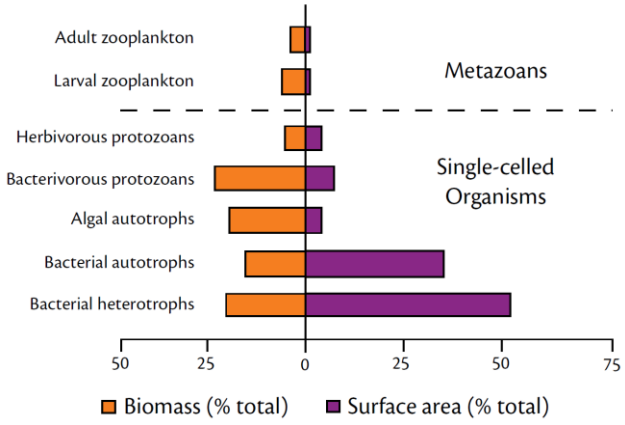


Figure 2-1
Biomass (mean of data derived from diverse marine sampling spots) and surface area (calculated hypothesizing spherical geometry) for different size classes of plankton in euphotic ocean areas (from Pomeroy et al., 2007)

Due to their very small cell size bacteria possess an advantageous surface to volume ratio which enables a fast response to altering environmental conditions. Prokaryotes dominate the flux of energy and carbon in marine ecosystems and therefore play an important role in biogeochemical cycling of organic and inorganic nutrients (Pomeroy et al., 2007). A major part of Earth`s oceans is aerobic and that is why the majority of marine

prokaryotes exhibit a chemoheterotrophic lifestyle, consuming organic matter, respiring $O_2$ and releasing $CO_2$ (Carlson et al., 2007).

Bacterial growth efficiency (BGE) is defined as the amount of biomass produced per unit of organic carbon consumed by heterotrophic bacteria (Sherr and Sherr, 1996; del Giorgio and Cole, 1998). Several factors have an effect on BGE, abiotic factors like temperature, toxic substrates or UV radiation as well as the energetic value of the nutrient source oxidized, lability of DOM, viral activity, bacterial diversity and nutrient limitation (Carlson et al., 2007). Bacterial growth efficiency is low when nutrients and energy are in short supply, when the stochiometry of DOM is imbalanced or when environmental conditions are unsuitable in other ways (Carlson et al., 2007). In general in oligotrophic ocean waters, like the Eastern Mediterranean Sea, BGE is low (< 10%) (del Giorgio and Cole, 1998). It has been proposed that a bacterial cell exploiting a number of nutrient sources needs an augmented capacity of enzymes leading to a higher energy demand for maintenance processes and therefore lowering energetic efficiency (Teixeira de Mattos and Neijssel, 1997). Other ATP energy-driven processes like cell motility might also lower BGE.

Heterotrophic bacteria are the largest contributors to community respiration in the Sea (Carlson et al., 2007) and are important players in global cycling of energy and nutrients. Even though microbial activity has a pivotal role for structuring the oceanic ecosystems, it is important to keep in mind that the actual dimensions of prokaryotic interactions with each other or with organic matter are nanometers to micrometers. Considering small scales, nutrients are not distributed homogeneously in the Sea but local nutrient gradients and hot spots do exist (Azam and Malfatti, 2007). Based on separation by filtration organic matter was traditionally separated into DOM (dissolved organic matter) and POM (particulate organic matter) which was retained on the filters. However, it has been suggested that organic matter in the oceans rather forms a gel-like structure (Koike et al., 1990) and that there likely exists a DOM-POM size-continuum (Verdugo et al., 2004). Marine gels potentially constitute nanoscale microenvironments which might provide habitats for bacteria (Verdugo et al., 2004; Azam and Malfatti, 2007). These microenvironments are probably characterized by comparably high nutrient concentrations (Verdugo et al., 2004). However, the precise role of marine gels in global biogeochemical cycling especially with respect to providing microhabitats has to be elucidated (Verdugo et al., 2004).

## 2.2 THE MEDITERRANEAN SEA

### 2.2.1 GEOGRAPHY AND OCEANOGRAPHY

The Mediterranean Sea constitutes the largest (area: 2969000 $km^2$) and deepest (average depth: 1460 m) enclosed Sea on Earth (Coll *et al*., 2010). In the West, it is connected to the Atlantic Ocean via the Strait of Gibraltar, in the Northeast through the Dardanelles to the Black Sea and the Sea of Marmara, while the Suez Canal in the Southeast links the Mediterranean Sea to the Red Sea and the Indian Ocean (Coll *et al*., 2010).

The Mediterranean Sea constitutes a concentration basin where freshwater loss exceeds freshwater input leading to an anti-estuarine circulation (Bethoux 1979; Menna and Poulain, 2009; Siokou-Frangou *et al*., 2010) (Figure 2-2). Cool, low salinity water from the Atlantic enters the Mediterranean Sea through the Strait of Gibraltar. Subsequently the Atlantic Water (AW) flows eastward passing the North African coast and finally enters the Eastern Mediterranean basin through the Strait of Sicily. The Atlantic surface water becomes increasingly saltier on its way. The increased density finally leads to sinking to a depth of 200 to 500 m. In this manner the Levantine Intermediate Water (LIW) is formed in the Levantine Sea. Along several pathways the LIW flows westwards underneath the inflowing AW and finally exits the Mediterranean Sea through the Strait of Gibraltrar. The exit of saltier and denser water is thus balanced by the entrance of surface water from the Atlantic ocean (Menna and Poulain, 2009; Siokou-Frangou, *et al*., 2010)(Figure 2-2).

A shallow ridge (400 m depth) divides the Mediterranean Sea into two sub-basins, the Western Mediterranean Sea and the Eastern Mediterranean Sea (Coll *et al*., 2010)(Figure 2-2). The Eastern Mediterranean Sea can further be divided into eight sub-basins: the North Adriatic Sea, the Central Adriatic Sea, the South Adriatic Sea, the Ionian Sea, the North Aegean Sea, the South Aegean Sea, the Levantine Basin, and the Gulf of Gabés, which are located East of the Strait of Sicily (Coll *et al*., 2010).

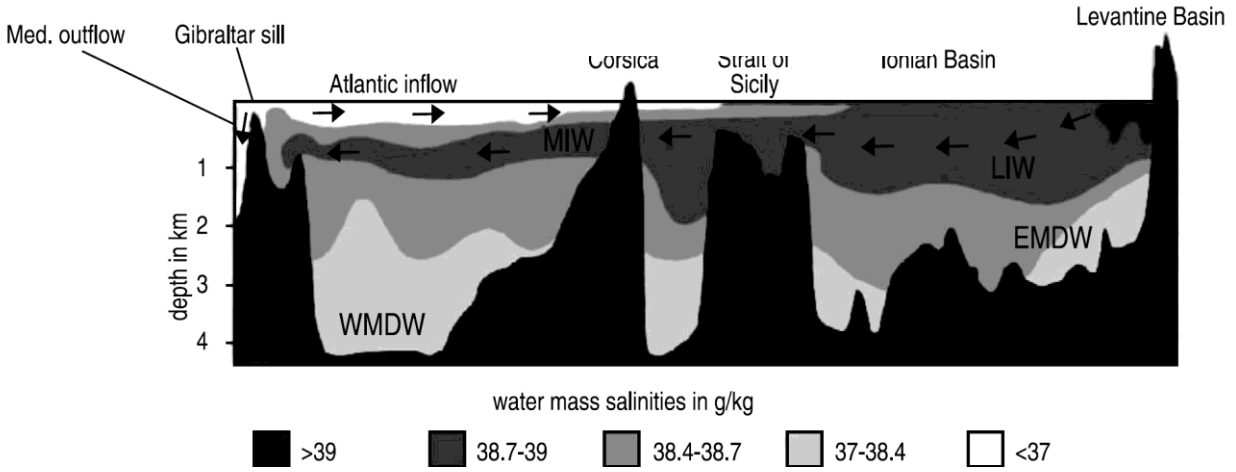


Figure 2-2

Water mass circulation in the Mediterranean Sea. LIW = Levantine Intermediate Water; EMDW = Eastern Mediterranean Deep Water; WMDW = Western Mediterraenan Deep Water; MIW = Mediterranean Intermediate Water (from Krijgsman, 2002)

The entire Mediterranean Sea, excluding its coastal regions, is oligotrophic (Bosc *et al.*, 2004). One major characteristic of the Mediterranean Sea is a strong West-East gradient regarding surface nutrient concentrations with a sharp change at the transition between the two basins so that the eastern basin is ultraoligotrophic whereas the western basin is oligotrophic (Siokou-Frangou *et al.*, 2010). The unusual low nutrient concentrations throughout the Eastern Mediterranean basin are caused by the anti-estuarine water circulation (Krom *et al.*, 2004). The Eastern Basin of the Mediterranean Sea (except the Adriatic Sea) is characterized by very low concentrations of algal biomass reflected by low overall Chl a concentrations and low levels of primary production (Bosc *et al.*, 2004) (Figure 2-3). Within the Eastern Mediterranean Sea the Ionian Sea and the Levantine Sea are the most nutrient-depleted provinces (Bosc *et al.*, 2004).

Deposition of nutrients by aerial dust accounts for up to 40% of new primary production while riverine nutrient input is less important with only three major rivers (Po, Rhone and Nile) flowing into the Mediterranean Sea (Siokou-Frangou *et al.*, 2010). The low contribution of riverine input to total nutrient input might be a reason for the very high N/P ratios as well as for the fact that the Mediterranean Sea is depleted in inorganic phosphate (Kress and Herut, 2001; Lebaron *et al.*, 2001; Krom *et al.*, 2004). N/P stoichiometries of 25-142 significantly exceed the Redfield ratio (Redfield, 1958; Kress and

Herut, 2001). The scarcity of inorganic phosphate is more pronounced in the Eastern basin than in the Western basin (Van Wambeke *et al.*, 2002).

Additional features of the Mediterranean Sea are its deep chlorophyll maximum (DCM), which generally does not exceed 1.5 µg Chl a $l^{-1}$ and which persists throughout the year in the whole basin (Siokou-Frangou *et al.*, 2010), a high homothermy (from 300 – 500 m to the bottom there are no thermal boundaries) and a high salinity (37.5 – 39.5 psu) (Coll *et al.*, 2010).

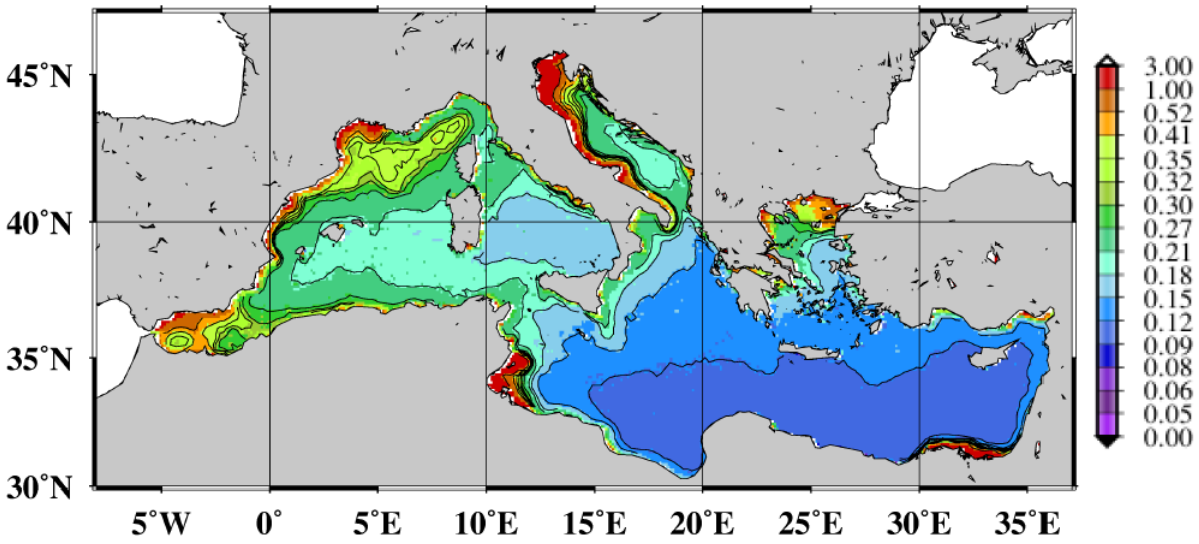


Figure 2-3

Chl a content in the Mediterranean Sea. Ten years climatological mean of the chlorophyll concentration in mg/$m^3$ (from Siokou-Frangou et al., 2010)

### 2.2.2 Bacterioplankton in the Mediterranean Sea

Water covers approximately 70% of Earth`s surface and 50% of global primary production occurs in the oceans (Field *et al.*, 1998). Biomass turnover of marine photosynthetic bacteria exceed turnover of plants by a factor of 700 (Overmann und Garcia-Pichel, 2000). Thus oceanic habitats are important in regard to global nutrient cycling.

A recent diversity analysis estimates roughly 17,000 species, 26% being of prokaryotic or eukaryotic microbial origin, populate the Mediterranean Sea (Coll *et al.*, 2010). However, data assessing bacterial and archaeal diversity are very limited to date and cannot yet be evaluated with the current techniques at hand (Coll *et al.*, 2010).

Picophytoplankton (*Cyanobacteria* like *Synechococcus* and *Prochlorococcus*) accounts for 60% of total Chl a content and for 65% of primary production (Vidussi *et al*., 2001). The most abundant primary producers in the oceans are *Cyanobacteria* (*Syncechococcus* sp. and *Prochlorococcus* sp.) (Azam and Worden, 2004). Especially in low-nutrient oceanic regions most of the fixed carbon is subsequently consumed by heterotrophic bacteria and therefore stays within the microbial loop. In the Eastern Mediterranean Sea, the fraction of photosynthetically fixed carbon entering the microbial loop is unusually high (85%) which is attributed to the superior efficiency of bacterial nutrient uptake under ultraoligotrophic conditions (Williams, 1998). As in most oligotrophic regions, low phytoplankton biomass in the Mediterranean Sea is linked to a high abundance of *Cyanobacteria* (Tanaka *et al*., 2007).

In the euphotic layer of the Levantine basin heterotrophic bacteria, ciliates and heterotrophic nanoflagellates account for 60% to 70% of the microbial carbon biomass (Tanaka *et al*., 2007). Heterotrophic nanoflagellates are dominated by cells smaller than 5 µm with an overall abundance of $10^5$ to $10^6$ cells $\cdot$ $l^{-1}$ (Christaki *et al*., 1999). In the Aegean Sea grazing by heterotrophic nanoflagellates is the major cause of bacterial mortality as bacteriovory balances bacterial production (Christaki *et al*., 2001). About $5 \cdot 10^2$ ciliates $l^{-1}$ are present in the Aegean Sea (Pitta and Giannakourou, 2000) and constitute important grazers and potential consumers of primary production (Dolan *et al*., 1999). Standing stocks of mesozooplankton are generally scarce and decrease from West to East (Dolan *et al*., 2002). Regarding its composition copepods dominate while other groups like crustaceans or cladocerans are less common (Siokou-Frangou *et al*., 2010). Mesozooplankton being a prey of small fish, is the link between plankton and fish production (Siokou-Frangou *et al*., 2010). Similarly to the invertebrates, a northwestern-to-southeastern gradient of species richness also exists for vertebrates which are characterized by a lower diversity than invertebrates (Coll *et al*., 2010).

On the whole the microbial food web (microbial loop) is dominant in most parts of the Mediterranean Sea (Turley *et al*., 2000). Heterotrophic to autotrophic biomass ratios are especially high in the eastern basin suggesting dominance of heterotrophs (Tanaka *et al*., 2007) (0.9 to 3.9 in the Aegean Sea (Siokou-Frangou *et al*., 2002)). Bacterial production is lower in the eastern basin than in the western basin following the gradient of oligotrophy (Van Wambeke *et al*., 2002). In addition bacterial growth efficiency (BGE) is low especially in the ultraoligotrophic eastern basin (Anderson and Turley, 2003). A low

BGE is suggested to exist due to scarcity of inorganic nutrients (Del Giorgio *et al*., 1997; del Giorgio and Cole, 1998). In the Eastern Mediterranean Sea growth of heterotrophic bacterioplankton is most likely limited by the availability of inorganic phosphate (Thingstad *et al*., 2005; Zohary *et al*., 2005). Heterotrophic bacteria thus play an important role in the marine food web and dominate in oligotrophic ocean systems such as the eastern basin of the Mediterranean Sea.

## 2.3 PHOSPHATE – AN ESSENTIAL NUTRIENT FOR MICROBIAL LIFE

Phosphate is an important macro-nutrient for microbial cells as it is an integral part of nucleotides, the phosphate-ester backbone of DNA and RNA and element of phosphoproteins and phospholipids (Figure 2-4).

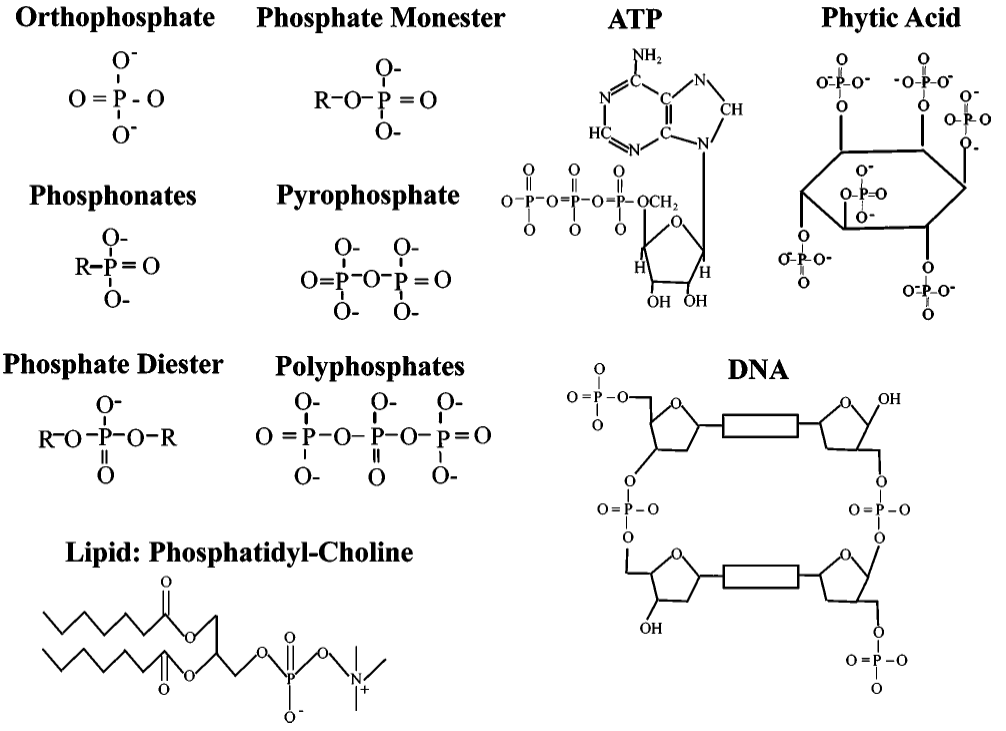


Figure 2-4
Important porkaryotic cell components containing inorganic phosphate (from Paytan and McLaughlin, 2007)

In the past time nitrogen has been suggested to represent the major limiting nutrient in oceanic regions and phosphate has been considered a limiting nutrient only over geological time scales when $N_2$-fixing organisms eventually fill up marine nitrogen pools (Redfield, 1958; Tyrrell, 1999). This view has changed since it has been found that phosphate and not nitrogen limits community production in oligotrophic open ocean environments in the Atlantic (Wu *et al*., 2000b; Vidal *et al*., 2003) the Pacific (Cotner *et al*., 1997; Karl and Yanagi, 1997) and especially the Mediterranean Sea (Thingstad *et al*., 1998; Krom *et al*., 2004), where phosphate was shown to be growth-limiting for autotrophic (Krom *et al*., 1991) as well as for heterotrophic (Zohary and Robarts, 1998) bacteria.

Phosphorus is recycled within the oligotrophic euphotic ocean zones and its availability affects primary production rates, bacterial species composition as well as the marine carbon cycle (Benitez-Nelson, 2000; Paytan and McLaughlin, 2007).

### 2.3.1 THE MARINE PHOSPHORUS CYCLE

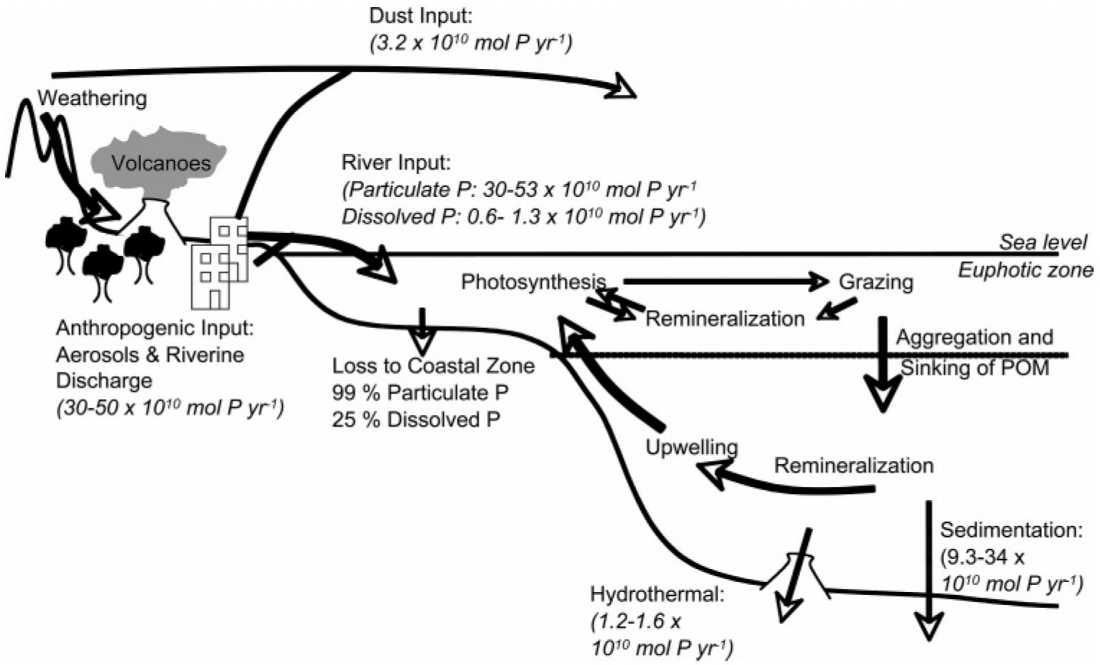


Figure 2-5
Overview of the marine phosphorus cycle (from Paytan and McLaughlin, 2007)

The major source for phosphorus in the ocean is continental weathering and subsequent input of particulate and dissolved phosphorus via riverine influx (Benitez-Nelson, 2000; Paytan and McLaughlin, 2007) (Figure 2-5). However, only about 10-30% of the total phosphorus is biologically available and only three quarters of this phosphorus finally reaches the open ocean (Compton *et al.*, 2000). There is a significant quantity of phosphorus from anthropogenic sources such as fertilizers for agricultural use that enter the ocean via rivers (Carpenter *et al.*, 1998). Atmospheric input of phosphorus with aeolian dust particles also takes place and is particularly important in off-shore marine regions where riverine input is small (Benitez-Nelson, 2000).

The major sink for phosphorus is sedimentation (Figure 2-5). Phosphorus is buried as sinking particulate organic matter or associated with metal oxides and hydroxides, via phosphorite burial and hydrothermal processes (Benitez-Nelson, 2000; Paytan and McLaughlin, 2007). Phosphorus present in the water column can exist in organic (P-esters,

P-diesters, phosphonates) or inorganic (orthophosphate, pyrophosphate, polyphosphate and phosphate containing minerals), dissolved (inorganic phosphate, organic phosphorus compounds, macromolecular colloidal phosphorus) or particulate (living and dead plankton, precipitates of phosphorus minerals, amorphous phosphorus phases) forms (Paytan and McLaughlin, 2007). Much of the cycling and transformation of the various forms of phosphorus takes place in the upper water column. Autotrophic and heterotrophic bacteria assimilate inorganic orthophosphate according to their metabolic needs and heterotrophic bacteria are responsible for a major part of the hydrolysis of dissolved organic phosphorus (Azam *et al*., 1983; Cotner and Biddanda, 2002). Biological phosphorus does not undergo redox processes but is present as phosphate with oxidation state +5. While nitrogen and carbon compounds undergo various redox reactions and occur in different oxidation states, phosphate usually cannot be used as electron acceptor (Hensen, 2006).

Dissolved organic matter (DOM) has been shown to be depleted in P relative to N or C with increasing depths suggesting a favored regeneration of P from organic matter implicating a more effective cycling of dissolved organic P (DOP) than dissolved organic C (DOC) or dissolved organic N (DON) (Paytan and McLaughlin, 2007).

Phosphate cycling rates appear to be very high in the upper water column so even low concentrations of phosphate can lead to high primary production (Paytan and McLaughlin, 2007). The primary sink for phosphate is sinking of particulate organic matter (POM) and subsequent burial within sediments (Paytan and McLaughlin, 2007). However, most of the P is remineralized within the water column and only 1% is finally buried within sediments (Paytan and McLaughlin, 2007). Organically bound phosphate must first be disconnected from its “carrier”- molecules before it can be utilized by microorganisms. The enzyme alkaline phosphatase hydrolyzes monoester-bounds, other enzymes like phosphodiesterases, nucleases, nucleotidases, kinases, phophonatases or C-P lyases catalyze the hydrolyzation of orthophosphate from diverse organic molecules (Benitez-Nelson, 2000; Paytan and McLaughlin, 2007; Martinez *et al*., 2010). The ecological importance of bacteria being able to metabolize phosphonates has been demonstrated recently (Dyhrman *et al*., 2006; Martinez *et al*., 2010). Phosphonate utilization pathways were shown to be common in marine bacteria and probably constitute a way to cope with low concentrations of phosphate (Martinez *et al*., 2010).

### 2.3.2 BACTERIA EXHIBIT VARIOUS STRATEGIES TO COPE WITH LOW CONCENTRATIONS OF INORGANIC PHOSPHATE

Bacteria have developed several different strategies to cope with the scarcity of phosphate (Figure 2-6).

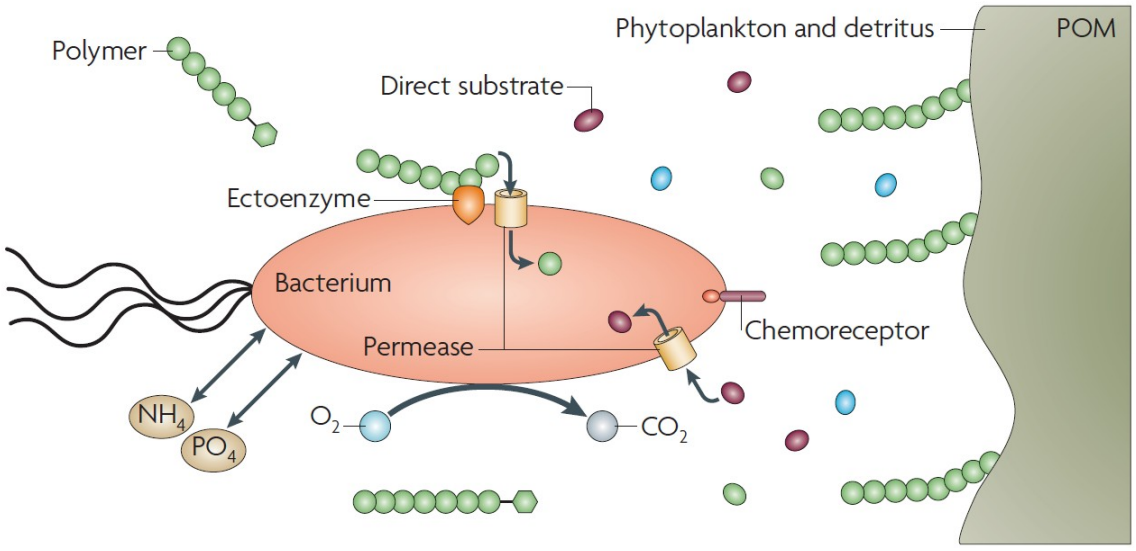


Figure 2-6

Adaptation of marine bacteria. The strategies, including chemotaxis/motility and expression of ectoenzymes and permeases are effective at dimensions of nanometer to millimeter and link bacteria and particulate organic matter (Azam and Malfatti, 2007)

Alkaline phosphatases hydrolyze phosphate ester bonds (C-O-P) separating phosphate from organic "carrier"-molecules making this nutrient accessible. Alkaline phosphatases like PhoA and PhoX are induced upon phosphate starvation in marine bacteria (Sebastian and Ammerman, 2009). Moreover, in a study conducted along a transect of the Mediterranean Sea, activity of the enzyme alkaline phosphatase was shown to be highest where phosphate concentrations are lowest (Levantine Sea) (Van Wambeke *et al.*, 2002). This makes expression of exoenzymes a potential strategy to cope with low availability of phosphate.

The ability to use organophophonates as an additional source of phosphate seems to be widespread among marine bacteria (Martinez *et al.*, 2010). Organophosphonates like 2-aminoethylphosphonate are characterized by a stable C-P bond (in contrast to the more labile C-O-P ester bond). Organophosphonates are mobilized by specific transporters and C-P lyases (Gilbert *et al.*, 2009) constituting an alternative P-source for marine bacterioplankton.

An additional strategy bacteria exhibit in answer to phosphate starvation comprises the expression of high affinity uptake systems for phosphate, like the membrane-bound ABC transporter PstSCAB (Gebhard *et al*., 2009). Recent metagenomic analyses indicate that the bacterioplankton residing in the Eastern Mediterranean Sea has adapted to nutrient limitation by employing high affinity phosphate uptake systems as well as by utilizing organophosphonates (Feingersch *et al*., 2010). The majority of the corresponding functional genes were affiliated with the *Alphaproteobacteria* including members of the SAR11 group. The latter represent oligotrophic bacteria that are known to harbor a disproportionately large number of substrate binding proteins for phosphate and phosphonate (Sowell *et al*., 2009) and typically are non-motile like e.g. *Pelagibacter ubique* (Rappé *et al*., 2002; Giovannoni *et al*., 2005).

Different strains of *Prochlorococcus* sp. up-regulate phosphate acquisition genes during periods of phosphate starvation as shown in microarray experiments (Martiny *et al*., 2006). Moreover, in the genomes of *Prochlorococcus* sp., a correlation of the presence of genes involved in phosphate uptake and those involved in the utilization of organic phosphorus sources, to ambient phosphate concentrations could be observed (Martiny *et al*., 2009). This correlation was shown to be independent of 16S rRNA phylogeny (Martiny *et al*., 2009).

A full genome microarray for *Synechococcus* sp. WH8102 showed several genes to be up- or down-regulated under P-deplete conditions. Genes being up-regulated include putative alkaline phosphatases and genes predicted to be relevant for transport (Tetu *et al*., 2009). Moreover, the two genes *swmA* and *swmB* coding for cell-surface proteins involved in swimming motility were significantly up-regulated (Tetu *et al*., 2009).

*Cyanobacteria* inhabiting marine environments that are characterized by low phosphate concentrations, substitute non-phosphorus membrane lipids for phospholipids and in doing so are able to decrease their cellular phosphorus content (Van Mooy *et al*., 2009). In contrast to phytoplankton, heterotrophic bacterioplankton in oligotrophic ocean regions did not contain substitute lipids (Van Mooy *et al*., 2009). However, the replacement of phospholipids with membrane-forming lipids devoid of phosphorus e.g. sulfolipids could also be shown to occur in the heterotrophic bacterium *Sinorhizobium meliloti* in response to phosphate starvation (Zavaleta-Pastor *et al*., 2010).

Chemotaxis toward inorganic phosphate in response to phosphate starvation has been observed in the two bacterial pathogens *Pseudomonas aeruginosa* (Kato *et al*., 1992;

Ohtake *et al*., 1998; Wu *et al*., 2000a) and *Enterobacter cloacae* (Kusaka *et al*., 1997), and more recently in the archaeon *Halobacterium salinarum* strain R1 (Wende *et al*., 2009). Phosphate directed chemotaxis has not yet been shown to occur in marine bacteria but nevertheless constitutes an additional strategy to cope with scarcity of inorganic phosphate. The adaptive potential of chemotaxis in bacterioplankton will be discussed in the next section and is a major focus of the following thesis.

## 2.4 CHEMOTAXIS IN MARINE BACTERIA

### 2.4.1 CHEMOTAXIS IN PROKARYOTES

The directed movement toward or away from a certain stimulus is called "taxis"(Lux and Shi, 2004). Many bacteria and archaea have been shown to be motile and to respond either positively or negatively to various metabolites, light, salinity, oxygen or the magnetic field (Armitage, 1999).

The best known chemotaxis system is that of the most important prokaryotic genetic model, the *Gammaproteobacterium Escherichia coli* (Szurmant and Ordal, 2004). The core of chemotaxis in *E. coli* is a two-component signaling system where the level of phosphorylation of a response regulator (CheY) mirrors the phosphorylation of a histidine autokinase (CheA) (Figure 2-7). Subsequently the phosphorylated response regulator (CheY-P) interacts with the switch of the bacterial motor protein (Szurmant and Ordal, 2004). In *E. coli* the protein CheZ dephosphorylates CheY (Rao *et al*., 2004). Receptor activity is regulated by the degree of methylation mediated by the proteins CheB (catalyzes demethylation) and CheR (catalyzes methylation) (Rao *et al*., 2004). The process of adaptation via receptor methylation allows bacteria to move up or down a concentration gradient (Szurmant and Ordal 2004). The protein CheW is essential for chemotactic activity coupling CheA to the receptor (Rao *et al*., 2004). The receptor sensing the attractant or repellent and leading to autophosphorylation of CheA is a methyl accepting protein (MCP) consisting of a conserved cytoplasmic domain and a highly variable periplasmic sensing domain (Szurmant and Ordal, 2004) (Figure 2-7). Chemoreceptors (which form dimers) are not distributed randomly in the cytoplasma membrane but do form complex, ordered, polar assemblages (Maddock and Shapiro, 1993; Briegel *et al*., 2009).

The chemotaxis mechanism found in the gram-positive genetic model organism *Bacillus subtilis* is different from that in *E. coli* and seems to be closer to that of the common ancestor of bacteria and archaea (Szurmant and Ordal, 2004). Mechanisms as well as molecular details of bacterial chemotaxis are diverse and differ from that of *E. coli* in many ways.

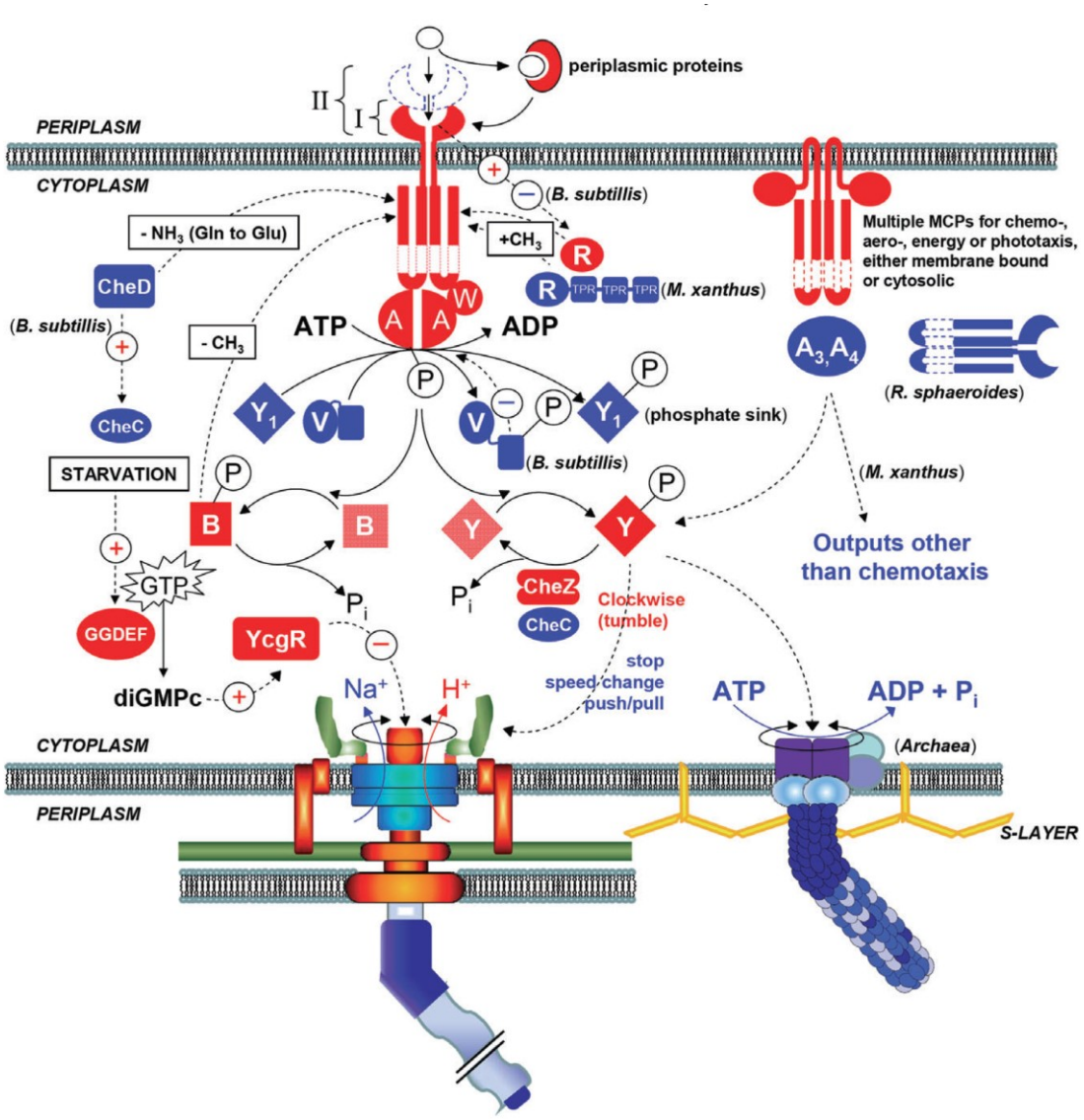


Figure 2-7

Chemotactic signaling in bacteria and archaea. Proteins present in E. coli are shown in red, proteins present in other bacteria are shown in blue (Krell et al., 2010)

While *E. coli* possesses 4 MCPs the number of putative chemoreceptor genes vary from just one in *Mesorhizobium loti* (Wadhams and Armitage, 2004) and bacterial pathogens, like for example *Yersinia pestis* Nepal516 (Lacal *et al.*, 2010) to 64 in *Magnetosprillum magnetotacticum* (Alexandre *et al.*, 2004). Large differences in regard to numbers of *mcp* genes per genome were found in an analysis including 244 bacterial and 20 archaeal strains (Lacal *et al.*, 2010). On average bacteria possess 13.9 *mcp* genes per genome whereas archaeal genomes contain 6.7 *mcp* genes (Lacal *et al.*, 2010). Bacteria exhibiting a complex lifestyle (for example interaction with other species, differentiation, magnetotaxis, bacterial predators) possess the most *mcp* genes (Lacal *et al.*, 2010).

The cytoplasmic signaling and adaptation domain of MCPs forms a dimeric α-hairpin (Park *et al*., 2006). The length of this signaling domain varies and all in all seven different major classes of MCP cytoplasmic domains can be distinguished based on an analysis of 152 genomes of bacteria and archaea (Alexander and Zhulin, 2007).

In *B. subtilis* additional signaling proteins are present including the additional CheY-P phosphatases CheC, FliY and CheX, the receptor deamidase CheD and CheV which is a CheW homologue (Krell *et al*., 2010) (Figure 2-7).

In *E. coli* the kinase CheA is inhibited as soon as an attractant binds to the receptor (Borkovich *et al*., 1989). In contrast, in *B. subtilis* an increase in CheA kinase activity occurs in response to ligand binding (Garrity and Ordal, 1997).

The protein CheY-P binds to the flagellar motor and controls its activity. In *E. coli* and in *Salmonella typhimurium* counterclockwise rotation of the motor leads to straight swimming (runs) while clockwise rotation leads to tumbling (Sowa and Berry 2008). In *Rhodobacter sphaeroides* the flagellum can only rotate clockwise and cells reorientate by Brownian motion during stop periods (Armitage and Macnab, 1987).

In bacteria energy for motor rotation is generated by electrochemical gradients. While flagellar motors of *E. coli*, *B. subtilis* and *R. sphaeroides* are driven by protons, *Vibrio alginolyticus* uses a $Na^+$-gradient (Krell *et al*., 2010) (Figure 2-7). Archaeal motors are either fueled by ATP hydrolysis or by an ATP-dependent ion gradient (Streif *et al*., 2008). Archaeal motors are functional analogues to bacterial motors and the respective proteins share no sequence homology (Figure 2-7) but archaeal flagella rather resemble to bacterial type IV pili (Krell *et al*., 2010).

These are merely some examples illustrating mechanisms of chemotaxis and motility to be highly variable among bacteria and archaea. The physiological bases for differences in chemosensory pathways are still poorly understood and will be a subject of future research.

### 2.4.2 CHEMOTAXIS AND ITS IMPLICATIONS IN THE MARINE ENVIRONMENT

At a behavioral level, chemotaxis helps bacteria to find favorable environments by moving toward attractants or by moving away from repellents. Chemoreceptors can be very sensitive and enable bacteria to sense changes in a few molecules in the presence of background concentrations that vary at least over five orders of magnitude (Wadhams and Armitage, 2004).

Marine bacteria face severe nutrient scarcity especially in open ocean environments. That is why a possible strategy for motile bacteria to respond to these adverse conditions would be moving toward the desired nutrient source. It has been suggested that a potential strategy of motile bacteria to cope with very low concentrations of a growth-limiting nutrient might be chemotaxis toward local nutrient hot-spots (Azam and Malfatti, 2007). In fact it could be shown that bacteria are attracted to patches of dissolved organic matter, which might allow the cells to maintain high growth rates independently of low average nutrient concentrations (Blackburn *et al*., 1998). The chemotactic response of the marine bacterium *Pseudoalteromonas haloplanktis* toward nutrient pulses and nutrient plumes is much faster than that of *Escherichia coli* suggesting that marine bacteria are specifically adapted to effectively exploit ephemeral nutrient patches (Stocker *et al*., 2008). As the chemotactic accumulation potentially results in the formation of hotspots of bacterial growth and carbon cycling, this microscale bacterial behavior represents an important new variable in biogeochemical models and may even determine whether the ocean acts as a carbon sink or source (Azam and Long, 2001).

To date, the relevance of chemotaxis has only been investigated with respect to its relevance for carbon cycling. Typical chemoattractants that have been identified for marine bacteria so far comprise amino acids (*Pseudoalteromonas haloplanktis*, *Silicibacter*, *Vibrio*; Barbara and Mitchell, 2003; Malmcrona-Friberg *et al*., 1990; Miller *et al*., 2004) glucose (*Vibrio*; Malmcrona-Friberg *et al*., 1990) and algal exudates (*P. haloplanktis*, *Silicibacter*; Miller *et al*., 2004; Stocker *et al*., 2008). Only a few marine bacterial isolates have been demonstrated to exhibit chemotaxis toward inorganic or organic nitrogen (Willey and Waterbury, 1989) or organic sulfur compounds (Miller *et al*., 2004; Seymour *et al*., 2010). In contrast, it is currently not known if heterotrophic marine bacteria are capable of a chemotactic response toward inorganic phosphate.

It has been observed that natural assemblages of marine bacteria as well as cultured marine isolates showed a different swimming behavior than the model organism *Escherichia coli* employing higher average swimming speeds of up to 144 μm $s^{-1}$ and a run-and-reverse swimming movement rather than a run-and-tumble movement (Mitchell *et al*., 1995b; Mitchell *et al*., 1995a; Johansen *et al*., 2002). This could be a strategy to cope with the unstable, locally changing marine environment and the enhanced effect of Brownian motion on small marine bacteria compared to larger *Enterobacteriaceae* (Mitchell, 1991; Mitchell *et al*., 1995b). However, the energetic costs of high-speed chemotactic motility also

have to be considered with respect to a probable competitive advantage (Stocker *et al*., 2008). In fact, *Pelagibacter ubique* the first cultured representative (Rappé *et al*., 2002) of the abundant and ubiquitous SAR11 cluster (Morris *et al*., 2002) is a non-motile bacterium characterized by a very small genome (Giovannoni *et al*., 2005). Indeed one of the genomic features suggested to define a copiotrophic (as opposed to an oligotrophic) lifestyle is the high abundance of clusters of orthologous groups (COG) of categories N (cell motility) and T (signal transduction) (Lauro *et al*., 2009).

## 2.5 THE ALPHAPROTEOBACTERIUM THALASSOSPIRA SP.

The first representative of the genus *Thalassospira* was isolated from off-shore seawater near Alicante, Spain in a continuous culture experiment designed to maintain oligotrophic conditions (filtered seawater was amended with fish-meal, Spirulina and *Artemia salina*.) (López-López *et al*., 2002). *Thalassospira lucentensis* is a gram-negative, vibriod to spiral, strictly halophilic, motile, strictly aerobic, chemoheterotrophic bacterium using carbohydrates, organic acids or amino acids as carbon source and ammonium or nitrate as nitrogen source (López-López *et al*., 2002). In the following years isolates of *Thalassospira* sp. were obtained from West Pacific Ocean sediments, surface water of a waste-oil pool in China (Liu *et al*., 2007) and from petroleum-contaminated seawater (Kamaishi Bay, Japan) (Kasai *et al*., 2002; Kodama *et al*., 2008). Bacteria belonging to the species *Thalassospira tepidiphila* can grow facultative anaerobic by using nitrate as final electron acceptor and are able to degrade polycyclic aromatic hydrocarbons like pyrene, benzopyrene or phenanthrene (Kodama *et al*., 2008). 16S rRNA gene sequences affiliated with *Thalassospira* sp. have been frequently detected in petrol-oil degrading "consortia" (Liu *et al*., 2007). Moreover, two new cyclic peptides (thalassospiramides A and B) showing immunosuppressive activity have been isolated from members of the genus *Thalassospira* (Oh *et al*., 2007).

*Alphaproteobacteria* are abundant members of marine planktonic communities. In surface waters of the Eastern Mediterranean Sea *Alphaproteobacteria* constitute an important component of free-living bacterioplankton (Acinas *et al*., 1999; Zaballos *et al*., 2006; Feingersch *et al*., 2010). Most *Alphaproteobacteria* in the Eastern Mediterranean Sea are either members of the ubiquitously present SAR11-clade (Morris *et al*., 2002; Feingersch *et al*., 2010) or belong to the *Rhodobacteracea* (Zaballos *et al*., 2006; Feingersch *et al*., 2010). *Thalassospira* sp. has not been found to be an abundant member of marine bacterial assemblages neither in the Mediterranean Sea nor in other oceanic regions. Due to its ability to grow in rather high- nutrient media and to form colonies on agar plates, *Thalassospira* sp. should be regarded as a copiotrophic prokaryote as opposed to oligotrophic bacteria like *Pelagibacter ubique* which can grow neither in rich media nor on agar plates (Rappé *et al*., 2002). The latter are thought to dominate in marine habitats (Morris *et al*., 2002; Lauro *et al*., 2009).

In spite of this, it has been found in regard to rank-abundance curves that those of bacteria, archaea and viruses are characterized by extremely long tails, suggesting that rare

species significantly contribute to microbial diversity (Sogin *et al*., 2006). These organisms belong to the so-called "rare biosphere" (Sogin *et al*., 2006) and their ecological function remains a matter of discussion (Galand *et al*., 2009; Sogin *et al*., 2010). A study of the microbial community of a freshwater lake revealed that rare taxa were more active than common taxa (Jones and Lennon, 2010). Even though the motile, anaerobic, phototrophic bacterium *Chromatium okenii* comprised only 0.3% of the total microbial community in the chemocline of Lake Cadagno, these bacteria were shown to be responsible for more than 40% of total uptake of ammonium and for 70% of total uptake of carbon (Musat *et al*., 2008). In the marine realm the copiotrophic *Gammaproteobacterium Alteromonas* sp. dominated the attached bacterial community in the Eastern Mediterraean Sea (Acinas *et al*., 1999) and could be found in high numbers in nutrient-amended enrichments (Allers *et al*., 2007; Allers *et al*., 2008) responding rapidly to nutrient addition (Eilers *et al*., 2000). In fact a recent transcriptomic analysis showed that upon supplementation with high molecular weight dissolved organic matter, transcripts enriched in nutrient-amended microcosms relative to the control originated to a large extent from bacteria affiliated with the order *Alteromonadales* (McCarren *et al*., 2010). Using a combination of BrdU magnetic beads immunocapture and the DGGE technique, the phylogenetic identity of actively growing bacteria in open ocean surface seawater along a north-south transect in the North Pacific was assessed. Band patterns of the total bacterial communities and the actively growing, BrdU-incorporating bacterial communities were dissimilar with the latter being dominated by culturable organisms belonging for example to the *Vibrionales*, *Alteromonadales* and *Roseobacter*-related bacteria (Taniguchi and Hamasaki, 2008). This indicated that some rapidly growing bacteria were not abundant enough to be detected in the total bacterial community by DGGE because their absolute abundance might be negatively influenced by selective grazing or viral lysis (Taniguchi and Hamasaki, 2008).

These examples show that even non-abundant members of the microbial community might play an important ecological role in nutrient cycling. Actually low coverage is a major drawback of modern metagenomic approaches with rare taxa and their probably critical genetic potential not being represented at all in genomic libraries (Warnecke and Hugenholtz, 2007). Even with modern techniques at hand there is still a need to investigate bacteria on physiological level in order to shed light on the way microbes shape their environments.

## 2.6 AIM OF THE PRESENT WORK

The Eastern Mediterranean Sea is one of the most oligotrophic oceanic habitats on Earth (Turley *et al*., 2000). Heterotrophic bacteria, which represent an important component of the marine carbon cycle, are therefore exposed to an environment characterized by very low nutrient concentrations. Especially the scarcity of phosphate being the major factor limiting microbial growth (Krom *et al*., 1991; Zohary and Robarts, 1998; Krom *et al*., 2004; Thingstad *et al*., 2005), forces bacteria to develop adaptive strategies to cope with these unfavorable conditions. Although recent metagenome studies on surface waters of the Eastern Mediterranean Sea indicate that heterotrophic bacterioplankton has adapted to phosphate limitation by employing high affinity phosphate uptake systems as well as by utilizing organophosphonates (Feingersch *et al*., 2010) knowledge of additional adaptive strategies especially on physiological level is still lacking. Lately it has been suggested that chemotaxis constitutes a potential adaptive strategy allowing bacteria to exploit nutrient hot-spots in a low-nutrient environment and possibly fueling the marine carbon cycle (Azam and Malfatti, 2007; Stocker *et al*., 2008).

In a first approach, on-board experiments (Meteor cruise M71/3) were conducted in order to reveal possible adaptive strategies of heterotrophic bacterioplankton in the Eastern Mediterranean Sea. Growth response of bacterioplankton in dialyses cultures and the measurement of exoenzyme activities suggested phosphate limitation as described previously (Krom *et al*., 2004).

The following thesis aimed to elucidate the *in situ* relevance of chemotaxis as an adaptive strategy of heterotrophic bacterioplankton in the ultraoligotrophic, phosphate-depleted Eastern Mediterranean Sea. A special emphasis was put on chemotactic response toward inorganic phosphate being the growth limiting nutrient. Moreover, not only the bulk response of marine bacteria toward potential attractants but also the phylogeny of the chemotactic community was of special interest. To this end a method needed to be developed to extract DNA from a very small amount of microorganisms present in few µl of seawater in order to be employed in subsequent PCR-based phylogenetic analyses.

In the course of the following study, it has been observed that bacteria affiliated with the alphaproteobacterial genus *Thalassospira* exclusively responded chemotactically toward inorganic phosphate. Hence these organisms might be specifically adapted to phosphate starvation by being chemotactic. Therefore, a representative of this genus was

chosen for cultivation. The obtained pure culture allowed detailed physiological analyses. The response of representatives of the genus *Thalassospira* including the novel isolate from the Eastern Mediterranean Sea toward phosphate starvation was analyzed with a special emphasis on chemotaxis. Moreover, the *in situ* abundance of *Thalassospira* sp. was assessed employing the quantitative PCR (qPCR) technique. These experiments aimed to clarify the role of *Thalassospira* as a representative of non-abundant heterotrophic marine bacterioplankton in its natural habitat.

# 3 MATERIAL AND METHODS

## 3.1 PHYSIOLOGICAL METHODS AND IN SITU MEASUREMENTS

### 3.1.1 SAMPLING LOCATIONS AND IN SITU MEASUREMENTS

Water samples were obtained during cruise M71 leg 3 of the R/V Meteor from Heraklion to Istanbul between January 17 and February 4, 2007. The six sampling stations H02 (21°0.046'E, 35°45.014'N), H03 (18°0.008'E, 35°45.003'N), H07 (17°44.969'E, 39°0.994'N), H10 (19°0.063'E, 39°55.142'N), Rho02 (27°42.026'E, 35°37.008'N) and Ier01 (26°12.103'E, 34°26.476'N) were located in the oligotrophic Eastern Mediterranean Sea and covered a distance of 880 km between the Eastern Ionian Basin (station H03) and the Rhodes basin (station Rho02) (Figure 4-1). Water samples were recovered from 5 m below the sea surface, from the chlorophyll (Chl) maximum, the salinity maximum of the Levantine intermediate water and from a water depth of 1000 m, employing a SBE911 plus CTD system (Sea-Bird Electronics Inc., Washington, USA) and a SBE32 rosette equipped with 24 ten-liter FreeFlow-bottles (Hydrobios). Conductivity, temperature and molecular oxygen concentrations were monitored in 1 m intervals (Rubino and Hainbucher, 2007). Water samples were processed immediately after collection. From each station and sampling depth, bacterial cells were collected by filtering 500 to 2000 ml of water on sterile 0.1 µm pore size polycarbonate filters (type VCTP, Millipore, Eschborn, Germany). To generate sufficient inoculum required for gradient agar tubes and for the determination of enzyme activities, bacterial cells in 100 liters of water obtained from the Chl *a* maxima were concentrated using a tangential flow filtration system (Pellicon 2, 0.1 µm pore size filtration cassette; Millipore). For molecular analyses 100 to 200 ml of concentrated seawater were filtered on sterile 0.1 µm pore size polycarbonate filters (type VCTP, Millipore). Filters were stored at -20 °C until analyses.

### 3.1.2 BACTERIAL CELL NUMBERS

Water samples for the determination of total bacterial cell numbers were fixed with glutardialdehyde at a final concentration of 2% (v/v). Bacterial cells were subsequently stained with 4',6-diamidino-2-phenylindol (DAPI) at a final concentration of 0.1 µg·ml$^{-1}$ for

10 minutes in the dark and collected on black polycarbonate filters (0.1 µm pore size, 13 mm diameter, type VCTP, Millipore). The filters were dried and embedded in DABCO antifading solution consisting of 25 mg of 1,4-diazabicyclo[2.2.2]octane in 1 ml of phosphate buffered saline (PBS, 137 mM NaCl, 2.7 mM KCl, 10 mM $Na_2HPO_4$, 2 mM $KH_2PO_4$, pH 7.4) buffer plus 9 ml of glycerol. Cell numbers were determined by epifluorescence microscopy (Zeiss Axiolab) (Hobbie *et al*., 1977) employing filter set 02 (G 365, FT 395, LP 420). In parallel, the numbers of red or orange autofluorescent, i.e. Chl a containing, cells were determined in the samples using the Zeiss filter set 09 (BP 450-490, FT 510, LP 515).

### 3.1.3 MEASURING OF EXOENZYMATIC ACTIVITIES USING FLUOROGENIC SUBSTRATE ANALOGUES

Enzyme kinetics of the exoenzymes alkaline phosphatase and leucine aminopeptidase were measured in concentrated seawater with the aid of fluorogenic substrate analogues (4-methylumbelliferone-phosphate/MUF-P and 7-amino-4-methylcoumarin-leucine/AMC-leucine; Sigma-Aldrich, Steinheim, Germany). Stock solutions (2 mM) of substrate analogues were prepared by dissolving the respective amount of substrate in 2.5 ml ethanol (100%) and subsequently filling up with sterile filtered seawater. The enzymatic reactions were initiated by adding substrate analogs at final concentrations of 100 µM ,50 µM, 20 µM, 10 µM, 5 µM ,2 µM, 1 µM and 0.5 µM (MUF-P) or 200 µM, 100 µM, 50 µM, 20 µM and 10 µM (AMC-leucine) respectively to 9.5 ml concentrated seawater. All reactions were incubated for 3 h at 15 °C in the dark on a shaking rotator. Blank control reactions for each substrate concentration consisted of boiled water (20 minutes, boiling water bath) with added substrate analogues, which were handled in the same way as sample reactions. After incubation, each sample and each negative control was diluted 1 in 100 (with sterile filtered seawater) and 1 ml of this dilution was subsequently transferred to cuvettes. The concentrations of free dissolved fluorophores were measured in a TD-700 fluorimeter (Turner Designs, Sunnyvale, USA) equipped with a 300-400 nm excitation filter (P/N 10-069R) and a 410-600 nm emission filter (P/N 10-110R-C) (maximum excitation MUF/AMC 365/380 nm, maximum emission MUF/AMC 460/440 nm). In the case of MUF-phosphate 3 µl 2M NaOH were added prior to measuring in order to stop the enzymatic reaction. Prior to each measurement different concentrations of 4-methylumbelliferone (MUF) and 7-amino-4-methylcoumarin (AMC) were used in order to calibrate the fluorimeter.

For graphic analyses Michaelis-Menten plots were constructed by plotting substrate concentration (x-axis) versus reaction velocity (y-axis), according to the following formula:

(1)

$$v\ (\mu M \cdot h^{-1}) = \frac{vmax \cdot A}{(Km + Sn) + A}$$

To obtain kinetic parameters of alkaline phosphatase activities as well as of leucine aminopeptidase activities, the Michaelis-Menten plots were linearized by using the formula of Wright and Hobbie (Wright and Hobbie, 1966):

(2)

$$\frac{A}{V} = \frac{Km + Sn}{vmax} + \frac{A}{vmax}$$

The ratio A/velocity versus A (concentration of substrate added) was plotted. The maximum hydrolysis rate (equivalent to vmax) is the slope of the resulting graph, while the turnover time, defined as (Km+Sn)/vmax is the y-axis intercept, which is also used for calculating Km+Sn:

(3)

$$\text{turnover time (h)} = \frac{\text{Km} + \text{Sn}}{\text{vmax}}$$

(4)

$$Km + Sn\ (\mu M)\ = \frac{\frac{Km + Sn}{vmax}}{vmax}$$

A denotes the concentration of substrate analogue added (MUF-P or AMC-leucine), v is the hydrolysis rate ($\mu M \cdot h^{-1}$) and Km+Sn (µM) is the sum of the Michaelis-Menten constant (Km) and the concentration of natural occurring substrate. The sum of Km+Sn is a maximum estimator for the half-saturating constant frequently used in microbial ecology.

### 3.1.4 GROWTH EXPERIMENTS IN DIALYSIS CULTURES

Stimulation experiments were conducted directly after water sampling employing the dialysis cultivation technique on board. Samples from the chl maximum at stations H02 and H03 were filtered through 2 µm pore size sterile polycarbonate filters (Isopore, type TTTP, Millipore) to remove protozoan grazers. 40 ml of the filtrate were dispensed in sterile dialysis bags (exclusion size, 12,000 – 16,000 Da) which were subsequently incubated in 1 liter of seawater at slow stirring, a constant temperature of 15 °C and in the dark for up to 108 h hours. In order to reduce bottle effects further and to replenish nutrients, the surrounding seawater was exchanged after 2 days. The seawater was amended with 50 µM inorganic N, 1 µM inorganic P, or a mixture of organic carbon

compounds that were each added at a final concentration of 20 µM or 200 nM organic C. N, P, and organic C were added from stock solutions. The stock solution of organic carbon (20 mM C of each substrate) contained an equal mixture of acetate, arabinose, benzoate, butanol, buyrate, citrate, ethanol, formate, glucose, lactate, malate, mannitol, methanol, N-acetyl glucosamine, 2-oxoglutarate, propanol, propionate, pyruvate, salicylate, succinate, valerate, and a combination of the 20 canonical amino acids. Yeast extract and Tween 80 were added to the 20 mM stock at a final concentration of 0.1% and 0.01% (w/v), respectively. The nitrogen stock solution consisted of a mix of $KNO_3$ and $NH_4Cl$ of 25 mM each (total nitrogen 50 mM) and the phosphorus stock solution contained 1 mM $KH_2PO_4$. With these amendments, concentrations of the respective substrates were increased by a factor of 50 compared to the *in situ* concentrations. A parallel incubation without substrate amendment served as control.

Cell numbers developing in the dialysis bags were determined by epifluorescence counting (see above) at six time points during the 108 hours of incubation. On-board dialysis cultures experiments were done by Dr. Karin Schubert. After the end of the experiment the remaining volume (30-35 ml) ml of the grown cultures was collected on polycarbonate filters (0.1 µm pore size, Millipore) and the total genomic DNA was extracted prior to PCR amplification, cloning and sequence analysis of the 16S rRNA genes (3.2.1, 3.2.2, 3.2.5).

### 3.1.5 IN SITU CHEMOTAXIS ASSAYS

Chemotactic responses of bacterioplankton were analyzed by capillary assays (Fröstl and Overmann, 1998; Overmann, 2005). The investigations focused on samples from the deep chlorophyll maximum (DCM) which is characterized by maximum primary productivity and phytoplankton concentrations (Acinas *et al*., 1999). At the three sampling stations, water samples were taken from the depth of the maximum chlorophyll concentrations. 20 ml of seawater were transferred to sterile 100 ml Meplats bottles which had 12 holes of 3 mm diameter each drilled through one side wall. Stock solutions of attractants were prepared with sterile filtered seawater (0.1 µm pore size, 25 mm diameter; Nalgene, Thermo Fisher Scientific, Schwerte, Germany) from the same water sample and contained either a mixture of carbon substrates (composition see below, final concentration of each substrate, 2 mM each, plus 0.1% (w/v) yeast extract and 0.01% (w/v) Tween 80), $KH_2PO_4$ (final concentrations 2 mM, 200 µM, 20 µM or 2 µM) or a mixture of $KNO_3$ and $NH_4Cl$ (final

concentrations, 2 mM each). Sterile filtered seawater without amendments served as a negative control. Flat rectangular glass capillaries (length 50 mm, inside diameter 0.1 x 2.0 mm; Vitrocom, New Jersey, USA) were filled by capillary action with the stock solutions, sealed at one end with plasticine (Münchner Künstler Plastilin, Munich, Germany), inserted into the holes of the Meplats bottles such that open ends of capillaries were immersed in seawater sample inside and the positions of capillaries in the holes were fixed with plasticine.

Chemotaxis assays were incubated at *in situ* temperature of 15 °C for 24 and 48 hours in the dark. Afterwards, capillaries were removed from the bottles and their contents expelled into 1.5 ml reaction tubes containing 5 µl of sterile Tris-buffer (2 mM Tris-HCl, pH 8.0). An aliquot of each sample was stained with DAPI (see above) for quantification of bacterial cell numbers. In order to sufficiently increase the sensitivity of the epifluorescent cell counting procedure, each stained subsample was filtered through a filter area of 7 $mm^2$ that had been generated by printing rings of paraffin (3 mm inner diameter) onto black polycarbonate filters (0.1 µm pore size, 25 mm diameter, type VCTP; Millipore). The remaining sample volume was stored at -20 °C until subsequent molecular analyses. On-board chemotaxis experiments were done by Prof. Dr. Jörg Overmann together with Martina Mayer.

Calculations were performed in order to assess whether the accumulation of bacteria in the capillaries could be caused by growth after cells had entered the nutrient richer attractant solutions in the capillaries. If bacterial cells enter the capillary at a constant rate $m$ (in units of cells · $h^{-1}$) and subsequently grow exponentially with a growth rate µ ($h^{-1}$), the instantaneous rate of change in cell numbers in the capillaries is described by the following differential equation:

(5)

$$\frac{dN}{dt} = \mu \cdot N + m$$

Integration of equation (5) yields

(6)

$$N_t = N_0 \cdot e^{\mu t} + \frac{m}{\mu}(e^{\mu t} - 1)$$

In chemotaxis experiments, capillaries were filled with sterile filtered seawater. Therefore, $N_0$= 0 and the first term in eq. 6 becomes zero. Prof. Dr. Dirk Metzler (Ludwig-Maximilians-Universität München) solved differential equations.

During incubation of the capillaries, the rapid diffusion of substrates into the surrounding medium causes a rapid decline in substrate concentration at the opening of the capillaries. Consequently, the bacteria found inside the capillary must have been capable of sensing lower concentrations than present at the opening of the capillary at the start of the chemotaxis experiment. In order to estimate the actual concentration sensed by the marine bacteria, additional diffusion experiments were conducted using fluorescein which has a diffusion coefficient ($D = 0.5 \cdot 10^{-9}$ $m^2 \cdot s^{-1}$; (Stocker *et al.*, 2008)) that is comparable to other low molecular weight substrates tested during the chemotaxis experiments. Capillaries were filled with 50 µM 5(6)carboxy-2',7'-dichlorofluorescein-diacetate dissolved in artificial seawater medium (ASW) (Manske *et al.*, 2005) with the pH set to 10 by addition of NaOH. 10% (v/v). DABCO solution (see above) was added to prevent rapid fading. Subsequently capillaries were sealed at one or both (control experiments) ends with plasticine to avoid evaporation. Capillaries were incubated in a Meplats-bottle filled with 20 ml artificial seawater salt solution for 24 hours in the dark. Control capillaries were incubated in the same way. Fluorescence intensity was analyzed by fluorecence microscopy (Zeiss Axiolab, filter set 10, FITC EX BP 450-490, EM 515-565) and fluorescence images were recorded with a digital camera (Hamamatsu Orca R2, C10500, Olympus, Munich, Germany). Control measurements demonstrated that the camera signal increased in a linear fashion with fluorescence intensity over the range used for measurements. Relative gray values corresponding to particular fluorescence intensities and exposure times were determined at different areas of the capillary opening employing the imaging software cell^F (Olympus).

### 3.1.6 Cultivation and isolation of Thalassospira sp. EM from the Eastern Mediterranean Sea

Cultivation trials were conducted in substrate gradient agar tubes. An equal mixture of 43 different carbon substrates were added [acetate, arabinose, benzoate, butanol, buyrate, citrate, ethanol, formate, glucose, lactate, malate, mannitol, methanol, N-acetyl glucosamine, 2-oxoglutarate, propanol, propionate, pyruvate, salicylate, succinate, valerate, a combination of the 20 canonical amino acids, at 20 µM each; and yeast extract and Tween

80 0.1% and 0.01% (w/v), respectively]. 1-ml bottom agar plugs contained ASW, substrates and 1% (w/v) washed agar (Oxoid, Cambridge, UK), and were overlaid with 4 ml of 1% washed agar without substrates. One l ASW basic medium was composed of 24.4 g NaCl, 10 g $MgCl_2$ x 6 $H_2O$, 1.5 g $CaCl_2$ x 2 $H_20$, 0.66 g KCl, 4 g $Na_2SO_4$ and 2.38 g HEPES; trace elements and vitamins were added according to Manske (Manske *et al*., 2005), while $KH_2PO_4$ (P source) and $NH_4Cl$ (N source) were omitted in basic medium. For gradient agar tubes P and N were added to a final concentration of 5 mM and 500 µM respectively. Gradients were produced by pre-incubation over a period of five days. Subsequently, the bacterial cell suspension generated by tangential flow filtration was used to inoculate the gradient agar tubes. The gradients were inoculated over their entire depth using a sterile Pasteur pipette, and incubated at 15 °C in the dark for up to five weeks.

For molecular analyses of bacteria enriched in gradient agar tubes, 1 $cm^3$ agar block containing enriched bacterial cells was removed with a sterile spatula and subsequently transferred into a falcon tube containing 5 ml sterile filtered tap water. After mixing thoroughly the resulting cell-agar-suspension was filtered (type SCWP, diameter 8 µm, Millipore) in order to get rid of agar particles. Filtrate was then centrifuged at maximum g-force (centrifuge 5417R, Eppendorf, Hamburg, Germany) for 20 minutes. Supernatant was discarded and cells were resuspended in 50 µl sterile filtered tap water. Cells were lysed by five consecutive rounds of freezing (ethanol-ice bath) and thawing (boiling water bath). Bacterial enrichments were screened by amplification of 16S rRNA gene fragments employing universal primers for *Bacteria*, separation of the fragments by denaturing gradient gel electrophoresis (DGGE), and sequencing of the excised bands (3.2.3).

In order to obtain bacterial pure cultures, subsamples of enriched bacteria grown in gradient agar tubes were streaked onto agar plates composed of ASW amended with carbon substrates (see above), 1 µM $KH_2PO_4$, 20 µM $KNO_3$ and 20 µM $NH_4Cl$ and solidified with 1.5% (w/v) purified agar. Agar plates were incubated at 15 ° C in the dark until growth became visible. Colonies of different morphology were then transferred to liquid artificial seawater medium of the same composition. Single colonies grown on ASW-agar plates were transferred into the wells of sterile 96-well polystyrene microtiter plates (Corning Inc., Corning, NY). Each well was filled with 180 µl ASW medium amended with N,C and P (see above). Plates were incubated at 15°C in the dark until growth occurred (one to two weeks). Growth was monitored microscopically.

Finally, a motile bacterium was purified by repeated streaking onto agar plates composed of Marine Broth (Difco, Becton Dickinson, Heidelberg, Germany) solidified with 1.5% agar. Routine cultivation of this strain was done in Marine Broth. For chemotaxis experiments, the isolate was cultured in artificial seawater medium containing 5 mM $KH_2PO_4$, 15 mM $NH_4Cl$, 10 mM glucose and 0.005% (w/v) yeast extract ($ASW_{GluNPYE}$). In order to obtain phosphate-depleted bacterial cells, $KH_2PO_4$ was omitted ($ASW_{GluNYE}$).

### 3.1.7 CHEMOTAXIS ASSAYS EMPLOYING BACTERIAL PURE CULTURES

*Thalassospira* sp. EM isolated from the Eastern Mediterranean Sea, as well as *Thalassospira lucentensis* DSM 14000$^T$ and *Thalassospira profundimaris* WP0211$^T$ (=DSM 17430$^T$) were maintained in ASW supplemented with phosphate, ammonia, glucose and trace amounts of yeast extract as described above. An initial series of cultivation experiments revealed that none of the *Thalassospira* strains was able to grow in defined mineral medium in the absence of trace amounts of yeast extract (see Results section). The concentration of yeast extract was minimized to 0.005% (w/v) in order to enable growth of the bacteria but at the same time to produce phosphate-starved cultures. Based on a phosphorus content of yeast extract of 1.34 weight% (Kirkbright and Marshall, 1973), this amount corresponds to starting concentrations of 21 µM of phosphate in the cultures. For the chemotaxis experiments, phosphate depleted cultures were generated by two consecutive passages in ASW lacking free phosphate. In the first passage, cells were starved in phosphate-free $ASW_{GluN}$ to deplete intracellularly stored P. To generate sufficient cell densities for chemotaxis experiments, the prestarved cells were then inoculated into ASW devoid of phosphate ($ASW_{GluNYE}$) for phosphate starvation and parallel into ASW containing 5 mM phosphate ($ASW_{GluNPYE}$) for phosphate-replete cultures.

Chemotactic responses of bacteria were analyzed by capillary assays (Fröstl and Overmann, 1998; Overmann, 2005). After reaching stationary growth phase, 15 ml aliquots of the *Thalassospira* cultures were transferred into sterile Meplats bottles that had twelve bore holes drilled through one side wall. Flat rectangular glass capillaries (length 50 mm, inside diameter 0.1 x 2.0 mm; Vitrocom) were filled by capillary action with the stock solutions, sealed at one end with plasticine (Münchner Künstler Plastilin), inserted into the holes of the Meplats bottles such that the open ends of the capillaries were immersed in the culture inside, and fixed in the holes with plasticine. Chemotaxis assays lasted for 1.5 h.

Different concentrations of $KH_2PO_4$ , 2-aminoethylphosphonate (2 mM), a mixture of amino acids (L-threonine, L-arginine, L-histidine, L-methionine, L-proline, glycine, L-lysine, L-valine, L-serine, L-alanine, L-glutamic acid, L-leucine, L-cysteine, L-asparagine, L-tryptophan, L-aspartic acid, L-glutamine; 2mM each), a mixture of sugars [α-D(+)-glucose, α-mannitol, L(+)-arabinose, D(+)-trehalose and (+)-xylose; 2 mM each], yeast extract (0.1% v/v) or peptone (0.1% v/v) were assessed as chemoattractants and for this purpose were dissolved in culture supernatant. Capillaries filled with culture supernatant alone served as negative control. Experiments were routinely carried out in two separate Meplats bottles as parallels. The entire content of three capillaries per Meplats bottle were recovered, their contents combined and fixed with glutardialdehyde (2% v/v) for cell counting.

In addition to chemotaxis experiments using stationary bacterial cell cultures, aliquots for analyzing bacterial chemotaxis toward phosphate were additionally removed from growing phosphate starved and phosphate saturated cultures. In total at four different time points (phosphate-deplete culture) or two different time points (control: phosphate-replete culture) 15 ml aliquots of the respective cultures were transferred into sterile Meplats bottles with twelve bore holes. Chemotaxis experiments were carried out as described.

### 3.1.8 DETERMINATION OF SWIMMING SPEED AND CELL SIZE

The mean swimming speed of *Thalassospira* sp. EM under various growth conditions was determined as described by Eschemann et al. (Eschemann *et al*., 1999). Bacterial cultures were diluted as required and a drop of the cell suspension was spotted onto a glass slide. The movement of cells was immediately traced under an inverted microscope (Axiovert, Zeiss) employing dark field at 400-fold magnification. Tracks of swimming cells were measured over an exposure time of 500 msec using a digital camera (Hamamatsu Orca R2, C10500) and the software package cell^F (Olympus). Mean swimming speed for each growth condition was determined based on measurements with up to 109 individual cells. The fraction of motile cells was determined based on the number of trajectories of motile cells and numbers of point-shaped traces of non-motile cells.

Bacterial cell sizes in phosphate-starved and phosphate-replete *Thalassospira* sp. cultures were determined by phase-contrast microscopy using fixed cells and 1250 fold magnification. Images of were recorded digitally (Hamamatsu Orca R2, C10500, Olympus) and analyzed with the software package cell^F (Olympus).

### 3.1.9 ANALYTICAL PROCEDURES

Growth was monitored by following optical density of subsamples at 580 nm (Genesys 20, Thermo Scientific, Dreieich, Germany) and cell counting in a Neubauer counting chamber (Marienfeld, Lauda-Königsdorf, Germany). For cell counting, 500 µl aliquots of the cultures were fixed with glutardialdehyde (final concentration 2% v/v). Numbers of colony forming units (CFU) were determined by plating on Marine Broth agar plates.

Total protein content was determined in triplicate using 2 ml culture aliquots. Cells were harvested by centrifugation at 20,800 x g (Eppendorf, 5417R) for 15 minutes. The pellets were washed once with artificial seawater medium devoid of glucose, resuspended in 250 µl of 0.1 N NaOH and lysed by boiling for 2 minutes. Subsequently 1 to 2 µl of the solution was used for measurements of protein concentrations using a bicinchoninic acid assay (Smith *et al*., 1985).

The presence of 19 different exoenzymes was assessed using API ZYM test strips (API systems, bioMérieux, Nürtingen, Germany). Bacterial cells from one ml of cultures were collected by centrifugation at 20,800 x g (Eppendorf, 5417R) for 15 minutes, the cells resuspended in one ml of 1 M NaCl and transferred into the API ZYM chambers. Test strips were incubated for 4 hours at room temperature. The activity of the different exoenzymes in seawater samples from the Eastern Mediterranean Sea was assessed using the bacterial cell concentrates obtained by tangential flow filtration (see above). In this case, incubation time was extended to 16 hours and the incubation temperature was adjusted to the *in situ* value of 15 °C.

## 3.2 MOLECULAR BIOLOGICAL METHODS

### 3.2.1 EXTRACTION OF DNA

Genomic DNA of chemotactically accumulated cells was extracted using the QIAamp DNA MicroKit (Qiagen, Hilden, Germany). The protocol of the manufacturer for the isolation of genomic DNA from small volumes of blood was modified as follows. After addition of proteinase K, samples were incubated for 3 hours at 55 °C on a thermoshaker (Thermomixer 5436, Eppendorf, Hamburg, Germany). Afterwards, 1 µg of carrier polyA RNA was added. For the final elution step, 2 mM Tris-buffer (pH 8.0) was applied instead of buffer AE. As negative controls, 15 µl of sterile-filtered seawater were subjected to the same extraction procedure. In a last step, the eluates were concentrated to a final volume of 4 µl using a Speed-Vac (SC100, Savant). The entire volume was used in subsequent PCR amplifications (3.2.2).

Filters containing either bacterial cells from dialysis cultures or bacteria present in seawater (concentrated or non-concentrated) were extracted according to a protocol modified after (Fuhrman *et al*., 1988). Filters were cut into small strips with a sterile scalpel and transferred into sterile 12 ml polypropylene tubes. After adding 4 ml STE-buffer (10 mM Tris-HCl, 1 mM EDTA, 100 mM NaCl, pH 8.0) as well as 0.4 ml 10% (w/v) sodiumdodecylsulfate the samples were boiled for 10 minutes. The liquid phases were transferred into fresh polypropylene tubes and the filter strips were washed with 2 ml TE-buffer (10 mM Tris-HCl, 1 mM EDTA, pH8.0). Both liquid phases of each sample were combined and extracted with 4 ml phenol/chloroform/isoamylalcohol (25:24:1, v/v/v). After centrifugation for 10 min at 6000 x g (Avanti J-25, rotor JA14; Beckman Coulter, Krefeld, Germany) the upper aqueous phases were extracted again with 4 ml chloroform and centrifuged again. Afterwards the aqueous DNA solutions were transferred to a Centricon-50 dialysis filtration unit (Millipore) and the DNA was purified according to the instructions of the manufacturer. DNA concentrations were determined by fluorescent dye binding with PicoGreen (Invitrogen, Karlsruhe, Germany). Of each extract 10 ng of DNA were used during subsequent PCR.

In order to obtain DNA of sufficient purity for quantitative PCR analyses (3.2.4) an additional purification step was included. DNA was precipitated by adding 0.1 volumes of 3 M sodium acetate and 2.5 volumes 100% ethanol followed by incubation at -20 °C

overnight. The DNA was sedimented by centrifugation at 18.000 x g for 30 minutes at 4° C (Eppendorf, 5417R), washed with ice cold 70% (v/v) ethanol and dried and resuspended in 10 µl of sterile Tris-buffer (2 mM Tris-HCl, pH 8.0). DNA concentrations were determined by fluorescent dye binding with PicoGreen (Invitrogen).

### 3.2.2 PCR AMPLIFICATION AND CLONING OF 16S rRNA GENES

All PCR amplifications were carried out in a GeneAmp PCR system 9700 (Applied Biosystems, Foster City, USA). PCR was either employed for generating a mixture of PCR products with GC-clamps suitable for subsequent DGGE analyses or to generate a mixture of PCR products with A-overhangs for subsequent cloning reactions. Additionally the nearly full-length 16S rRNA gene of the novel bacterial isolate *Thalassospira* sp. EM was amplified by means of PCR for subsequent sequence analysis.

For PCR amplification in preparation for DGGE (denaturing gradient electrophoresis) either 1 µl of lysed cell solution or 500 ng extracted DNA were employed in each PCR reaction. The variable region of 16S rRNA genes was amplified using the universal bacterial primer GC341f and 907r (Muyzer and Ramsing, 1995). To ensure stable melting behavior of PCR-generated DNA fragments primer 341f contained a 40 bp-long GC-clamp at its 5' end. Cycling conditions were as followed. A hot start at 96 °C for 4 minutes was followed by a step-down PCR protocol: 10 cycles with denaturation at 94 °C for 30 sec, primer annealing at 58 °C for 45 sec and elongation at 72 °C for 1 min were followed by 20 cycles with denaturation at 94 °C for 30 sec, primer annealing 53 °C for 45 sec and elongation 72 °C for 1 min. A final extension at 72 °C for 7 min was included to ensure maximum amount of correct-sized PCR products. Each 50 µl reaction contained additionally to 1 µl cell suspension or DNA solution 1 µl primer GC341f (50 µM), 1 µl primer 907r (50 µM), 5 µl PCR buffer containing 15 µM $MgCl_2$ (10x, Qiagen GmbH, Hilden Germany), 1 µl dNTP-Mix (final concentration 200 nM each, Qiagen), 10 µl Q-solution (5x, Qiagen) 500 nmoles $MgCl_2$ 40 µg bovine serum albumin, 1.25 units taq polymerase (Qiagen) and 27 µl PCR-grade water. PCR-products were analyzed by standard agarose gel electrophoresis prior to DGGE.

PCR amplifications in preparation for cloning were carried out as followed. Each 25 µl PCR reaction contained template DNA (3.2.1), 25 pmoles of each primer 8f and 1492r (Lane, 1991), 1x GeneAmp PCR Gold buffer (Applied Biosystems), 100 pmoles of each dNTP, 87.5 nmoles $MgCl_2$, 2 µg of bovine serum albumin and 0.625 units of AmpliTaq Gold

LD DNA polymerase (Applied Biosystems). The cycling conditions comprised an initial 5 minutes at 95 °C for DNA denaturation and activation of the hot start polymerase, followed by 10 cycles of denaturation at 94 °C for 30 seconds, primer annealing at 59 °C for 45 seconds and elongation at 72 °C for 1 minute. During the subsequent 25 cycles, the primer annealing temperature was decreased to 54 °C. To generate A-overhangs for the subsequent cloning reaction a final elongation step at 72 °C for 7 minutes was included. PCR-products were analyzed by standard agarose gel electrophoresis.

Prior to cloning, all PCR products generated were gel purified using the NucleoSpin Extract II Kit (Machery-Nagel, Düren, Germany). The cloning reaction was performed with the TOPO TA cloning Kit (Invitrogen) according to the instructions of the manufacturer. Plasmids were extracted with alkaline lysis and the presence of inserts of the correct size was verified by digestion of the purified plasmids with the restriction enzyme EcoRI (New England Biolabs, Frankfurt, Germany).

For phylogenetic analyses of the isolated bacterial strain, the almost full length 16S rRNA gene was amplified by colony PCR with universal bacterial primers 8f and 1492r (Lane, 1991) as described. PCR products were purified using the NucleoSpin Extract II Kit (Machery-Nagel).

### 3.2.3 DENATURING GRADIENT GEL ELECTROPHORESIS (DGGE)

Denaturing gradient gel electrophoresis was employed in order to determine the identity of bacteria enriched in gradient agar tubes. In another experiment this fingerprint technique was used to analyze the natural bacterial community present in concentrated (using tangential flow filtration) and non- concentrated seawater (chl maxima) throughout the Eastern Mediterranean Sea.

To separate the mixture of PCR-products according to their GC content, 6% (w/v) polyacrylamidegels in 1X Tris-acetate-EDTA (pH 8.0) and a urea/formamide gradient from 70% to 30% where 100% denaturant is defined as 7M urea and 40% (v/v) formamide, were used (Muyzer *et al*., 1993). Electrophoresis was performed in an Ingeny phoU system (Ingeny International BV, Goes, The Netherlands). Electrophoresis buffer was preheated to 60 °C. Electrophoresis was run at 150 V for 16 h. Gels were subsequently stained in a rotating buffer bath containing 1 in 10000 diluted SYBRgold (MoBiTec, Göttingen, Germany) for 45 minutes. Bands were visualized on a UV transilluminator (LTF Labortechnik, Wasserburg, Germany) and gels were then photographed (Visitron Systems

GmbH, Puchheim, Germany). For sequence analyses gel bands were excised with a sterile scalpel and immediately transferred to 1.5 ml reaction tubes each containing 25 µl Tris-HCl-buffer (10 mM, pH 8.0), DNA was eluated by incubating the reactions for to 2 h at 65 °C and reamplification reactions were conducted using the same primer set without GC-clamp and the same PCR cycling conditions. PCR products were purified using the QIAquick SpinKit (Qiagen) and sequenced as described.

In order to compare DGGE profiles a cluster analysis was performed. First bands were defined using GelAnalyzer (http://www.gelanalyzer.com/). Subsequently a binary matrix was constructed assessing the presence (1) or absence (0) of a band in a certain lane. This binary matrix was transformed into a distance matrix using the Jaccard coefficient and next a dendrogram was constructed applying the unweighted-pair group method (UPGMA). Therefore, the software PAST was employed (Hammer, 2001).

### 3.2.4 QUANTITATIVE PCR

The relative abundance of bacteria of the genus *Thalassospira* in water samples was determined by a specific quantitative PCR. The genus-specific PCR primers Tha585f (5´-CGGTCTTGCCAGTCAGGG-3´) and Tha655r (5´-CACCACCCTCTCCTAGTC-3´) were designed using the ARB software package (Ludwig *et al*., 2004). Primer specificity was checked using the probe match tool of the RDP database (Cole *et al*., 2009) and by qPCR trials with genomic DNA of *Thalassospira* sp. EM as a positive control and 5 ng of genomic DNA from the six *Alphaproteobacteria Rhodospirillum rubrum* DSM107, *Rhizobium radiobacter* DSM30147$^T$, *Sphingomonas kaistensis* DSM16846$^T$, *Hyphomonas jannaschiana* DSM5153$^T$, *Ruegeria pomeroyi* DSM15171$^T$ and *Erythrobacter citreus* DSM14432$^T$ as negative controls. Genomic DNA of *Thalassospira* sp. EM served as standard for calibration and was employed as a tenfold dilution series ranging from 5 fg to 500 pg. All reactions were run at least in triplicates using an iCyclerQTM Multi-Color Real Time Detection System (Bio-Rad, Munich, Germany). Each 25 µl reaction consisted of 8.5 µl PCR-grade water, 20 µg bovine serum albumine, 12.5 µl IQ SYBR Green Supermix (Bio-Rad), 2 µmoles each of primers Tha585f and Tha655r and the respective DNA template. The cycling conditions included an initial denaturation at 94 °C for 2 minutes, followed by 40 cycles of denaturation at 94 °C for 15 seconds, annealing at 65 °C for 20 seconds and elongation at 72 °C for 20 seconds. After completion, the melting behavior of products was analyzed to assess their specificity. Evelyn Lage-Sonntag helped with initial qPCR experiments.

### 3.2.5 SEQUENCING, PHYLOGENETIC ANALYSES OF 16S RRNA GENES AND DIVERSITY ESTIMATES

Inserts and purified PCR products were sequenced by the dideoxynucleotide method (Sanger *et al*., 1977) on an ABI Prism 3730 genetic analyzer (Applied Biosystems) using either standard M13 primers or primers 8f, 1492r and 907r and the AmpliTaq FS Big Dye terminator cycle sequencing kit. Sequences were edited and assembled with the Vector NTI computer package (Invitrogen). All sequences were finally checked for the presence of chimeric sequences using the software program "Pintail" (Ashelford *et al*., 2005). Suspicious sequences were subsequently removed.

The closest relatives of all sequenced 16S rRNA gene fragments obtained from clone libraries were identified and retrieved from the GenBank database employing blastn (Altschul *et al*., 1997). Nearly full-length sequences of the station H02 clone library were imported into the ARB program package (Ludwig *et al*., 2004) and subsequently aligned to close relatives from the SILVA database (Pruesse *et al*., 2007). Phylogenetic trees were constructed using the FastdnaML maximum likelihood algorithm as implemented in the ARB software package. Since sequences of 800 bp length were sufficient for the classification into genera, only partial sequences were obtained for four different clone libraries established for station Ier01. These shorter sequences were included in the phylogenetic analysis using the parsimony tool and the termini-filter of the ARB software package. Sequences were judged as identical based on the sequencing error of 0.1% which was calculated from the fidelity value of the taq polymerase ($2.28 \cdot 10^{-5}$), the length of the PCR fragment and the number of amplification cycles (http://www.finnzymes.com/pcr/fidelity_calc.php).

For diversity analyses, distance matrices for sequences in the clone libraries were calculated using the ARB software package (Ludwig *et al*., 2004). These matrices were subsequently analyzed employing the software mothur package (Schloss *et al*., 2009). The diversity estimator Chao1 and the coverage of the clone libraries obtained were calculated using the default settings of mothur. For these analyses the cutoff value was set to 99.9%.

For detailed phylogenetic analysis of the novel isolate *Thalassospira* sp. EM the obtained sequence was imported into the ARB program package (Ludwig *et al*., 2004). Additionally 16S rRNA gene sequences of *Thalassospira* spp. were retrieved from the GenBank database and imported into the ARB program package. After alignment, phylogenetic trees were constructed using the FastdnaML maximum likelihood algorithm as implemented in the ARB software package.

### 3.2.6 NUCLEOTIDE SEQUENCE ACCESSION NUMBERS

The 16S rRNA gene sequence of *Thalassospira* sp. EM has been deposited in the GenBank database under accession number JF292454. The 16S rRNA gene sequences obtained from clone libraries have been deposited in the GenBank database under accession numbers HQ161359 to HQ161632. Sequences obtained from excised DGGE bands are listed in the supplement (II).

# 4 RESULTS

## 4.1 EXOENZYMATIC ACTIVITIES, COMPOSITION AND GROWTH RESPONSE OF BACTERIOPLANKTON

### 4.1.1 PHYSICOCHEMICAL CONDITIONS AND VERTICAL DISTRIBUTION OF BACTERIA AT THE STUDY SITES

The sampling stations (Figure 4-1) were located in the ultraoligotrophic eastern part of the Mediterranean Sea where molar N:P ratios of 25.6 to 141.6 were measured concomitantly (Emeis *et al.*, 2010).

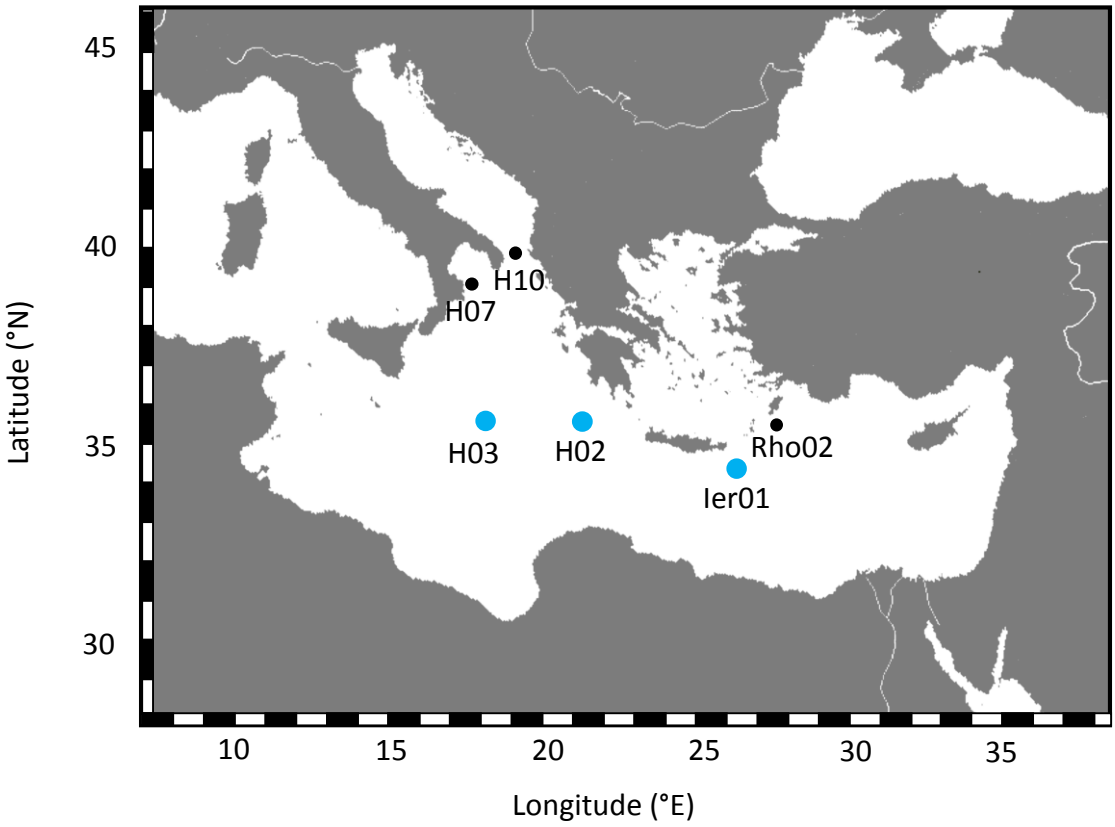


Figure 4-1
Position of sampling locations in the Eastern Mediterranean Sea. Blue and black circles show the location of main sampling stations (chemotaxis experiments were performed at these stations) and additional sampling stations, respectively

Winter surface temperatures in this layer of Modified Atlantic Surface Water (Rubino and Hainbucher, 2007) ranged from 16.7 and 17 °C at stations H02 and H03 and reached 19 °C

at station Ier01 (Figure 4-2 A). At stations H02 and H03 a thermocline and halocline was present between 100 and 200 m depth (Figure 4-2 A. B). These gradients mark the upper boundary of Levantine and Cretan Intermediate Water which has an increased salinity, forms during winter time and occupies depths between 200 and 500 m at these two stations. Within the Modified Atlantic Surface Water, smaller but significant vertical differences in temperature and salinity were determined, indicating that in this part of the water column vertical mixing occurred only to a limited extent. At station Ier01, however, the upper 250 m of the water column exhibited constant temperature and salinity values demonstrating vertical mixing over this entire depth interval. Water temperatures reached 13.8 °C below 1000 m depth. The uniform values of temperature and salinity (38.7 psu; Figure 4-2 B) at this depth at all three stations are characteristic for the homogenous Eastern Mediterranean Deep Water that originates in the Adriatic Sea (Rubino and Hainbucher, 2007). Whereas chlorophyll (chl) *a* fluorescence remained almost constant within the mixed upper water column at station Ier01, vertical differences in Chl a existed within the Modified Atlantic Surface Water at station H02, and a pronounced maximum was detected at a depth of 60 m at station H03 (Figure 4-2 C).

Total cell numbers and numbers of red autofluorescent cells at the surface of station H03 surpassed the numbers at the other stations (Figure 4-3 A, B) but decreased steeply through the salinity maximum. Total cell numbers measured at the chlorophyll maximum and the salinity maximum were lowest at station H07 (Figure 4-3 A, B). All in all cell numbers were in the same order of magnitude at all stations investigated. Cell numbers in the deep water samples were approximately one order of magnitude lower than in the upper layers.

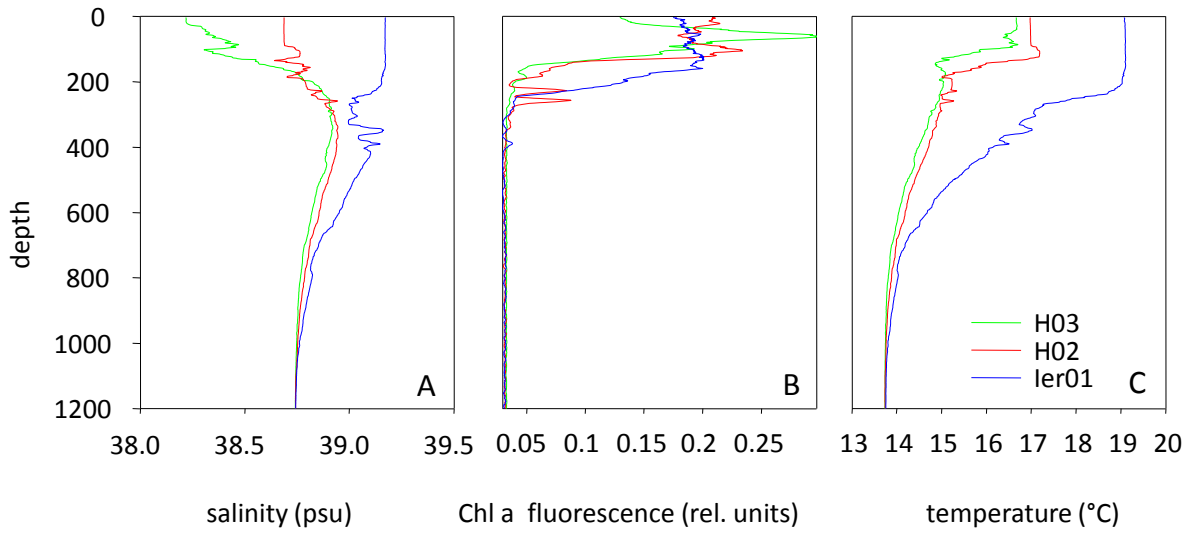


Figure 4-2

Vertical distribution of physicochemical parameters at the three main sampling locations. A. Temperature. B. Salinity (given in potential salinity units, psu). C. Fluorescence

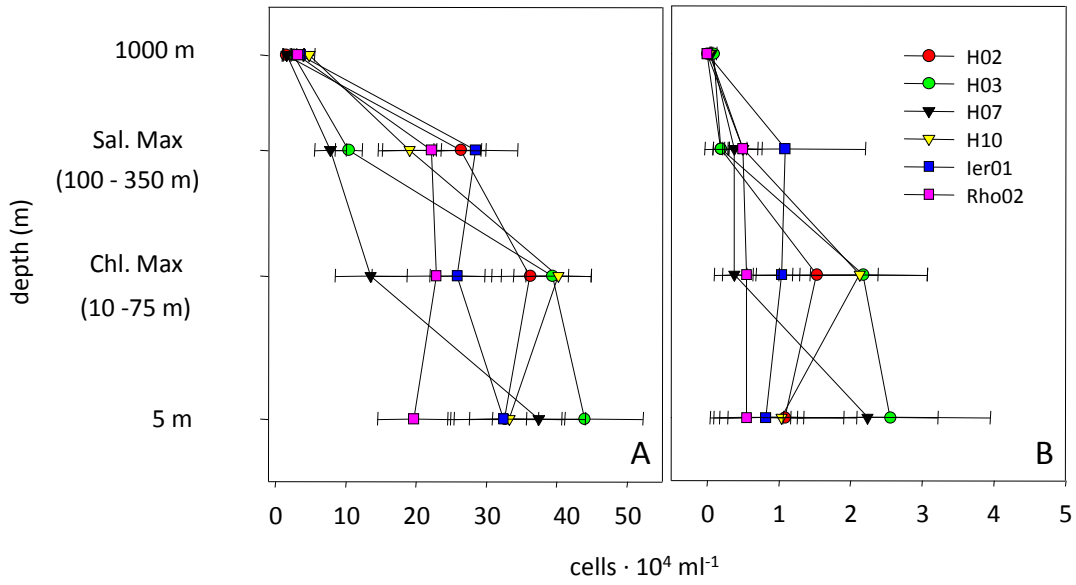


Figure 4-3

Bacterial biomass at the sampling stations. A. Total bacterial cell numbers. B. Numbers of red and orange autofluorescent cells. Horizontal bars in A. and B. indicate one standard deviation

### 4.1.2 EXOENZYMATIC ACTIVITIES

In order to measure enzymatic parameters of alkaline phosphatase and leucine aminopeptidase the fluorigenic substrate analogues MUF-P and AMC-leucine were employed. When utilizing untreated (non-concentrated) seawater out of the chlorophyll maxima at stations H02, H10, Ier01 and Rho02 (Figure 4-1) even long incubation times did not allow accurate measurement of kinetic parameters, due to low overall bacterial biomass and activity in the ultraoligotrophic Eastern Mediterranean Sea (Thingstad *et al*., 2005). Thus concentrated seawater was used for determining activities of alkaline phosphatase and leucine aminopeptidase. Concentration of biomass was achieved by means of tangential flow filtration.

Experiments were run in two parallels for each substrate concentration. Standard deviations (fluorescence intensities) were usually below 15% (Figure 4-4).

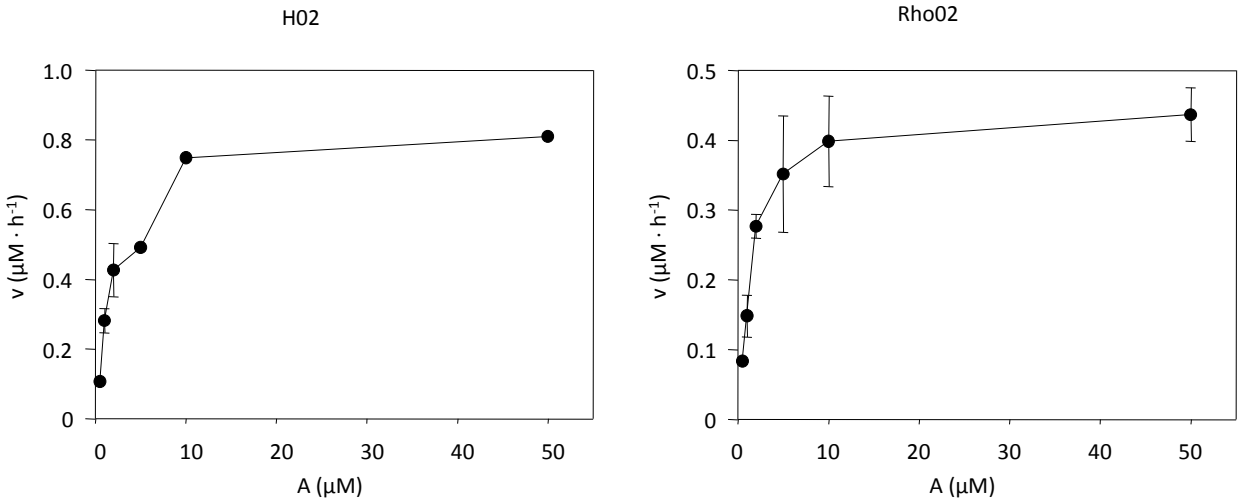


Figure 4-4

Alkaline phosphatase activity. Michaelis-Menten kinetics of alkaline phosphatase activity measured at station H02 and station Rho02. Concentrated seawater out of the respective chlorophyll maximum was used for analyses. Error bars indicate one standard deviation.

Alkaline phosphatase activity (Figure 4-4) as well as leucine aminopeptidase activity (data not shown) follow Michaelis-Menten kinetics. Consequently relevant kinetic parameters could be calculated according to Wright and Hobbie (Wright and Hobbie, 1966) (Table 4-1; equations 2-4).

Table 4-1

Kinetic Parameters measured for alkaline phosphatase (A) and leucine aminopeptidase (B) at four different sampling stations[a].

A. Alkaline phosphatase

| Station | H02 | H10 | Ier01 | Rho02 |
|---|---|---|---|---|
| **Kt+Sn (µM)** | 2.552 | 2.050 | 0.673 | 1.681 |
| **Vmax (µM · $h^{-1}$)** | 0.854 | 1.149 | 1.025 | 0.452 |
| **Turnover time (h)** | 2.990 | 1.784 | 0.657 | 3.718 |
| **Affinity ($h^{-1}$)** | 0.269 | 0.269 | 1.522 | 0.269 |
| **Specific rate (pmol $cell^{-1}$ · $h^{-1}$)** | 0.739 | 0.756 | 2.614 | 0.617 |

B. Leucine aminopeptidase

| Station | H02 | H10 | Ier01 | Rho02 |
|---|---|---|---|---|
| **Kt+Sn (µM)** | 87.160 | 27.943 | 36.963 | 266.278 |
| **Vmax (µM · $h^{-1}$)** | 1.923 | 0.453 | 0.823 | 3.668 |
| **Turnover time (h)** | 45.323 | 61.634 | 44.963 | 72.602 |
| **Affinity ($h^{-1}$)** | 0.022 | 0.016 | 0.016 | 0.016 |
| **Specific rate (pmol $cell^{-1}$ · $h^{-1}$)** | 1.787 | 0.298 | 2.098 | 5.442 |

[a]For measuring exoenzymatic activities water out of the DCM was used. The exact position of the four stations is depicted in Figure 4-1

Alkaline phosphatase activity was high at all four stations investigated (Figure 4-4; Figure 4-1). Affinity constants (Km+Sn values) covaried with maximum hydrolysis rates (vmax). For this reason turnover times and affinity values stayed constant over the covered transect. Calculated turnover times were distinctly shorter and affinity values were higher than those reported previously (11-74 h and 0.012-0.036 $h^{-1}$ respectively; (Van Wambeke *et al*., 2002). When compared with alkaline phosphatase affinity, values measured for leucine aminopeptidase were lower reaching only 0.016 – 0.022 $h^{-1}$ and turnover times were significantly longer (44.9 –72.7 h) (Table 4-1). Once more turnover times and affinity values stayed constant over the whole transect.

### 4.1.3 COMPOSITION OF BACTERIAL COMMUNITIES IN CONCENTRATED AND NON-CONCENTRATED SEAWATER

The DGGE technique was chosen in order to evaluate the impact of the concentration process (tangential flow filtration) on composition of the bacterial community. Resulting fingerprints of bacteria present in concentrated and non-concentrated seawater show that all in all 46 different melting types could be clearly distinguished (Figure 4-5). The number of bands derived from bacteria present in concentrated seawater varied between 13 and 17 and was therefore significantly lower than the number of melting types originating from bacteria present in non-concentrated seawater (between 19 and 24 bands; Figure 4-5).

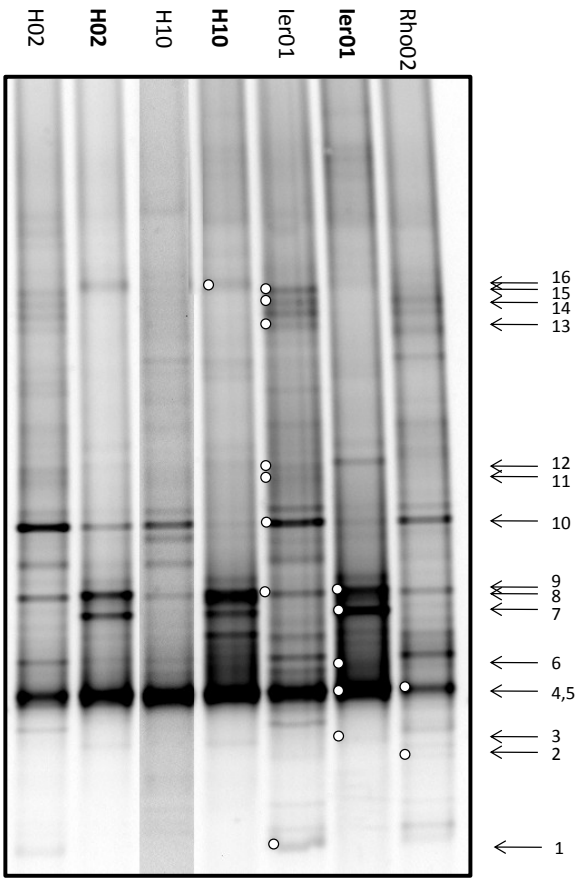


Figure 4-5

DGGE profile of bacteria present *in situ* in concentrated and non-concentrated seawater. Bacteria present at four different stations (Figure 4-1) were investigated. Circles denote bands which were excised and subsequently sequenced. Arrows indicate the position within the gel of each band excised. Each sequenced band was given a number, which was also used in consecutive phylogenetic analyses (Table 4-2). Bold print indicates samples derived from concentrated seawater and normal print indicates samples derived from non-manipulated seawater

To assess similarity of the resulting banding patterns, cluster analyses using the Jaccard coefficient were performed (Figure 4-6).

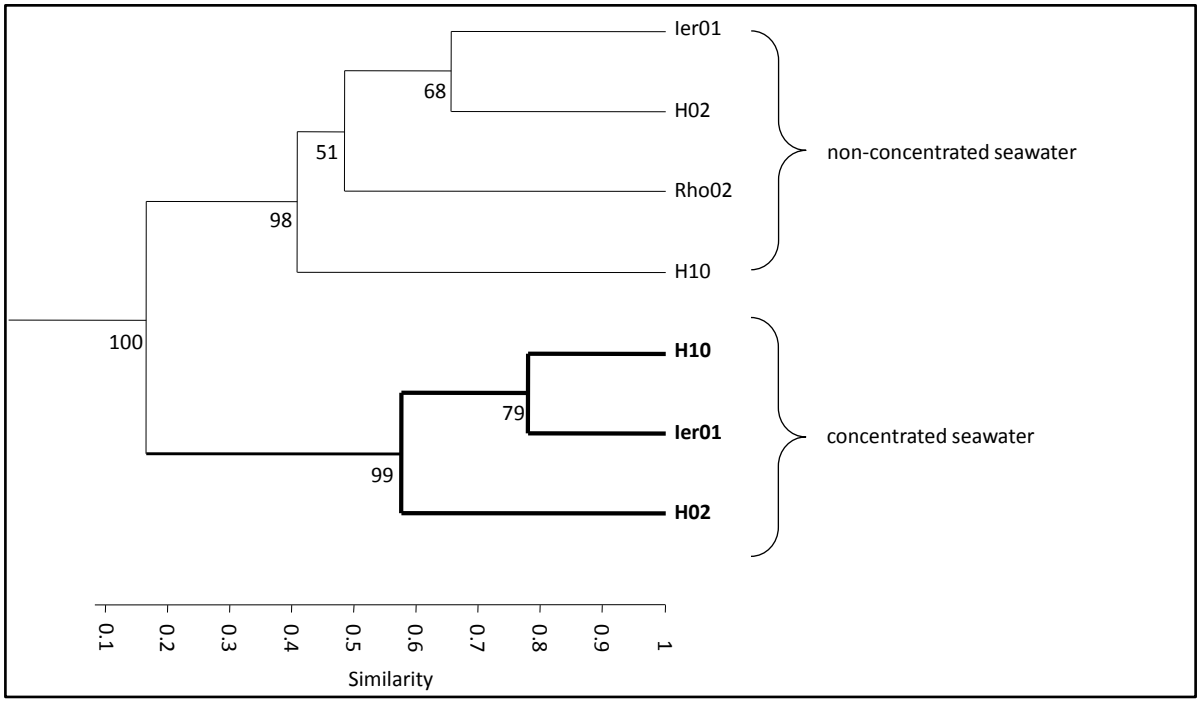


Figure 4-6

Similarity of bacterial communities in concentrated and non-concentrated seawater. Similarity is based on Jaccard coefficient. UPGMA-based dendrograms were constructed using 100 bootstrap resamplings. Bootstrap values > 50% are indicated at nodes

DGGE analysis of bacteria yielded several DNA fragments which were exclusively present in samples derived from concentrated seawater (band 7, band 16; Figure 4-5) whereas other fragments were detected exclusively in samples derived from non-concentrated seawater (band 1, band 13, band 15; Figure 4-5).

Similarity analysis (Figure 4-6) demonstrates that bacterial fingerprints cluster dependent on status of concentration rather than dependent on geographical origin of the samples analyzed. Thus a distinct cluster is formed by fingerprints derived from bacteria present in concentrated seawater, independent of geographical location (Figure 4-6). This implies that bacterial populations present in concentrated seawater of different stations were more similar to each other than to those present in non-concentrated seawater from the same station. Therefore, the concentration process apparently influenced bacterial composition significantly. For a more detailed phylogentetic analysis, 16 prominent bands

were excised from the gels (as indicated by arrows in Figure 4-5) and subsequently sequenced (Table 4-2).

Table 4-2

Phylogenetic affiliation of bacteria present in concentrated and non-concentrated seawater of the Eastern Mediterranean Sea analyzed by means of DGGE

| Band no.[a] | Origin[b] | Closest match (Accession no.) | Sequence similarity (in %)[c] |
|---|---|---|---|
| **1** | Ier01 | Uncultured delta proteobacterium HF0130_05G09 (GU474912) | 99 |
| **2** | Rho02 | Uncultured firmicute clone AEGEAN_182 (AF406552) | 99 |
| **3** | **Ier01** | *Methylobacterium* sp. SuP53 (EU912448) | 99 |
| **4** | **Ier01** | Uncultured *Synechococcus* sp. clone JL-ESNP-I5 (AY664232) | 99 |
| **5** | Rho02 | Uncultured cyanobacterium clone A8W_30 (HM057799) | 99 |
| **6** | **Ier01** | Uncultured cyanobacterium clone A8W_30 (HM057799) | 99 |
| **7** | **Ier01** | *Rhodobacteraceae* bacterium p1rh1 (FN811289) | 97 |
| **8** | Ier01 | *Brevundimonas* sp. FXJ8.080 (HQ622513) | 98 |
| **9** | **Ier01** | Uncultured *Roseobacter* sp. clone BG29-9 (AY904508) | 97 |
| **10** | Ier01 | Uncultured marine bacterium clone SPOTSAUG01_5m49 (DQ009139) | 99 |
| **11** | Ier01 | Uncultured alpha proteobacterium isolate DGGE band BL03-band33 (DQ778288) | 94 |
| **12** | Ier01 | Uncultured bacterium clone PROA27B_8 (GQ915632) | 98 |
| **13** | Ier01 | Uncultured Flavobacteria bacterium 16S rRNA gene, clone Vis_St18_1 (FN433412) | 99 |
| **14** | Ier01 | Uncultured bacterium clone PROA09B_43 (GQ916083) | 94 |
| **15** | Ier01 | Uncultured Flavobacterium sp. clone ARTE1_211 (GU230407) | 99 |
| **16** | **H10** | *Flexibacter* sp. UST991130-045 (AF465362) | 98 |

[a]Number of band excised from DGGE gel as indicated with arrows and circles in Figure 4-5

[b]Stations (Figure 4-1) from where water samples originate. Bold print designates water samples concentrated using tangential flow filtration in order to increase biomass · $ml^{-1}$.

[c]Degree of similarity of analyzed sequences to closest match in GenBank database. Sequences corresponding to the respective band can be found in the supplement

Bacteria present in concentrated (six bands) and non-concentrated (10 bands) seawater were affiliated with the *Alphaproteobacteria*, the *Gammaproteobacteria*, the *Deltaproteobacteria*, the *Bacteriodetes*, the *Firmicutes* and the *Cyanobacteria* (specifically uncultured bacteria belonging to *Synechococcus* sp.). Only two out of six bacteria present in concentrated seawater samples, were closely related with not yet cultivated bacteria (taking into account the first 100 blast hits), while 8 out of 10 bacteria present in non-concentrated seawater samples were related to uncultured organisms (according to the first 100 blast hits).

Table 4-3

Proportion of autotrophic cells in concentrated and non-concentrated seawater

| Station | H02[a] | H10[a] | Ier01[a] | Rho02[a] |
|---|---|---|---|---|
| **NC[b] (% autotrophs/TCC)** | 4.2 | 5.3 | 4.0 | 2.4 |
| **C[c] (% autotrophs/TCC)** | 11.3 | 11.4 | 8.7 | 8.0 |

[a]All water samples were derived from the respective chlorophyll maxima (Figure 4-1).
[b]NC: non-concentrated seawater
[c]C: concentrated seawater (concentration by means of tangential flow filtration)

Along the entire transect the percentage of autotrophs was twice as high in concentrated seawater samples as in non-concentrated seawater samples (Table 4-3). Autotrophic bacteria seem to be concentrated more efficiently than heterotrophic bacteria, as the former are more abundant in concentrated water samples than in untreated water from the same station.

### 4.1.4 GROWTH IN DIALYSIS CULTURES

In order to assess the limitation of growth of heterotrophic bacterioplankton by inorganic nutrients and organic carbon, bacterial growth rates in samples from the chl maxima at the two sampling stations H02 and H03 (Figure 4-1) were determined after addition of inorganic nutrients and carbon compounds at concentrations corresponding to the 50-fold of ambient values.

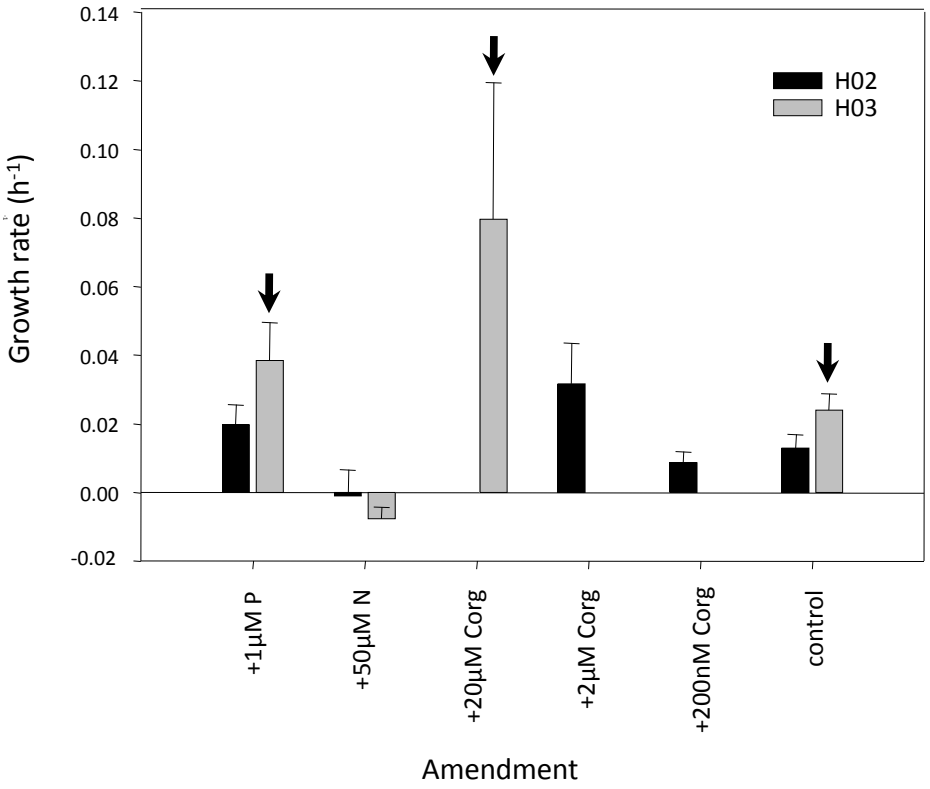


Figure 4-7

Growth response of heterotrophic bacterioplankton at two different stations (H02 and H03). Growth rates were determined in dialysis cultures established with water from the chlorophyll maxima. $NH_4Cl$ and $KNO_3$ were added at final concentrations of 25 µM each. Concentrations of organic carbon compounds indicate concentration of each individual compound within the mixture. Vertical bars indicate standard deviations. Arrows denote dialysis cultures which were subjected to subsequent phylogenetic analyses

After an initial lag phase of approximately 20 hours, cells started to proliferate and finally reached stationary phase after 40 hours of incubation. Bacteria in unamended seawater grew at a rate of (0.013 ± 0.004)$h^{-1}$ at station H02 and of (0.024 ± 0.005)$h^{-1}$ at station H03, respectively (Figure 4-7). Addition of 1 µM inorganic phosphate resulted in growth rates of (0.02 ± 0.006)$h^{-1}$ measured at station H02 and (0.04 ± 0.01)$h^{-1}$ measured at station H03. The latter value significantly exceeded that observed for the respective control

($p < 0.05$). In contrast, the addition of nitrogen compounds ($NH_4Cl$ and $KNO_3$, 25 µM each) led to a decrease in total cell numbers with time and hence yielded negative growth rates of $(-0.001 \pm 0.008)h^{-1}$ for station H02 and $(-0.008 \pm 0.003)h^{-1}$ for station H03 (Figure 4-7). The addition of a mixture of organic carbon substrates increased growth rates of heterotrophic bacterioplankton when concentrations between 5 and 20 µM C for each individual substrate were employed whereas no stimulation occurred after addition of 200 nM of organic carbon at station H02. The highest growth rates were observed in the presence of 20 µM of organic carbon and reached $(0.08 \pm 0.04)h^{-1}$ at station H03 (Figure 4-7).

Phylogenetic affiliation of bacteria grown in stimulation experiments was assessed for the cultures established with samples from station H03. Three clone libraries of 16S rRNA genes were prepared from cultures grown in the presence of 20 µM organic carbon substrates, of 1 µM phosphate and the negative controls (indicated by arrows in Figure 4-7). Most sequences could be assigned to the *Gammaproteobacteria* (Figure 4-8). All three dialysis cultures were clearly dominated by *Alteromonas* sp., with 56% to 79% of all analyzed sequences belonging to this genus. Much less abundant were members of the genera *Vibrio* sp., *Marinobacterium* sp., *Neptuniibacter* sp., *Colwellia* sp., *Pseudoalteromonas* sp. Only four sequences were affiliated with the genera *Tenacibaculum* sp., *Cytophaga* sp. or *Flexibacter* sp. of the phylum *Bacteriodetes*. Only two sequence types affiliated with the alphaproteobacterial genera *Erythrobacter* sp. and *Phaeobacter* sp. were present in the clone libraries whereas no single 16S rRNA gene sequence of *Thalassospira* sp. was detected among the total of 75 sequences analyzed from growth stimulation experiments (compare 4.2).

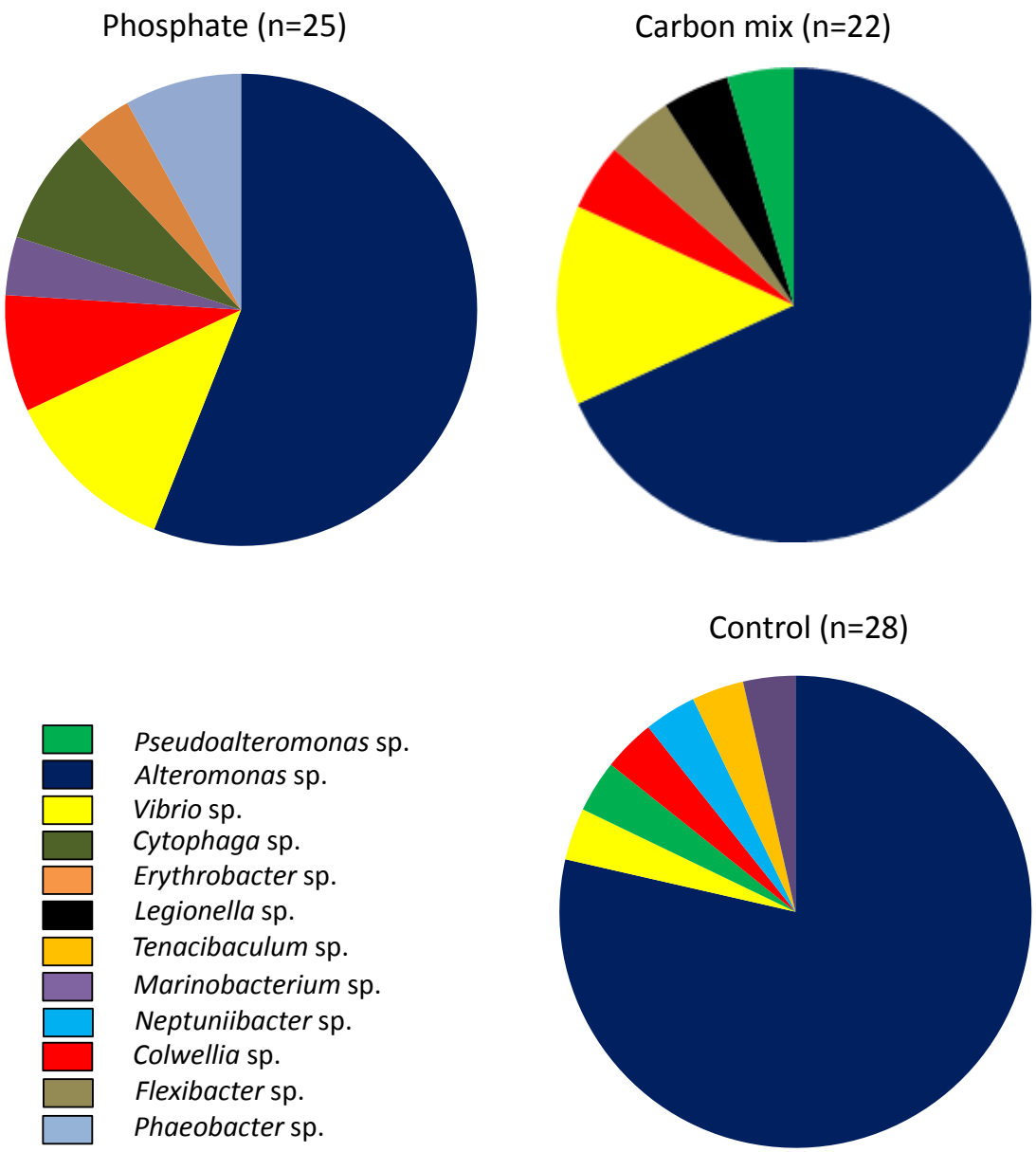


Figure 4-8

Phylogenetic affiliation of bacteria grown in dialysis cultures. Bacterial cultures contained either 20 µM of organic carbon substrates or 1 µM of phosphate (compare arrows in Figure 4-7).The negative control contained no additional substrates. Clone libraries of 16S rRNA genes were generated and the resulting sequences analyzed by comparisons with the GenBank database using blastn. Affiliation to individual genera was based on the criterion of ≥ 95% sequence identity of the 16S rRNA gene (Rosselló-Mora and Amann, 2001). The number of sequences analyzed is denoted by “n”

## 4.2 CHEMOTAXIS OF NATURAL BACTERIOPLANKTON

### 4.2.1 CHEMOTACTIC RESPONSE OF BACTERIOPLANKTON

As phosphate has been suggested to limit microbial growth in the Eastern Mediterranean Sea, the chemotactic response of bacterioplankton toward $KH_2PO_4$ was tested and compared to the responses toward a variety of organic carbon substrates and toward inorganic nitrogen compounds ($NH_4Cl$+$KNO_3$). In initial experiments, direct observations of motile bacterial cells inside the capillaries did not yield a visible increase in cell numbers after incubation for less than 10 h. Therefore, incubation periods of 24 and 48 hours were chosen for subsequent chemotaxis assays.

One series of measurements was conducted to determine the dependence of the bacterial chemotactic response on phosphate concentration. Capillaries were filled with $KH_2PO_4$ solutions with final concentrations of 2 mM, 200 µM, 20 µM or 2 µM. After an incubation period of 24 hours, bacterial cell numbers in capillaries containing 2 mM of phosphate exceeded the controls by (6 ± 1.5) fold. Cell numbers in capillaries containing 200 µM phosphate reached a relative enrichment of (3.4 ± 0.5) fold, whereas lower concentrations did not evoke a chemotactic response (Figure 4-9). However, the bacteria must have actually sensed lower concentrations that are caused by the initially rapid diffusion of substrates out of the capillary into the medium (see Materials and Methods). Using 50 µM fluorescein as a tracer, a decrease in concentration by a factor of (6.4 ± 1.2) was determined in the rectangular capillaries. Based on this significant decrease in concentration during incubation of the chemotaxis experiments, the actual threshold concentration evoking a chemotactic response of bacterioplankton toward phosphate was estimated to be in the order of 30-40 µM (instead of 200 µM) phosphate in Eastern Mediterranean surface waters.

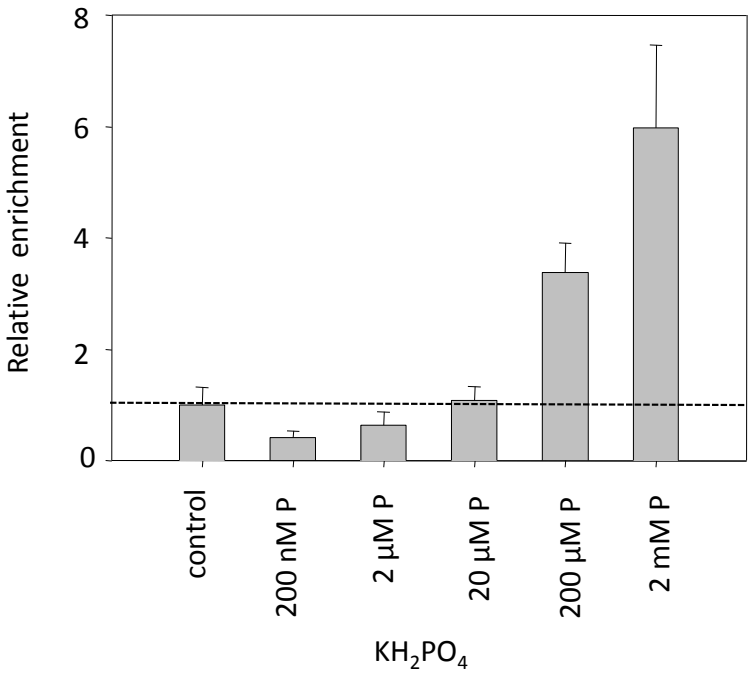


Figure 4-9

Chemotactic response of bacterioplankton at station Ier01 toward different concentrations of phosphate. The incubations lasted for 24 h. Data are given as relative enrichment as compared to the bacterial cell numbers in control capillaries (containing only filtered seawater without any substrate amendments). Vertical bars indicate one standard deviation

In water samples from station Ier01 and after an incubation time of 24 hours, bacteria showed a (15.6 ± 3.6) fold enrichment in capillaries loaded with organic carbon substrates, a comparable enrichment of (14.8 ± 4.1) fold in capillaries containing phosphate, but only a (5 ± 1.4) fold enrichment in capillaries loaded with ammonium and nitrate (Figure 4-10 A). After a total incubation time of 48 hours, bacterial cell numbers in capillaries with organic carbon had further increased, whereas they had decreased in capillaries containing either phosphate or inorganic nitrogen compounds (Figure 4-10 A). In contrast to station Ier01, bacterial cells at station H02 did not exhibit a pronounced response toward organic carbon substrates [(2.5 ± 0.6) fold] or inorganic nitrogen [(1.6 ± 0.5) fold] as attractants during the first 24 hours, while a considerable enrichment [(15.8 ± 2.4) fold] was found in capillaries containing phosphate (Figure 4-10 B). After 48 hours of incubation, especially the capillaries containing organic carbon substrates but also those loaded with phosphate contained significantly higher bacterial cell numbers (Figure 4-10 B). A third experiment at station H03 which lasted for 48 hours yielded responses toward organic carbon substrates and phosphate comparable to those observed at station Ier01

and station H02 (Figure 4-10 C). The highest number of bacterial cells that was found to accumulate during 24 hours in a single chemotaxis capillary was 26,803. This accumulation was observed for bacterioplankton from station H02 which had been exposed to phosphate.

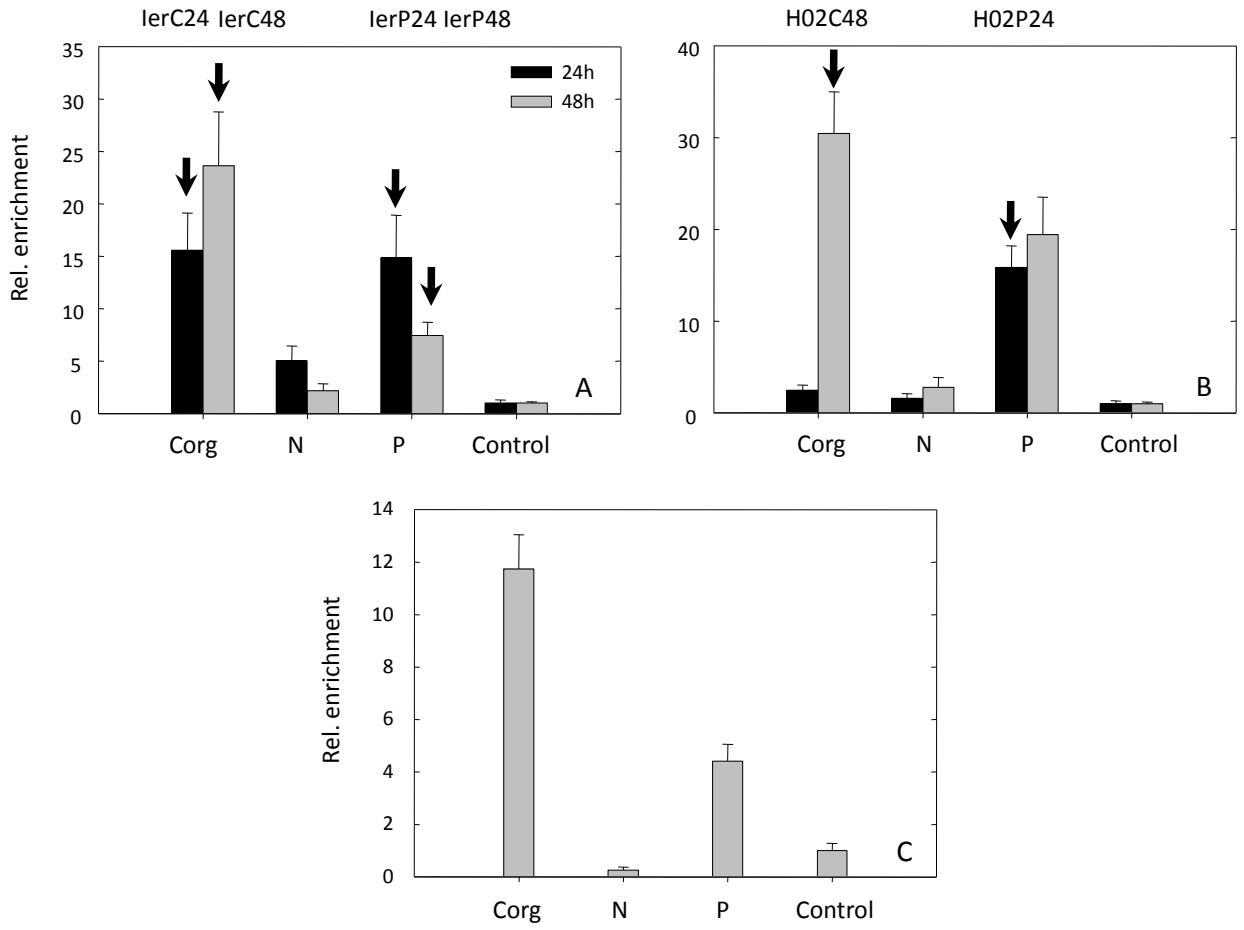


Figure 4-10

Chemotactic response of bacterioplankton from three different sampling locations toward organic carbon substrates, $KH_2PH_4$, or inorganic nitrogen compounds ($NH_4Cl$ and $KNO_3$). Incubations lasted for 24 (black bars) or 48 h (grey bars). Data are given as relative enrichment as compared to the bacterial cell numbers in control capillaries (containing only filtered seawater without any substrate amendments).Vertical bars indicate one standard deviation. Arrows indicate enrichments which were analyzed by cloning and sequencing of 16S rRNA genes

The chemotactic accumulation of bacterial cells in capillaries containing 2 mM of phosphate differed between different batches of seawater (compare Figure 4-9 and Figure 4-10 A) by a factor of 2.5 which could mostly be attributed to the fact that the number of cells accumulating in the control capillaries was 3 fold higher in the phosphate

concentration series experiment (Figure 4-9). Likewise, the numbers of cells entering the capillaries with phosphate concentrations ≤ 20 µM varied by a factor of two (Figure 4-9). Obviously, the numbers of cells entering the capillaries just by chance varied considerably but in a stochastic way and may reflect a patchy distribution of the bacterial cells in the medium. Consequently, only accumulations exceeding an enrichment factor of three were taken as evidence for a positive chemotactic response.

Epifluorescence microscopy of bacterial cells from the capillaries showed that single cells were present after 24 hours of incubation (Figure 4-11 A). In contrast, dividing stages of bacteria appeared after 48 hours of incubation, indicating that bacteria had started to multiply (Figure 4-11 B). Accordingly, only cells accumulating during the initial 24 h were generally used for subsequent phylogenetic analyses. However, the 48 h assays containing organic carbon substrates at station H02 was included in these analyses due to its delayed response (Figure 4-10).

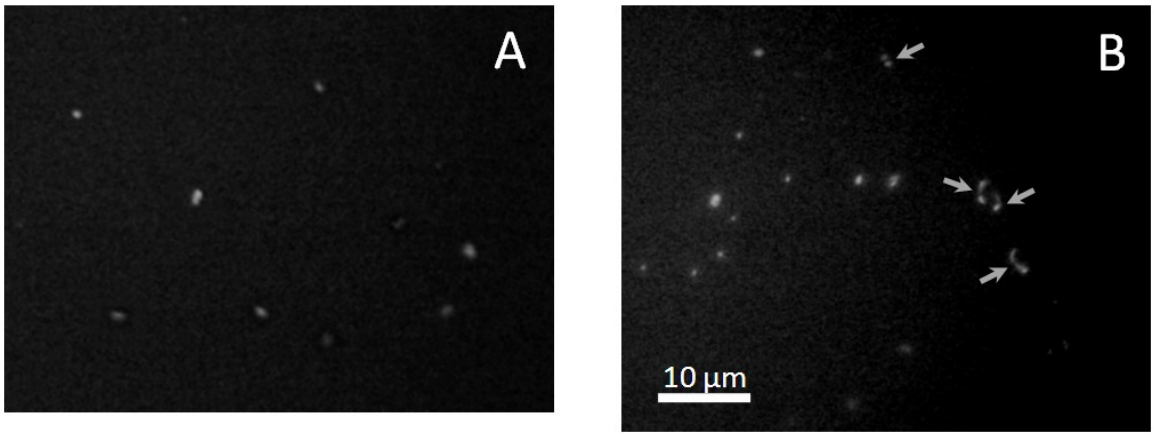


Figure 4-11
Epifluorescent photomicrographs of bacterial cells observed after A. 24 h and B. 48 h in the chemotaxis capillaries containing organic carbon substrates. The results shown in this figure were obtained using samples from station H02. Grey arrows mark dividing cells. Bar represents 10 µm

Because of time constraints, sampling time at individual stations was limited. Therefore, further chemotaxis experiments with different organic carbon substrates had to be conducted at additional station H10. The response of bacteria toward amino acids, sugars, tricarboxylic acid cycle intermediates, short chain fatty acids and alcohols was assessed. Within 24 h, planktonic bacteria at this station only responded to amino acids with a relative enrichment of (4.1 ± 0.6) fold. All the rest of carbon compounds did not elicit any response (Figure 4-12).

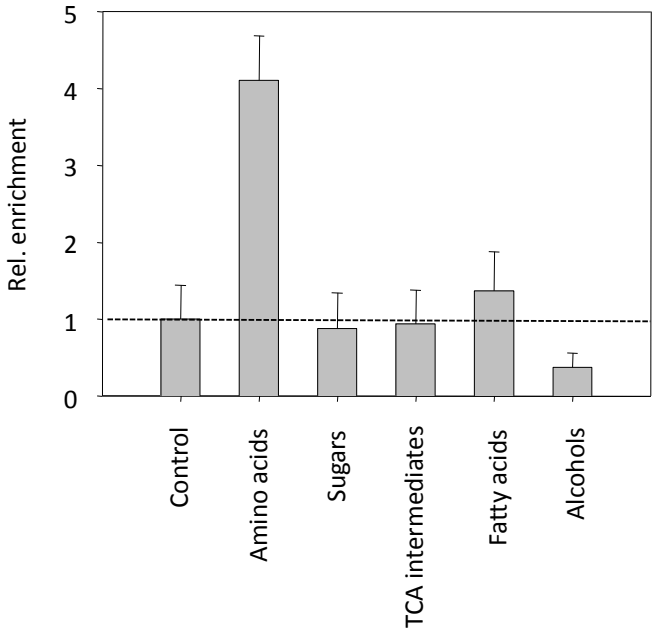


Figure 4-12

Accumulation of bacterial cells from station H10 in capillaries containing amino acids (20 canonical amino acids), sugars (glucose, N-acetyl glucosamine, arabinose, and mannitol), tricarboxylic acid cycle intermediates (2-oxoglutarate, pyruvate, lactate, succinate, malate, and citrate), short chain fatty acids (formate, acetate, propionate, buyrate, and valerate) and alcohols (methanol, ethanol, propanol, and butanol) at final concentrations of 2 mM each. The incubations lasted for 24 h. Data are given as relative enrichment as compared to the bacterial cell numbers in control capillaries (containing only filtered seawater without any substrate amendments). Vertical bars indicate one standard deviation

### 4.2.2 PHYLOGENETIC IDENTIFICATION OF IN SITU CHEMOTACTICALLY ACTIVE BACTERIA

After determination of bacterial cell numbers, remaining volumes of the three parallel capillaries containing the same attractant were combined and a sensitive DNA extraction and amplification technique was employed in order to determine the phylogenetic affiliation of chemotactically accumulated cells. This approach reliably yielded PCR products for samples containing ≥ 20,000 cells whereas samples containing ≤ 5000 bacterial cells were not sufficient to be included in this analysis. Overall, six different clone libraries were constructed for samples from stations Ier01 and H02 ( indicated by arrows in Figure 4-10).

For an initial classification, the generated sequences were analyzed by comparison with the GenBank database (Altschul *et al*., 1997) using blastn. Sequences were attributed to individual bacterial genera based on the criterion of ≥ 95% sequence identity of the 16S rRNA gene (Rosselló-Mora and Amann, 2001) (Figure 4-13). Bacterial assemblages responding to phosphate during a 24 h time interval were dominated by the *Gammaproteobacterium Alteromonas* sp. which accounted for up to 75% of all sequences analyzed. These samples also harbored the *Alphaproteobacterium Thalassospira* sp. as the second most frequent bacterium that contributed 8 to 25% of all clones (Figure 4-13). A major shift in composition of bacterial assemblages was observed after 48 h of incubation in capillaries containing phosphate when *Pseudoalteromonas* sp. had become dominant. Bacteria affiliated with the gammaproteobacterial genera *Alcanivorax*, *Oleispira* and *Marinobacterium* were detected only rarely while *Vibrio* sp. was never detected in capillaries containing phosphate.

In contrast, assemblages recovered from capillaries loaded with organic carbon substrates at station Ier01 contained mostly members of the gammaproteobacterial genus *Pseudoalteromonas* (up to 93% of all sequences) plus some *Vibrio* sp. and *Alteromonas* sp. Howerver, at station H02, 56% of bacteria attracted by organic carbon compounds belonged to the genus *Vibrio*. In contrast to chemotaxis assays using phosphate as attractant, *Alteromonas* sp. accounted for only 4 to 32% of all analyzed sequences in these samples. Most remarkably, assays with organic carbon compounds were always devoid of *Thalassospira* sp.

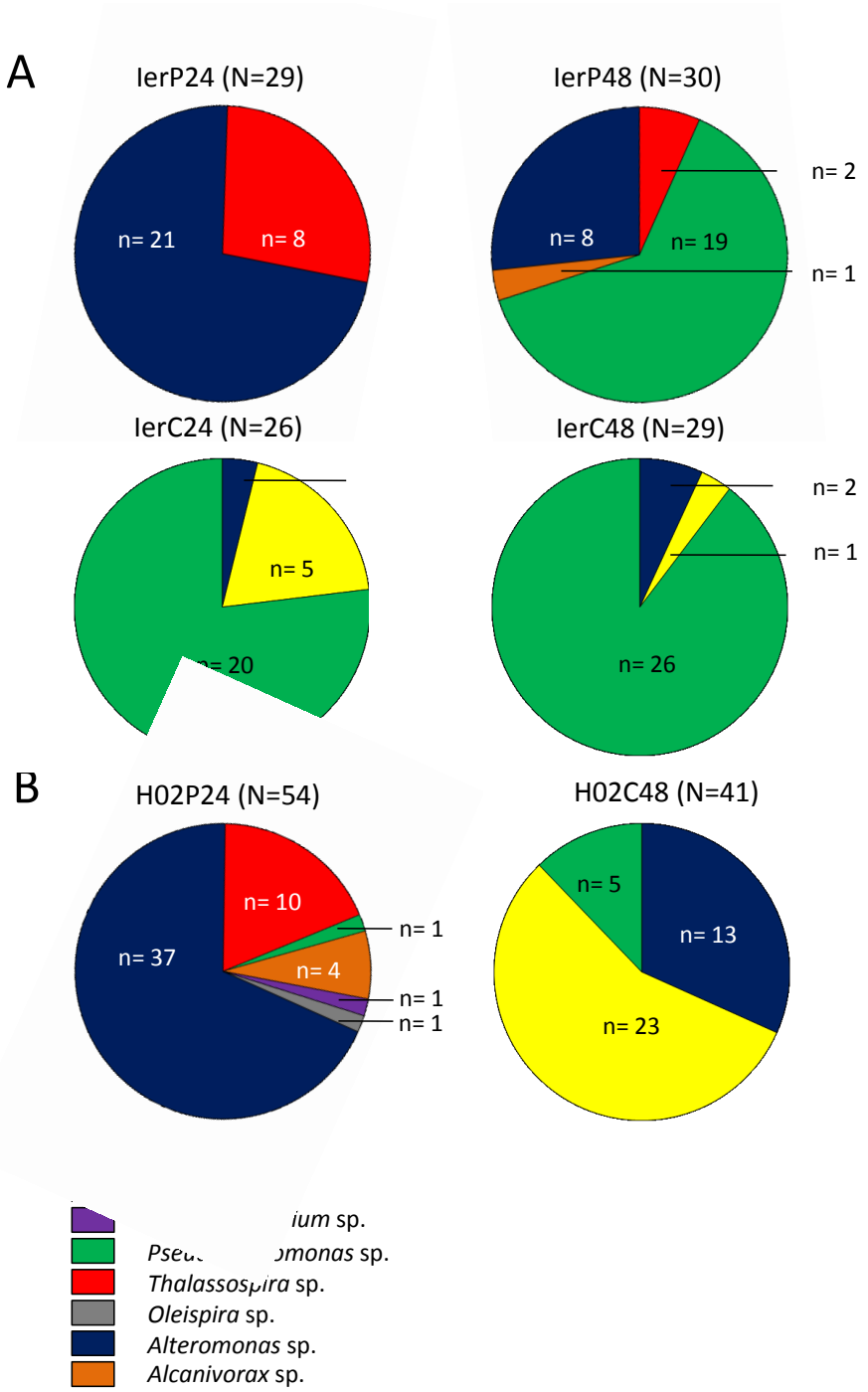


Figure 4-13

Phylogenetic composition of bacteria enriched in capillaries from stations A. Ier01 and B. H02 (compare arrows in Figure 4-10). The pie charts depicts the affiliation to individual genera based on database comparisons of 16S rRNA gene clone libraries with the GenBank database using blastn and employing the criterion of ≥ 95% sequence identity of the 16S rRNA gene (Rosselló-Mora and Amann, 2001). For each library, the number *N* of clones analyzed are provided. Values within pie charts (*n*) denote number of sequences affiliated with the particular genus

Chao1 estimates of the diversity of bacterial genera and values for their coverage by the clone libraries suggested that all major genera of chemotactically active bacteria had been detected in our analysis (coverage C=100%, not shown in Figure 4-14). Similar calculations for individual 16S rRNA gene sequence types based on a 1% cutoff value (99% identity; Figure 4-14) also indicated a limited total sequence diversity of 10 to 16 and a coverage of 94 to 90%, respectively, by our clone libraries (Figure 4-14). Detailed phylogenetic analysis of the 16S rRNA gene sequences obtained for members of the genera *Thalassospira* (Figure 4-15) and *Alteromonas* (Figure 4-16) revealed that the majority of the sequences from the different clone libraries formed tight clusters of ≥ 99% sequence identity.

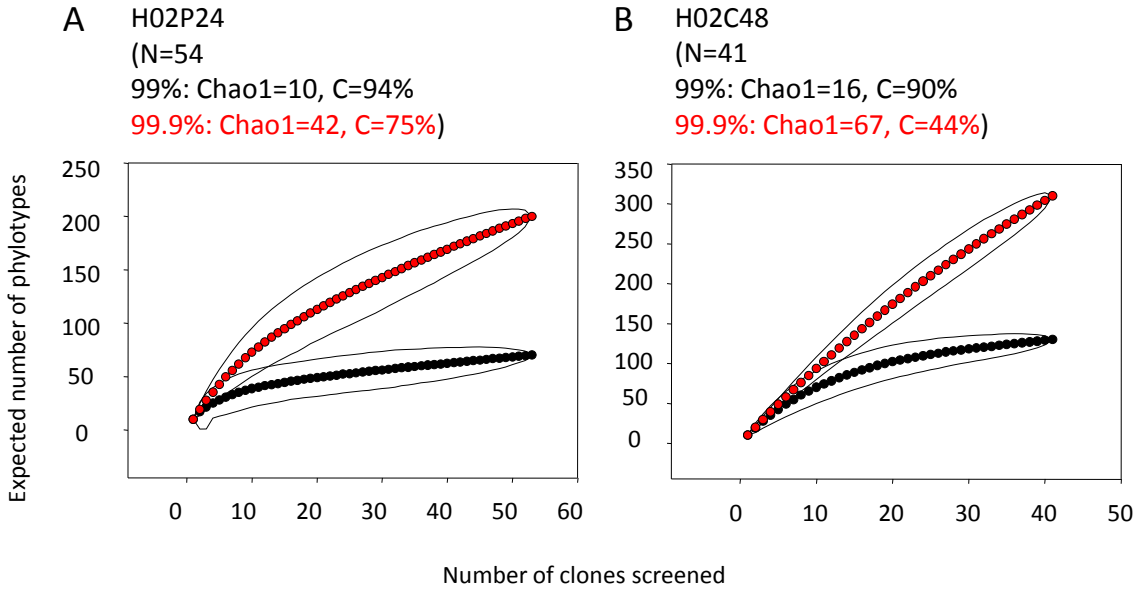


Figure 4-14

Rarefaction analyses of chemotactically active bacteria. Rarefaction curves for clone libraries derived from station H02 (Figure 4-10; Figure 4-13 B). A: H02P24 (phosphate) and B: H02C48 (carbon compounds) based on cutoff values of 0.01% (red dots) and 0.1% (black dots) difference. Straight lines represent the upper and lower 95% confidence intervals. Chao 1 estimates and coverages of the respective clone libraries are given in parentheses

All *Thalassospira* sequences obtained in the present study were very closely related to each other (> 99% sequence identity; Figure 4-15). The sequences were also highly similar to cultured *Thalassospira* strains obtained from algae and dinoflagellate cultures (*Emiliana huxleyi*, *Fragilariopsis* sp. and *Gymnodinium catenatum*), from seawater from the north-western Mediterranean Sea and from an oil spill (Costa da morte, Spain; Alonso-Gutiérrez *et al*., 2009). Overall, six very closely related clones could be discerned. It is also notable, that identical *Thalassospira* phylotypes were enriched at the two different stations (compare H02P24-14 with IerP48-24 and others; Figure 4-15). The closest cultured and described relative of sequences from these bacteria that responded chemotactically toward phosphate was *Thalassospira lucentensis* DSM 14000 isolated from the Western Mediterranean Sea (López-López *et al*., 2002).

Sequences of chemotactically active *Alteromonas* sp. were closely related or even identical at the different sampling stations (Figure 4-16). With the only exception of *Alteromonas macleodii* AD45, the chemotactically active *Alteromonas* from stations H02 and Ier01 were most closely related to, but still distinct from, cultured isolates of *Alteromonas* sp. that were isolated from coastal Mediterranean seawater of the Bay of Blanes (Spain) (Allers *et al*., 2007), from Mediterranean seawater at a station off Banyuls-sur-Mer (France) (Pukall *et al*., 1999), from the open Eastern Mediterranean Sea (Pinhassi and Berman, 2003), as well as from the chemocline of the Urania Basin in the Eastern Mediterranean Sea (Sass *et al*., 2001) (Figure 4-16).

Phylogenetic analyses of the remaining 16S rRNA sequences yielded an affiliation of the chemotactically enriched *Pseudoalteromonas*, *Vibrio*, *Marinobacterium*, *Oleispira* and *Alcanivorax* with sequences previously reported from the Mediterranean (Figure 4-17). Chemotactically active bacteria were for example related to *Pseudoalteromonas* sp. associated with sponges inhabiting the Mediterranean Sea (Hentschel *et al*., 2001; Muscholl-Silberhorn *et al*., 2008) as well as to colony-forming bacteria from the open Eastern Mediterranean Sea (Pinhassi and Berman, 2003).

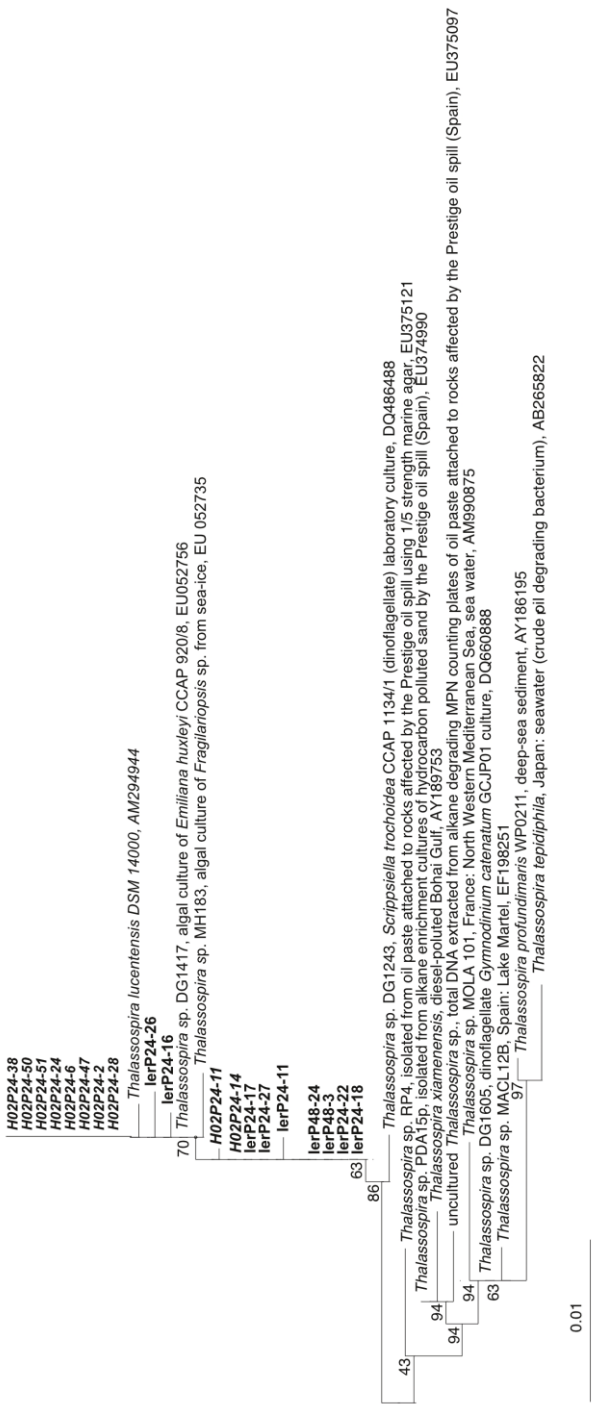


Figure 4-15

Phylogenetic tree constructed for all sequences from clone libraries (labeled in bold; bold and italic: sequences from station Ier01; bold: sequences from station H02) affiliated with the genus *Thalassospira* and their closest relatives from the Mediterranean Sea. The tree was constructed using the FastdnaML maximum likelihood algorithm as implemented in the ARB software package. Short sequences were added using the parsimony interactive tool. Bootstrap values derived from 100 resamplings are indicated at the nodes. Bar depicts 0.01 fixed point mutations per nucleotide position

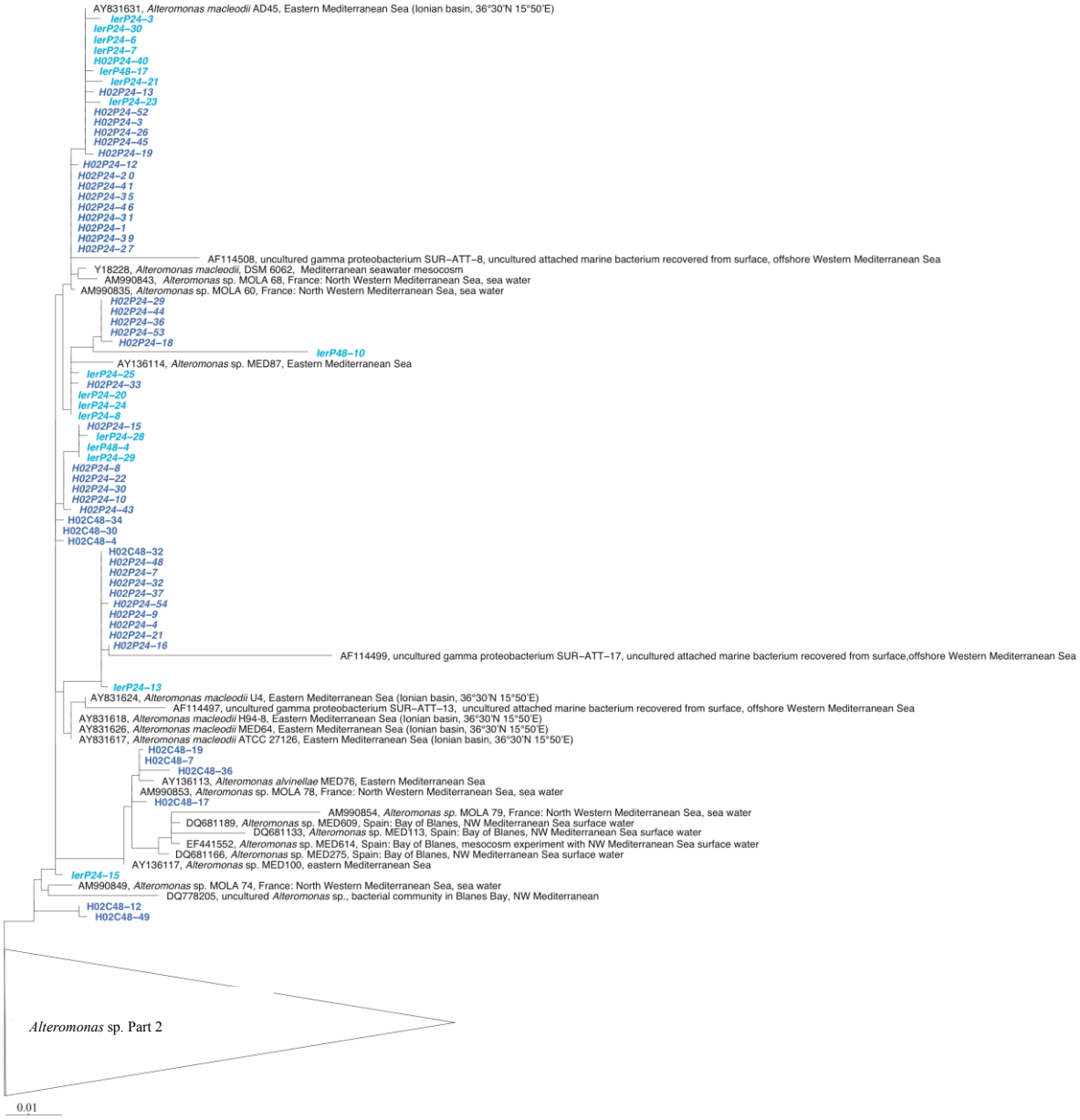


Figure 4-16

Phylogenetic tree constructed for sequences affiliated with the genus *Alteromonas*. The tree was constructed using the FastdnaML maximum likelihood algorithm as implemented in the ARB software package. Short sequences were added using the parsimony interactive tool. Sequences found in clone libraries of station Ier01 are labeled in turquoise and those found in clone libraries of station H02 are labeled in blue. Sequences found in clone libraries derived from chemotaxis assays with phosphate are depicted in italics. Bar depicts 0.01 fixed point mutations per nucleotide position

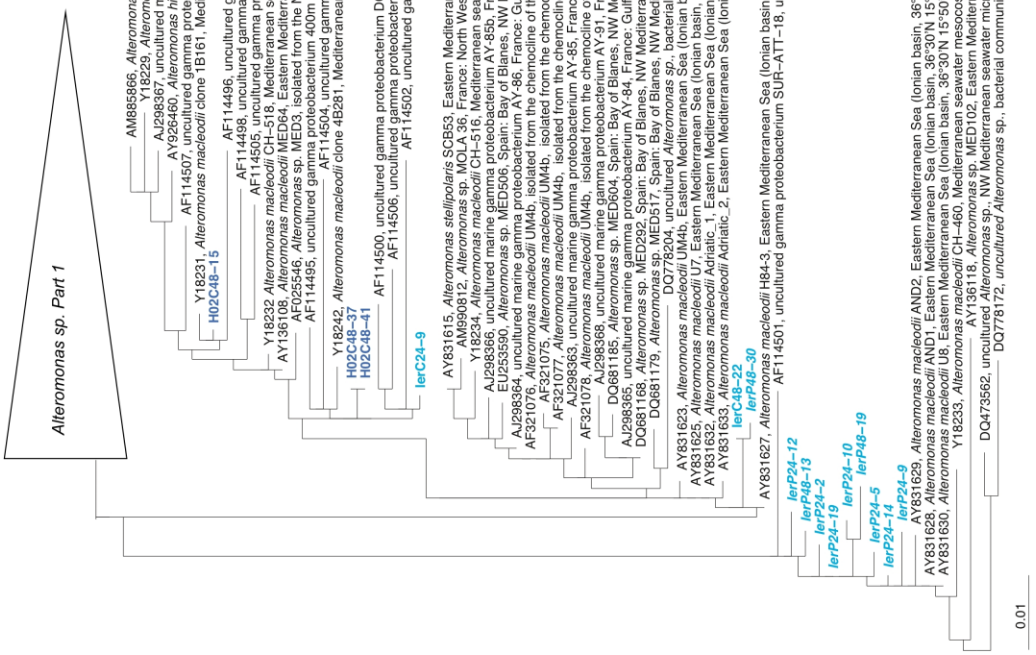


Figure 4-16 continued

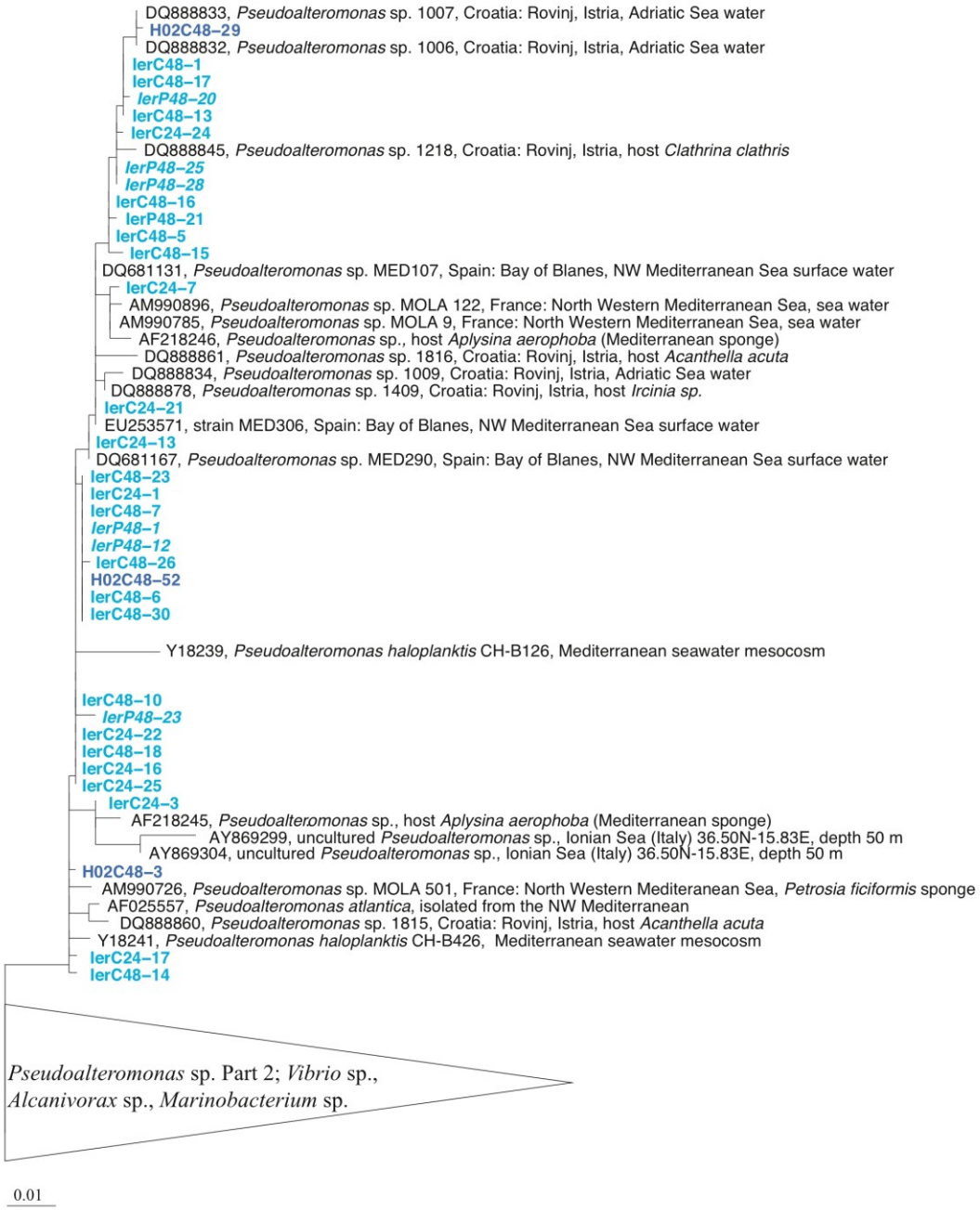


Figure 4-17

Phylogenetic tree constructed for sequences affiliated with the genera *Pseudoalteromonas*, *Vibrio*, *Oleispira*, *Marinobacterium* and *Alcanivorax*. The tree was constructed using the FastdnaML maximum likelihood algorithm as implemented in the ARB software package. Short sequences were added using the parsimony interactive tool. Sequences found in clone libraries of station Ier01 are labeled in turquoise and those found in clone libraries of station H02 are labeled in blue. Sequences found in clone libraries derived from chemotaxis assays with phosphate are depicted in italics. Bar depicts 0.01 fixed point mutations per nucleotide position


Figure 4-17 continued


Figure 4-17 continued

## 4.3 ISOLATION AND CHEMOTACTIC BEHAVIOR OF THALASSOSPIRA SPP.

### 4.3.1 ISOLATION, CULTIVATION AND PHYLOGENY OF THALASSOSPIRA SP. EM

Bacteria affiliated with the genus *Thalassospira* sp. are constituents of the oligotrophic Eastern Mediterranean bacterioplankton that exhibit a selective chemotactic response toward phosphate (Figure 4-13). In order to isolate a representative strain for detailed investigations of its chemotactic behavior, gradients of multiple organic carbon substrates were inoculated with concentrated bacterioplankton samples. The enrichments were screened by DGGE fingerprinting and sequencing of the separated 16S rRNA gene fragments (Figure 4-18).

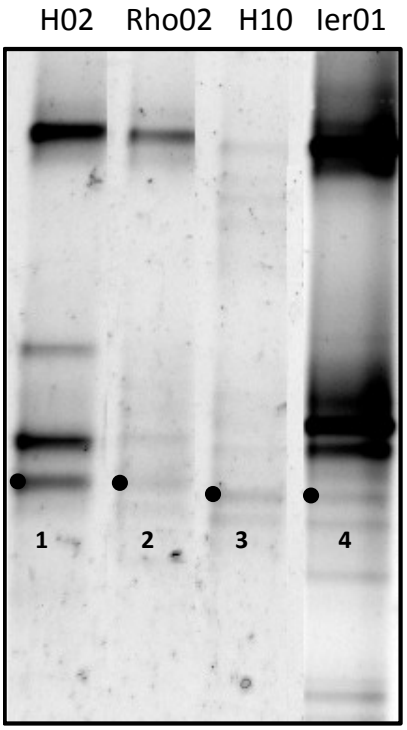


Figure 4-18
DGGE profile of bacteria enriched in gradient agar tubes. Bacteria enriched in gradient agar tubes were analyzed using DGGE. Bacteria present at four different stations (Figure 4-1) were investigated. Filled circles denote bands which were excised and identified as *Thalassospira* sp. in subsequent sequencing reactions

Bacteria affiliated with the genus *Thalassospira* were present in enrichments obtained from four different sampling stations (filled circles in Figure 4-18 denote melting types affiliated with *Thalassospira* sp.). Multiple sequence alignments using ClustalX (Larkin *et al*., 2007) indicate all melting types affiliated with *Thalassospira* sp. to be nearly

identical according to the 16S rRNA fragment analyzed (Figure 4-19). Differences between the sequences are most likely attributed to low sequence quality of sequences obtained from excised DGGE-bands. Thus the enriched phylotype affiliated with the genus *Thalassospira* exists throughout the Eastern Mediterranean Sea.

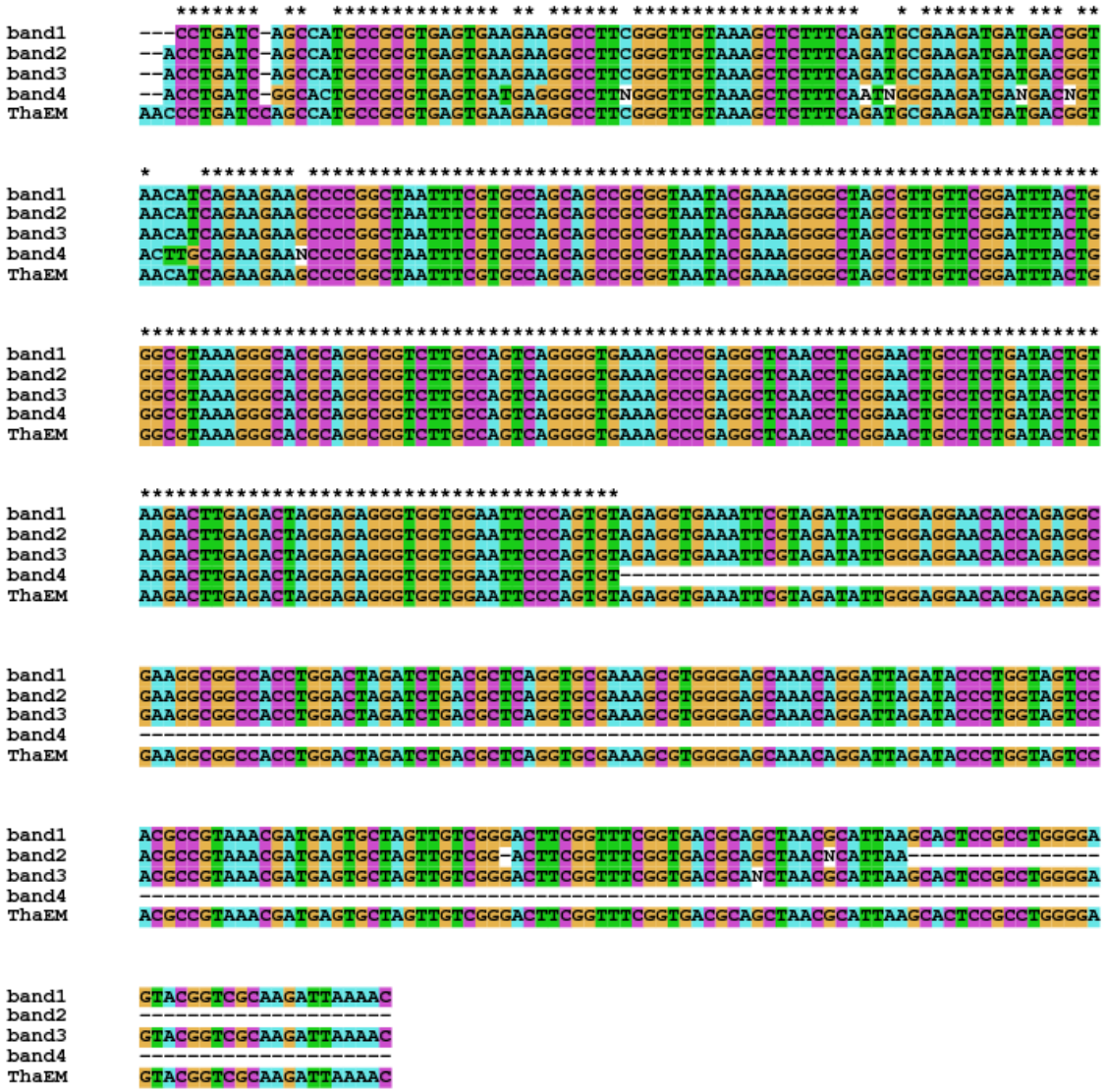


Figure 4-19

Alignment of partial 16S rRNA gene sequences obtained from excised bands (indicated by filled circles and numbers in Figure 4-18) affiliated with the genus *Thalassospira*. The 16S rRNA gene sequence of the purified isolate *Thalassospira* sp. EM was additionally included. Multiple sequence alignments were carried out using ClustalX (Larkin *et al.*, 2007). Identical bases in all five sequences analyzed are marked with asterisks

Further purification of the enrichments finally led to the isolation of a bacterial pure culture of a motile bacterium. Strain EM could be isolated from station Ier01 after several rounds of purification on agar-solidified artificial seawater medium (ASW) supplemented with a combination of 43 organic carbon substrates at low concentrations. Cells of this strain were short, highly motile spirilla (Figure 4-24).

Phylogenetic analyses of the 1349 bp-long 16S rRNA gene sequence placed strain EM within the radiation of the genus *Thalassospira* (Figure 4-20). At a sequence similarity of > 99.5%, the closest relatives were *Thalassospira tepidiphila* 1-1BT isolated from petroleum-contaminated seawater (Kodama et al., 2008) and *Thalassospira profundimaris* WP0211$^T$ (Liu et al., 2007) isolated from deep-sea sediment of the West Pacific Ocean. The 16S rRNA gene sequence of *Thalassospira lucentensis* DSM 14000$^T$ (López-López et al., 2002) clusters separately (subgroup 1; Figure 4-20) together with environmental clones from the Eastern Mediterranean bacterioplankton (Figure 4-20).

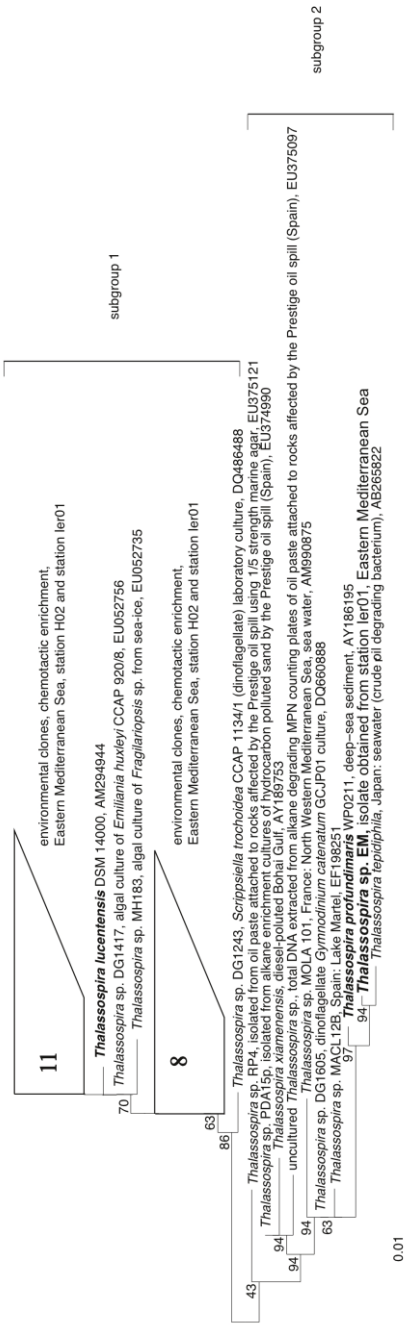


Figure 4-20

Maximum likelihood phylogenetic tree of 16S rRNA gene sequences of cultured and not-yet-cultured bacteria affiliated with the genus *Thalassospira*. The tree was constructed using the FastdnaML maximum likelihood algorithm as implemented in the ARB software package. Short sequences were added using the parsimony interactive tool. Clusters of sequences obtained from chemotactically active bacterioplankton in the Eastern Mediterranean Sea (Figure 4-16) are included for comparison. Strains analyzed in the following are marked in bold. Bar depicts 0.01 fixed point mutations per nucleotide position. Bootstrap values derived from 100 resamplings are indicated at the nodes

### 4.3.2 IN SITU ABUNDANCE OF THALASSOSPIRA SP.

So far, *Thalassospira* sp. has never been reported to occur in marine bacterioplankton assemblages but has only been recovered from other environments, in particular the marine littoral, algal cultures, or hydrocarbon-affected environments (Figure 4-20). Investigations of chemotactically active bacterioplankton yielded strong and specific enrichments of *Thalassospira* sp. in capillaries containing phosphate (Figure 4-10; Figure 4-13). Therefore, the *in situ* abundance of bacteria belonging to the genus *Thalassospira* in the water column of the Eastern Mediterranean Sea was determined by a quantitative PCR assay employing newly developed genus-specific primers.

Five different sampling locations were studied (Figure 4-1). Measurements were performed for the same water masses at all locations to reach a better comparability. Accordingly, water samples were recovered from 5 m below the sea surface within the Modified Atlantic Surface Water, and from the chlorophyll maximum positioned at 50, 40, 10, 40 and 45 m depth at stations H07, H10, H02, Ier01, Rho02, respectively. Deeper layers represented the salinity maximum of the Levantine intermediate water which forms during winter time and were sampled at 300, 300, 350, 100 and 180 m depth at stations H07, H10, H02, Ier01, Rho02, respectively. The deepest samples originated from a water depth of 1000 m within the homogenous Eastern Mediterranean Deep Water that forms in the Adriatic Sea (Rubino and Hainbucher, 2007).

The relative abundance of *Thalassospira* sp. ranged from very low values of (0.0004 ± 0.0001)% of total genomic DNA at station Rho02 (salinity maximum at 180 m water depth) to (1.2 ± 0.08)% at station H02 (deep water at 1000 m depth) (Figure 4-21). In the upper part of the water column the relative abundance of *Thalassospira* was highest at station H07, but no apparent pattern with respect to distance from the coast or to depth was detected. Based on these results, members of the genus *Thalassospira* sp. are present throughout the Eastern Mediterranean Sea and in some layers constitute a well discernable component of bacterioplankton.

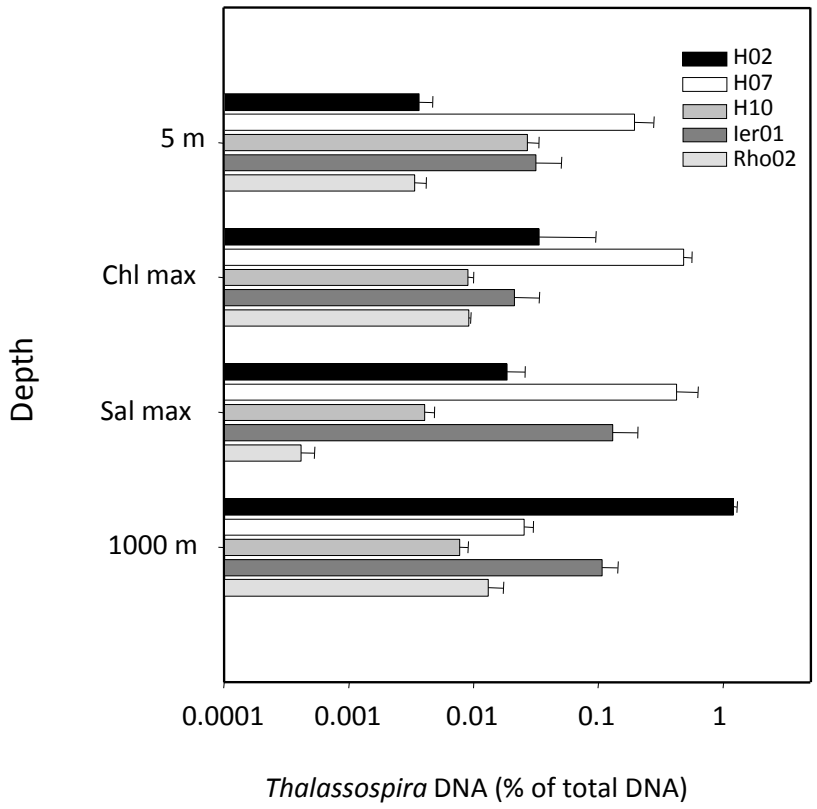


Figure 4-21

Relative abundance of *Thalassospira* sp. in bacterioplankton at five different sampling stations as determined by genus-specific qPCR. Error bars represent one standard deviation. Water samples were collected near the surface (5 m depth), at the chlorophyll maxima (positioned at 50, 40, 10, 40 and 45 m depth at stations H07, H10, H02, Ier01, Rho02, respectively), at the salinity maximum (positioned at 300, 300, 350, 100 and 180 m depth at stations H07, H10, H02, Ier01, Rho02, respectively) and at a depth of 1000 m

### 4.3.3 RESPONSE OF THALASSOSPIRA SP. EM TOWARD PHOSPHATE STARVATION

Bacterioplankton growth in the Eastern Mediterranean Sea is assumed to be limited by the availability of inorganic phosphate (Krom *et al*., 2004; Thingstad *et al*., 2005). Since cells of *Thalassospira* sp. EM were observed to rapidly lose their motility during centrifugation, phosphate-starved cultures could not be obtained by simply concentrating, washing and starving dense suspension of routinely grown laboratory cultures. Instead, phosphate depleted and phosphate-replete laboratory cultures were generated in parallel and their chemotactic response analyzed directly in order to elucidate the effect of phosphate starvation on chemotaxis. While the Eastern Mediterranean isolate *Thalassospira* sp. EM grew rapidly in Marine Broth, it was not capable of growing in defined minimal artificial seawater medium $ASW_{GluNP}$ containing glucose as the sole organic carbon source.

However, the strain resumed growth upon transfer in defined mineral medium supplemented with small amounts of yeast extract ($ASW_{GluNPYE}$ ; Figure 4-22), indicating a requirement of growth factors in addition to the 10 vitamins and 12 trace elements that are present in ASW. Contrary to previous reports (López-López *et al*., 2002; Liu *et al*., 2007), this obligate dependence on unidentified growth factors was also observed for *Thalassospira lucentensis* DSM 14000$^T$ and *Thalassospira profundimaris* WP0211$^T$. Consequently, all cultures for subsequent chemotaxis experiments had to be routinely grown in the presence of 0.005% (v/v) yeast extract.

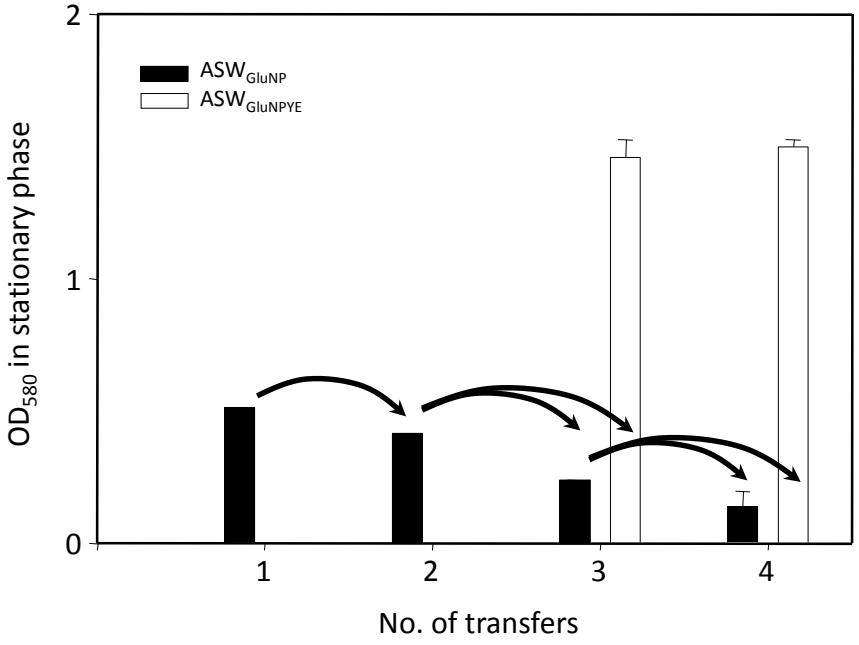


Figure 4-22

Growth factor requirement of *Thalassospira* sp. EM. In the first passage, a single colony grown on a MB agar was transferred into $ASW_{GluNP}$ containing 5 mM $KH_2PO_4$, 15 mM $NH_4Cl$ and 10 mM glucose. Subsequent passages were inoculated with 1% of the culture volume. After the second passage, artificial seawater medium without ($ASW_{GluNP}$) and with yeast extract ($ASW_{GluNPYE}$) were inoculated (arrows). After cultures had reached stationary phase the optical density was read at 580 nm. Error bars represent one standard deviation

Phosphate depleted ($ASW_{GluNYE}$) and phosphate-replete ($ASW_{GluNPYE}$) laboratory cultures of *Thalassospira* sp. EM reached doubling times of 6.2 hours during the exponential growth phase that lasted for 22 h under the cultivation conditions chosen (Figure 4-23 A). In phosphate-replete media, total cell numbers reached in the stationary phase significantly surpassed those in phosphate-depleted cultures by a factor of almost 3 and the protein content was 1.7 fold higher than that of phosphate-depleted cultures

(compare last three time points in Figure 4-23 A, Table 4-4). However, $OD_{580}$ values in the stationary phase remained similar in the two types of cultures (Table 4-4).

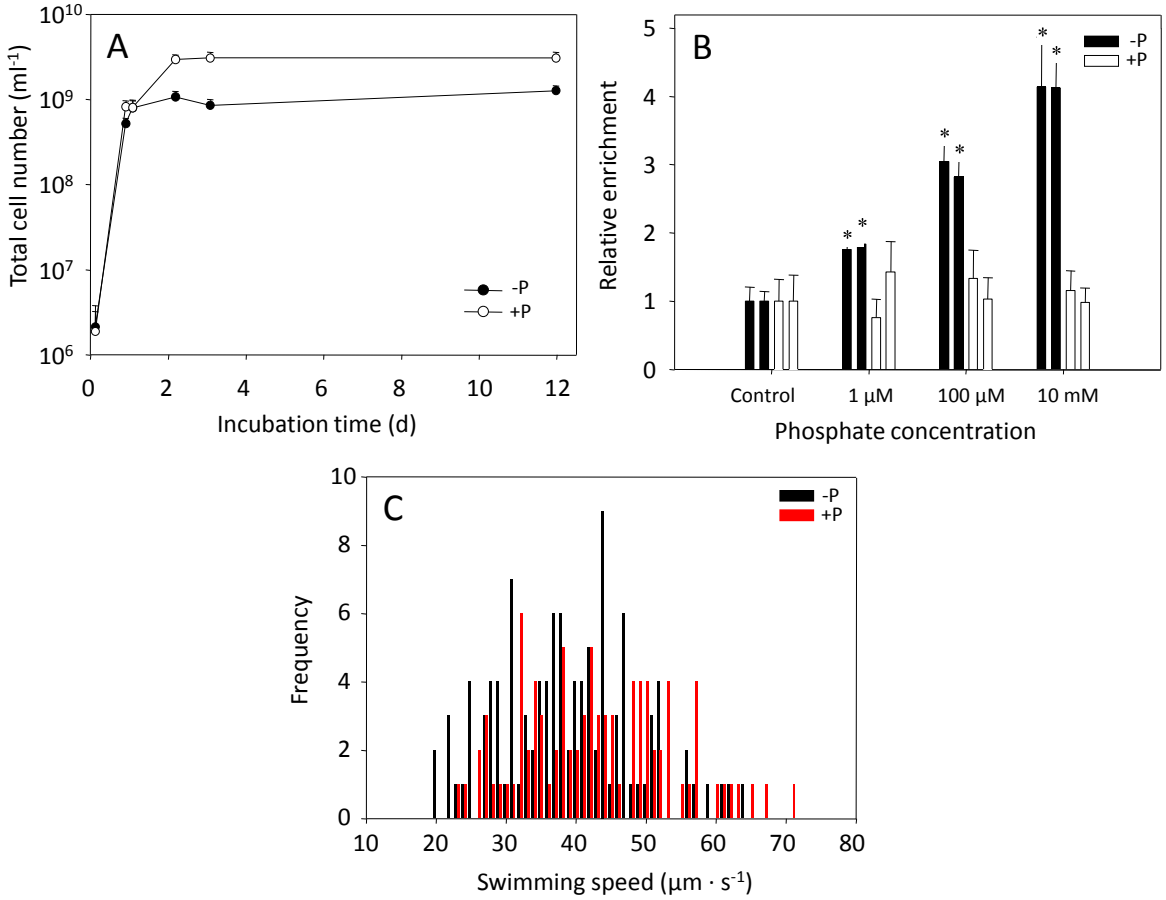


Figure 4-23

A. Growth curves of *Thalassospira* sp. EM grown in phosphate-depleted artificial seawater medium $ASW_{GluNP}$ (-P, black circles) and phosphate-replete medium $ASW_{GluNPYE}$ (+P, hollow circles). The time course of total cell numbers is shown. Cultures were inoculated with pre-starved starter cultures (see Material and Methods). B. Accumulation of phosphate-starved (black columns) and phosphate-replete (white columns) cells of *Thalassospira sp.* EM in chemotaxis capillaries loaded with artificial seawater containing different concentrations of $KH_2PO_4$. Incubation lasted for 1.5 hours. Control capillaries were devoid of phosphate. Error bars represent one standard deviation. Asterisks indicate values that differ significantly ($p < 0.001$) from negative controls based on a standard t-test. C. Frequency distribution of swimming speeds determined for phosphate-starved (black columns) and phosphate-replete (white columns) cells of *Thalassospira sp.* EM determined after 52.5 h of incubation. Frequencies per classes of 1 µm · $s^{-1}$ are given. The resulting mean swimming speeds are listed in Table 4-4)

Table 4-4 Characteristics of Thalassospira sp. EM cultivated in phosphate-depleted ASWGluNYE (-P) and phosphate-replete ASWGluNPYE (+P) medium*

| Time (h) | $OD_{580}$ | | Total cell count ($10^8$ cells·ml$^{-1}$) | | Colony forming units ($10^7$ cfu·ml$^{-1}$) | | Mean swimming speed (µm·s$^{-1}$) | | Protein content (µg·ml$^{-1}$) | | Motile cells (%) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | +P | -P | +P | -P | +P | -P | +P | -P | +P | -P | +P | -P |
| 0 | 0.002 | 0.001 | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* |
| 3.0 | 0.004 | 0.004 | 0.018 (0.013) | 0.021 (0.017) | 0.014 (0.003) | 0.015 (0.0012) | 35.1 (11.2) | 41.1 (12.8) | *n.d.* | *n.d.* | 87.2 (0.4) | 89.0 (2.5) |
| 21.8 | 0.307 | 0.187 | 8.2 (1.4) | 5.2 (0.83) | 7 (0.63) | 3.6 (0.25) | 45.1 (14.9) | 39.4 (14.2) | 58.7 (11.7) | 22.1 (10.0) | 82.9 (1.7) | 85.1 (0.3) |
| 26.0 | 0.460 | 0.253 | 8.0 (1.6) | 7.9 (1.9) | *n.d.* | 3.1 (0.99) | 51.4 (15.9) | 41.4 (14.1) | 78.9 (11.8) | 71.5 (23.6) | 83.4 (4.5) | 84.2 (0.8) |
| 52.5 | 2.48 | 0.785 | 29 (4.4) | 11 (1.9) | 28 (17) | 11 (4.8) | 42.4 (11.0) | 38.3 (9.6) | 228 (66.2) | 109 (47.9) | 50.7 (4.1) | 77.7 (1.8) |
| 74.0 | 2.30 | 1.27 | 30 (4.8) | 8.5 (1.5) | 16 (6.6) | 2.2 (0.62) | 62.6 (19.4) | 62.2 (14.3) | 294 (209) | 172 (56.9) | 37.3 (11.3) | 46.5 (2.5) |
| 170.8 | 2.08 | 1.86 | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | *n.d.* | 361 (25.2) | 190 (56.9) | *n.d.* | *n.d.* |
| 287.5 | 2.02 | 2.12 | 31 (5.3) | 12 (1.8) | 26 (4.2) | 19 (0.19) | 60.1 (14.4) | 53.8 (12.9) | 281 (135) | 205 (85.9) | 27.3 (10.3) | 42.5 (1.8) |

* Standard deviations are given in parentheses. *n.d.*, not determined

Individual cells in phosphate-depleted cultures were significantly elongated compared to cells in phosphate-replete media (Figure 4-24). As a result, mean cell volumes reached (2.8 ± 0.8) $\mu m^3$ for phosphate-starved cells and hence were 2 fold larger than the cells grown under phosphate-replete conditions that attained only (1.4 ± 0.2) $\mu m^3$. Correspondingly, the cellular protein content of phosphate-starved cells was (182 ± 75) fg · $cell^{-1}$ as compared to (94 ± 58) fg · $cell^{-1}$ for phosphate-replete cells. This cellular protein content of *Thalassospira* falls into the range determined for various bacterial cultures (60-330 fg · $cell^{-1}$), but surpasses that of bacterioplankton (average, 24 fg · $cell^{-1}$) (Zubkov *et al*., 1999). The numbers of colony forming units were about one order of magnitude lower than total cell numbers under all growth conditions but this fraction of culturable cells did not change with the amount of available phosphate (Table 4-4).

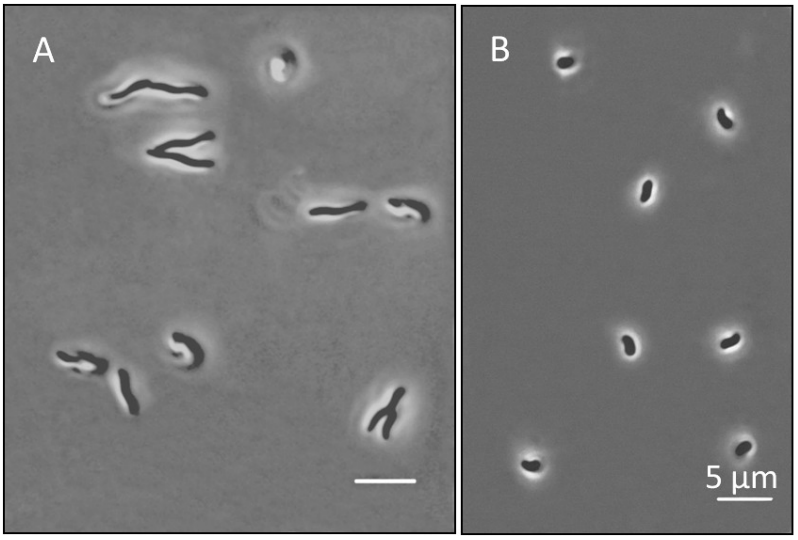


Figure 4-24

Morphology of cells of *Thalassospira* sp. EM grown under A. phosphate limitation and B. phosphate-replete conditions. Phase contrast photomicrographs of cells in the stationary phase are shown. Exponentially growing cells in phosphate replete and phosphate-depleted cultures exhibited the morphology as shown in B. Scale bars represent 5 µm

Cells in stationary cultures of phosphate-starved and phosphate-replete *Thalassospira* sp. EM exhibited a similar spectrum of exoenzyme activities that included alkaline phosphatase, acid phosphatase, C4-esterase and leucine arylamidase (Table 4-5). In addition, the phosphate-starved cells showed C8-esterase lipase activity whereas weak activities of valine arylamidase and naphthol-AS BI-phosphohydrolase were observed for phosphate-replete cultures. By comparison, concentrated bacterioplankton samples from the Eastern Mediterranean Sea also exhibited alkaline phosphatase as the dominant exoenzyme activity and in addition clear naphthol-AS BI-phosphohydrolase activity (Table 4-5). Only weak activities were determined for C8 esterase lipase, valine arylamidase and acid phosphatase at all stations, whereby C8 esterase lipase activities were higher at the easternmost stations Ier01 and Rho02.

The amount of yeast extract added had to be minimized in a manner to obtain phosphate starved cells but at the same to generate cell numbers that were sufficient for the chemotaxis experiments. The protein content of phosphate-depleted cultures in the stationary phase amounted to 189 $\mu g \cdot ml^{-1}$ (Table 4-4 mean of last three measurements). Based on the typical protein:dry weight ratio of 0.55 (Neidhardt, 1996) and a phosphorus content of 15 $mgP \cdot (g\ dry\ weight)^{-1}$ of *Escherichia coli* cells (Damoglou and Dawes, 1968), the demand of phosphorus to synthesize the biomass observed for *Thalassospira* sp. EM is expected to be 161 µM. However, the concentration of phosphate available in the culture medium in the form of yeast extract amounted to only 21 µM (see Material and Methods; 3.1.6.). The decreased biomass determined in $ASW_{GluNYE}$ thus is commensurate with the conclusion that growth of *Thalassospira* in this medium was limited by phosphate and resulted in the formation of phosphate-starved cells. Compared to *E. coli*, the cellular phosphorus demand of *Thalassospira* sp. EM is lower which suggests that the novel isolate is capable of decreasing its intracellular phosphorus concentrations and/or capable of substituting part of phosphorus-containing molecules during its adaptation to phosphate starvation.

The phosphate-replete growth medium $ASW_{GluNPYE}$ contained C:N:P in a molar ratio of 62:16:5 when accounting also for the amounts of nutrients added with the small amount of yeast extract. Assuming that half of the organic carbon substrate is respired, the ratio of nutrients available for biomass formation in this medium is C:N:P = 31:16:5. Based on the average composition of the bacterial cells of $C_{43.5}N_{11.4}P_{1.0}$ (Battley, 1995), the $ASW_{GluNPYE}$

medium thus provides limiting amounts of organic carbon for bacterial growth and produces cells starved for organic carbon.

Table 4-5

Exoenzyme activities in stationary phase cultures of Thalassospira EM and in bacterioplankton concentrated from water samples of four sampling stations in the Eastern Mediterranean Sea as determined with the API ZYM test system

| Enzyme | EM *+P* | EM *-P* | H10 | H02 | Ier01 | Rho02 |
|---|---|---|---|---|---|---|
| **Control** | - | - | - | - | - | - |
| **Alkaline Phosphatase** | ++ | +++ | ++ | ++ | ++ | ++ |
| **Esterase (C4)** | (+) | + | - | - | - | - |
| **Esterase Lipase (C8)** | - | + | (+) | (+) | + | + |
| **Lipase (C14)** | - | - | - | - | - | - |
| **Leucine arylamidase** | ++ | + | - | - | - | - |
| **Valine arylamidase** | (+) | - | (+) | (+) | (+) | + |
| **Cystine arylamidase** | - | - | - | - | - | - |
| **Trypsin** | - | - | - | - | - | - |
| **α-Chymotrypsin** | - | - | - | - | - | - |
| **Acid phosphatase** | +++ | ++ | (+) | (+) | (+) | (+) |
| **Naphthol-AS-BI-phosphohydrolase** | (+) | - | + | + | + | + |
| **α-Galactosidase** | - | - | - | - | - | - |
| **β-Galactosidase** | - | - | - | - | - | - |
| **β-Glucuronidase** | - | - | - | - | - | - |
| **α-Glucosidase** | - | - | - | - | - | - |
| **β-Glucosidase** | - | - | - | - | - | - |
| **N-Acetyl-β-glucosaminidase** | - | - | - | - | - | - |
| **α-Mannosidase** | - | - | - | - | - | - |
| **α-Fucosidase** | - | - | - | - | - | - |

* +P, cells grown in $ASW_{GluNPYE}$ ; -P, cells grown in $ASW_{GluNYE}$ ; –, no activity; (+), weak activity; +, activity; ++, intense activity; +++, very intense activity

### 4.3.4 CHEMOTAXIS OF *THALASSOSPIRA* SPP.

#### *Characterization of phosphate-directed chemotaxis*

Stationary phase cells of *Thalassospira* sp. EM cultivated in $ASW_{GluNP}$ and $ASW_{GluNPYE}$ were assessed for their chemotactic behavior toward different concentrations of $KH_2PO_4$ (Figure 4-23 B). In these assays, phosphate-replete *Thalassospira* cells never accumulated in capillaries containing inorganic phosphate irrespectively of the concentrations offered. Cells of *Thalassospira* sp. EM exhibited a broad range of swimming speeds (Figure 4-23 C). Maximum values of 63 $\mu m \cdot s^{-1}$ were reached in stationary phase (Table 4-4). Mean swimming speeds did not vary significantly between phosphate-starved and phosphate-saturated cells. On the contrary, a significant accumulation relative to control capillaries containing ASW medium was only observed for the phosphate-starved cells. The minimum phosphate concentration evoking a positive response was determined to be as low as 1 µM. The accumulation of *Thalassospira* cells observed in the capillaries has to be attributed to chemotaxis but not growth because of the following reasons. Firstly, the *Thalassospira* strains employed in the present study are not capable of growing without yeast extract that was omitted in some of the chemotaxis assays (experiment in Figure 4-27). Secondly, cell numbers increased in the capillaries by > fourfold within 1.5 hours whereas the doubling time in this medium (if supplemented with yeast extract) was 6.2 hours.

An additional experiment was carried out in order to investigate the response of *Thalassospira* sp. EM toward inorganic phosphate in relation to growth phase (Figure 4-25). The degree of enrichment and threshold concentration of $KH_2PO_4$ evoking a positive response in both parallel experiments slightly varied with growth phase. While in the beginning of the experiment (bacteria in lag-phase and in early stationary phase, Figure 4-25) there was no significant enrichment in both parallels in capillaries containing 1 µM $KH_2PO_4$, bacteria were enriched in capillaries containing as less as 1 µM phosphate as described above (bacteria in stationary phase, Figure 4-25).

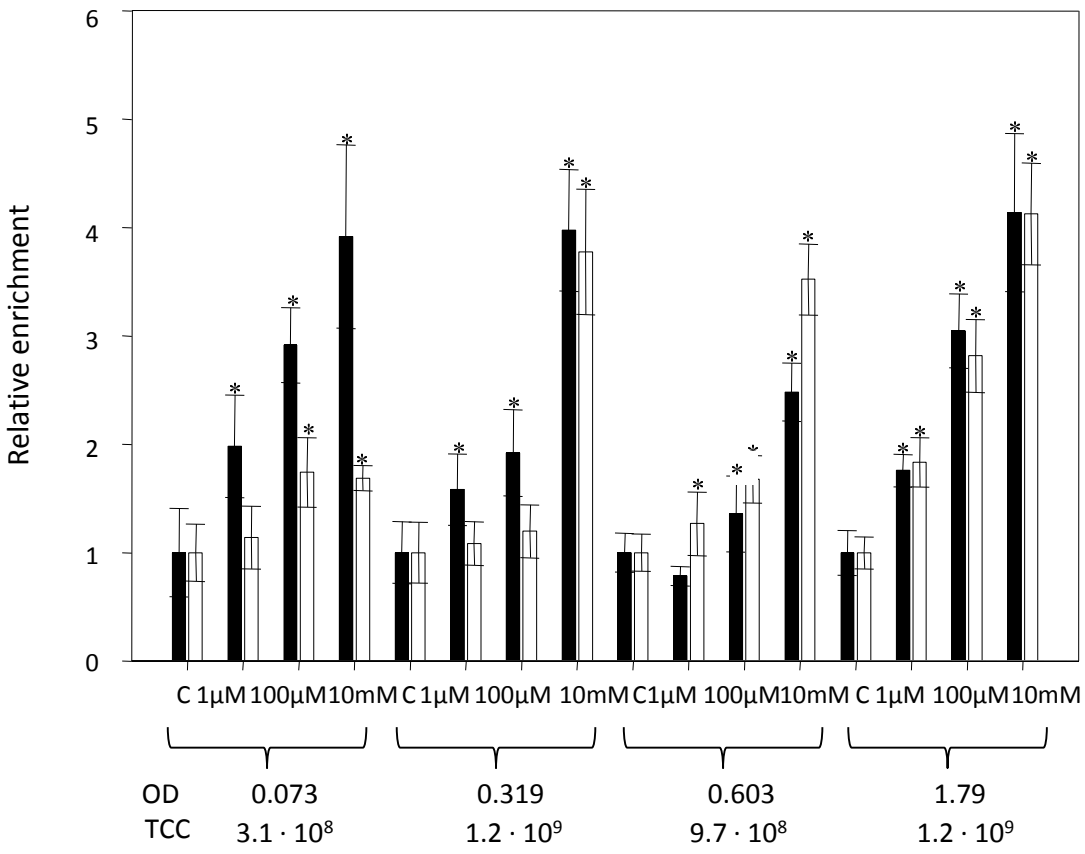


Figure 4-25

Accumulation of phosphate-starved cells of *Thalassospira sp.* EM in chemotaxis capillaries loaded with ASW containing different concentrations of $KH_2PO_4$. Incubation lasted for 1.5 hours. Control capillaries were devoid of phosphate. Error bars represent one standard deviation. White and black bars denote parallel experiments. Asterisks indicate values that differ significantly ($p < 0.001$) from negative controls based on a standard t-test. The experiment was carried out with cells in different growth phases. Optical densities (OD) and total cell numbers (TCC) of the cultures employed are given

### *Chemotaxis toward different substrates*

In a subsequent series of experiments, the chemotactic responses of *Thalassospira* sp. EM toward phosphate, organic phosphate and carbon substrates were compared. Cells from two consecutive passages in $ASW_{GluN}$ were tested for their chemotactic response toward $KH_2PO_4$ (2 mM), 2-aminoethyl phosphonate (AEP; 2 mM), a mixture of the amino acids (L-threonine, L-arginine, L-histidine, L-methionine, L-proline, glycine, L-lysine, L-valine, L-serine, L-alanine, L-glutamic acid, L-leucine, L-cysteine, L-asparagine, L-tryptophan, L-aspartic acid, L-glutamine, 2mM each), a mixture of sugars (α-D(+)-glucose, α-mannitol,

L(+)-arabinose, D(+)-trehalose and (+)-xylose; 2 mM each), yeast extract (0.1% v/v) or peptone (0.1% v/v) (Figure 4-26).

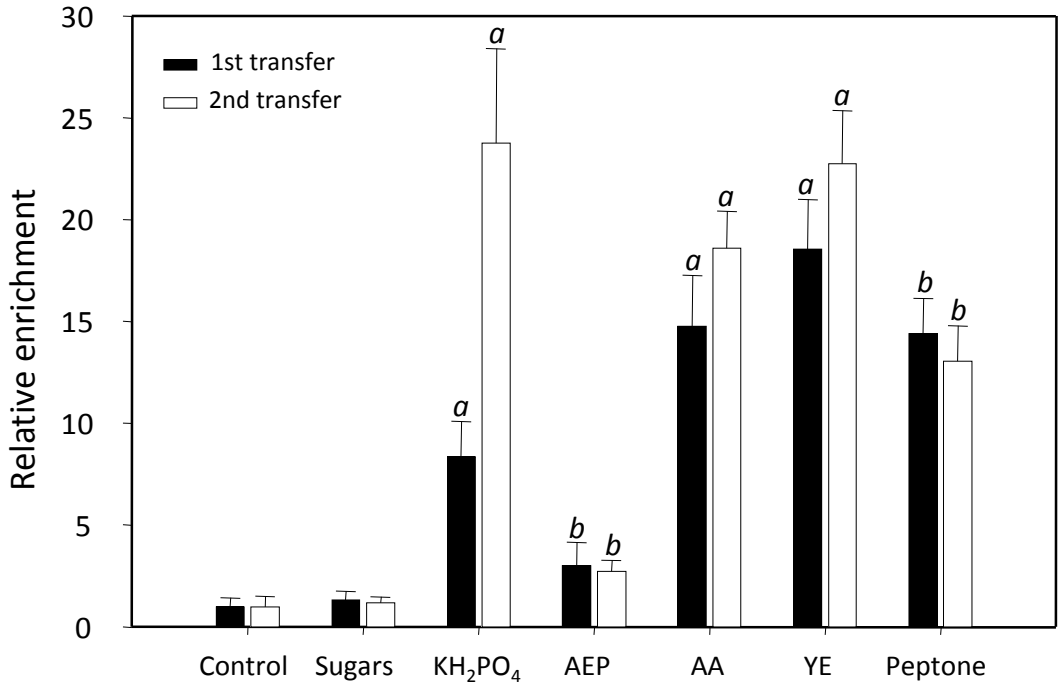


Figure 4-26

Chemotaxis of *Thalassospira* sp. EM toward different phosphorus and organic carbon compounds. AEP, 2-aminoethyl phosphonate; AA, mixture of amino acids, YE, yeast extract. The composition of the sugar and AA mixtures is given in the text. The relative chemotactic enrichment of bacterial cells from the first (black columns) and the second (white columns) transfer as compared to controls without substrates is given. Error bars represent one standard deviation. *a*, enrichment factors significantly different between the first and second transfer and from the control; *b*, enrichment factors similar between the first and second transfer but significantly different from the control (t-test; $p < 0.001$)

*Thalassospira* sp. EM showed no response toward sugars and only weakly responded toward the organic phosphate compound AEP. In contrast, strong responses toward inorganic phosphate, amino acids, yeast extract and peptone could be observed. Whereas the chemotactic responses toward inorganic phosphate was significantly stronger after the second transfer in $ASW_{GluN}$, and increased slightly but significantly for the amino acid mixture and yeast extract, an increase in enrichment factors was not observed for any of the other three chemoattractants. These results suggest that chemotaxis particularly toward inorganic phosphate is selectively induced under conditions of phosphate starvation.

### *Comparative analysis of phosphate-directed chemotaxis within the genus Thalassospira*

In a final set of chemotaxis experiment, the chemotactic response of the three available representatives of the genus *Thalassospira* toward inorganic phosphate was compared.

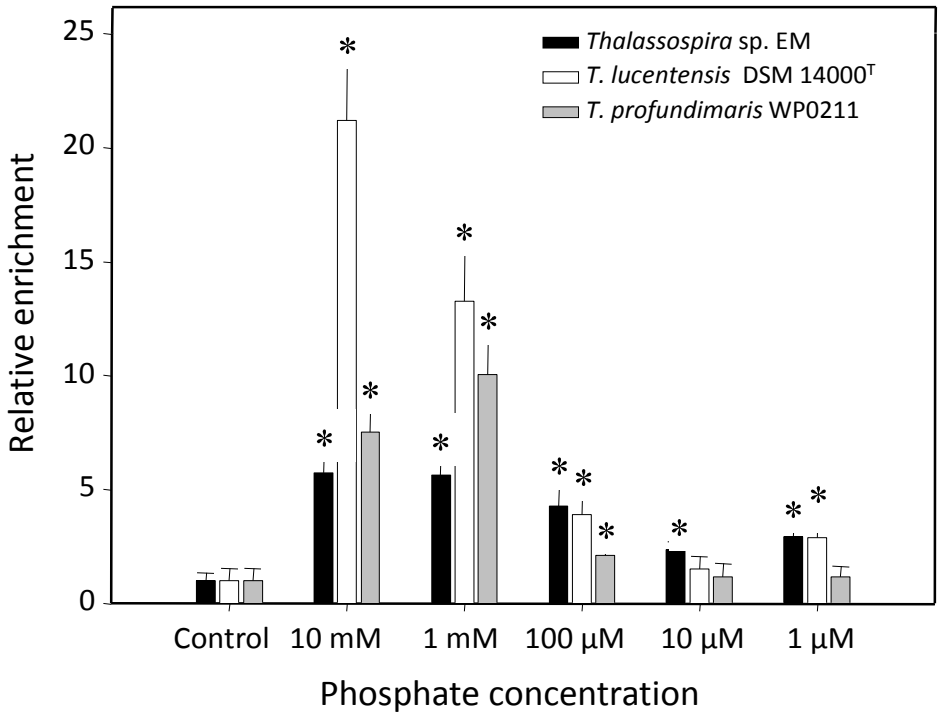


Figure 4-27

Chemotactic accumulation of three different *Thalassospira* strains in capillaries loaded with ASW containing different concentrations of phosphate. Prior to chemotaxis assays, all cultures were starved for phosphate. Error bars represent one standard deviation. Asterisks indicate significant differences to negative controls without phosphate (t-test; $p < 0.001$)

*Thalassospira lucentensis* (López-López *et al.*, 2002), *Thalassospira profundimaris* (Liu *et al.*, 2007) and the newly obtained isolate *Thalassospira* sp. EM were starved for phosphate in $ASW_{GlucN}$ for one week. Cells of all three strains accumulated in capillaries containing 10 mM, 1 mM or 100 µM $KH_2PO_4$ (Figure 4-27). In addition, *Thalassospira* sp. EM showed a significant positive response toward 10 µM as well as 1 µM $KH_2PO_4$ and *Thalassospira lucentensis* toward 1 µM $KH_2PO_4$.

# 5 DISCUSSION

The overall objective of this thesis was to assess adaptive strategies of heterotrophic bacterioplankton in the Eastern Mediterranean Sea. A special emphasis was put on chemotaxis. In addition growth response of heterotrophic bacteria in grazer-free dialyses cultures, composition of the bacterial community and exoenzymatic activities were analyzed. Most of the experiments were carried out using seawater out of the respective chlorophyll maxima. Albeit primary production in general is very low in the eastern basin of the Mediterranean Sea (Bosc *et al*., 2004), a stable deep chlorophyll maximum (DCM) which persists throughout the year is characteristic (Siokou-Frangou *et al*., 2010). These investigations focused on samples from the DCM because primary productivity and phytoplankton concentrations reaches its maximum at the DCM (Acinas *et al*., 1999) and hence nutrient limitation and cycling is expected to be of particular relevance at this depth.

## 5.1 BACTERIAL DIVERSITY AT DIFFERENT STATIONS AND IN DIFFERENTLY TREATED SEAWATER

Recent analyses indicate that on average 500 different species are present in a marine bacterioplankton community (Acinas *et al*., 2004). In the Western Mediterranean Sea natural bacterial communities were highly diverse and were dominated by *Alphaproteobacteria*, especially by the SAR11 group, as analyzed with clone libraries (Acinas *et al*., 1999). However, the attached fraction of bacteria was less diverse and was dominated by the *Gammaproteobacterium Alteromonas macleodii* (Acinas *et al*., 1999). There are only few analyses available which focus on the diversity at species level in offshore Eastern Mediterranean seawater. Zaballos and coworkers showed that the bacterial community in the Ionian Sea was different in composition compared with the bacterioplankton community in the oligotrophic Sargasso Sea (Zaballos *et al*., 2006). Clone libraries derived from a depth of 50 m were dominated by *Gammaproteobacteria* (including the SAR86 cluster, *Alteromonas* and *Pseudoalteromonas*). Additionally *Alphaproteobacteria* (including SAR11 but to a low percentage, bacteria belonging to the *Rhodospirillales* and to the genera *Erythrobacter* and *Pseudovibrio*), *Deltaproteobacteria*,

*Actinobacteria*, *Bacteriodetes*, *Cyanobacteria* and archaea were present to a lesser extent (Zaballos *et al*., 2006).

In this study a general overview of bacterial diversity was achieved with a DGGE analysis using universal bacterial primer. The sequences obtained from prominent bands belonged to the *Proteobacteria* (mainly the alpha subgroup), the *Bacteriodetes* and the *Cyanobacteria*. While *Gammaproteobacteria* were found to be present in Eastern Mediterranean seawater none of the obtained sequences was affiliated with *Alteromonas*, which dominated the clone libraries obtained from Ionian seawater previously (Zaballos *et al*., 2006). Moreover, none of the sequences belonged to the ubiquitous and highly abundant SAR11 cluster which is thought to dominate bacterioplankton communities at surface water layers (Morris *et al*., 2002) and which was also detected in high percentages in the Western Mediterranean Sea (Acinas *et al*., 1999). However, in many studies which rely on DGGE analyses when assessing bacterial diversity, SAR11 type bacteria could not be found (Schauer *et al*., 2003; Sánchez *et al*., 2009). High microdiversity as well as primer mismatches might cause this dominant bacterial group not to be retrieved in DGGE analyses (Sánchez *et al*., 2009). In a metagenomic library constructed from concentrated (tangential flow filtration) Eastern Mediterranean surface waters, genomes from *Alphaproteobacteria* (SAR11 and *Rhodobacterales*), *Cyanobacteria* (*Synechococcus* and *Prochlorococcus*) and diverse uncultured *Gammaproteobacteria* were dominant (Feingersch *et al*., 2010). However, only few 16S rRNA genes were retrieved when sequencing the BAC library and nearly 70% of these sequences were affiliated with *Cyanobacteria* (Feingersch *et al*., 2010).

Remarkably, in this thesis the most prominent band in DGGE analysis of seawater samples was that affiliated with *Synechococcus* sp. This band was even more pronounced in concentrated seawater samples at all stations investigated.

Clone libraries would have been better suited for analyzing bacterial diversity in more detail. However, the DGGE technique allowed analyses of samples from many stations in parallel and allowed to compare the composition of bacterial communities in concentrated and non-concentrated seawater from the same station (5.2).

## 5.2 TANGENTIAL FLOW FILTRATION SELECTS FOR STRONG PRODUCERS OF EXOENZYMES

Exoenzymes make nutrients accessible and thus represent a potential adaptive strategy of microorganisms to scavenge scarce nutrients (Azam and Malfatti, 2007; Arnosti, 2011). Alkaline phosphatase is one of the key enzymes in marine environments (Hoppe, 2003). To date few data analyzing bulk alkaline activity in offshore waters of the phosphate-depleted Eastern Mediterranean Sea are available (Van Wambeke *et al*., 2002; Van Wambeke *et al*., 2008). Kinetic parameters of the enzymes alkaline phosphatase and leucine aminopeptidase were analyzed in waters out of the deep chlorophyll maxima in order to determine potential adaptive strategies of the inhabiting bacterioplankton communities. Due to low overall bacterial biomass (maximum number of cells per ml in chlorophyll maxima did not exceed $4.0 \cdot 10^5$), water samples had to be concentrated prior to determining kinetic parameters for the enzymes alkaline phosphatase and leucine aminopeptidase. For this purpose concentration by means of tangential flow filtration was chosen. This method provided sufficient enrichment of bacterioplankton present in 100 l of water to allow measurement of exoenzyme activities. Turnover times measured for alkaline phosphatase varied between 1.8 and 3.7 hours over the transect covered and enzyme activities reached values between 0.26 and 1.5 $h^{-1}$. These values exceed the values measured previously (Van Wambeke *et al*., 2002) by a factor of 10. For leucine aminopeptidase the turnover times were between 44.9 and 72.7 h and affinities varied between 0.016 and 0.022 $h^{-1}$.

What might have caused the elevated values in comparison to literature?

A possible explanation for the elevated values (turnover times and enzyme affinities) in comparison to literature might be the release of intracellular enzymes due to fragmentation of cells during the pumping process. Indeed, it is known that intracellular cytoplasmatic alkaline phosphatases are widespread and abundant among marine bacteria (Luo *et al*., 2009). However, enzymes require certain conditions to be fully functional and it is highly unlikely that intracellular enzymes, released due to cell lysis keep their activity in seawater. Moreover, it could be shown that a major part of the loss of latex beads during tangential flow filtration was due to retention of those beads within filtration components (Rodríguez *et al*., 1998).

Considering the community fingerprint, it could be observed that the composition of bacteria underwent significant changes during the concentration process. For this reason bacterial species present in concentrated seawater samples obviously do not perfectly represent the *in situ* bacterial community. This may explain the elevated enzyme activities, affinities and turnover times. Tangential flow filtration has been used to concentrate marine bacteria since the late 1980s. Although the recovery rate of picoplankton was as low as 37%, electron microscopy revealed no change in bacterial morphotypes in comparison with the original non-concentrated seawater (Giovannoni *et al*., 1990). Therefore the authors believed this method to be suitable for concentrating representative bacterial members in oceanic waters (Giovannoni *et al*., 1990). Recently tangential flow filtration was used to generate sufficient amount of biomass for constructing a BAC library in order to assess community genomics in the Eastern Mediterranean Sea (Feingersch *et al*., 2010). However, it was argued that tangential flow filtration should not be used for quantitative studies (Rodríguez *et al*., 1998). Not only recovery rates but also size distribution and abundance of phytoplankton were not reproducible and different from those of non-concentrated samples (Rodríguez *et al*., 1998). Moreover, loss rate was elevated for smaller cells (Rodríguez *et al*., 1998). In this study sequences retrieved from DGGE fingerprints of concentrated seawater samples often showed high similarities to cultured representatives of bacterioplankton, while the fraction of sequences affiliated with uncultured bacteria was higher in non-concentrated seawater samples. Typical inhabitants of bacterioplankton communities are often characterized by very small cell sizes like SAR11 (Rappé *et al*., 2002). Maybe cells with larger cell volume which might be only a minor constituent of the total bacterial community *in situ* contribute significantly to bulk alkaline phosphatase activity. This hypothesis is supported by the finding that bacteria attached to marine snow are strong producers of phosphatase (Smith *et al*., 1992). In fact, the attached fraction of bacteria in offshore Western Mediterranean Sea mainly comprises *Alteromonas macleodii* (Acinas *et al*., 1999) – a well characterized copiotroph with comparable large cell size.

The percentage of autotrophic cells was twice as high in concentrated seawater compared to non-concentrated seawater. $N_2$-fixing, autotrophic *Cyanobacteria* produce significant amounts of alkaline phosphatase (Orchard *et al*., 2009) and genes coding for alkaline phosphatase are present in the genomes of marine *Cyanobacteria* (Su *et al*., 2007).

In conclusion, the high activity values measured for alkaline phosphatase in this study were likely caused by a selection process leading to an enrichment of certain bacteria during the filtration procedure. A subset of “strong producers” of alkaline phosphatase within a given planktonic community might provide the remaining community with inorganic phosphate.

## 5.3 DOMINANCE OF COPIOTROPHIC BACTERIA IN DIALYSIS CULTURES

Growth rates of bacteria grown in grazer-free dialysis cultures exhibited after an initial lag phase of 20 hours ranged from 0.013 $h^{-1}$ to 0.08 $h^{-1}$ resembling those reported previously for marine bacteria (Rappé *et al*., 2002). While phosphate as well as organic carbon compounds clearly stimulated bacterial growth, a decrease in bacterial cell numbers could be observed in cultures supplemented with nitrogen compounds, indicating a limitation of bacterial growth due to C or P supply which is in accordance with literature (Van Wambeke *et al*., 2002).

Phylogenetic identification of bacteria which responded to nutrient-addition as well as those grown in control experiments (no nutrients added) revealed that the vast majority of bacteria belonged to the gammaproteobacterial genus *Alteromonas*. *Alphaproteobacteria*, *Bacteriodetes* and bacteria belonging to the genera *Vibrio*, *Marinobacterium*, *Colwellia, Neptuniibacter* and *Pseudoalteromonas* (*Gammaproteobacteria*) did only contribute to a minor extent. Typical copiotrophs were even dominating the non-amended control culture. While no nutrients were added to the seawater used for our experiments, so called "bottle"-effects changing bacterial diversity and favoring copiotrophic bacteria are known to occur in non-treated water (Eilers *et al*., 2000a; Allers *et al*., 2007; Van Wambeke *et al*., 2009). Bacteria affiliated with the genus *Alteromonas* were often found to be dominant in mesocosm experiments (Pukall *et al*., 1999; Lebaron *et al*., 2001; Schafer *et al*., 2001; Allers *et al*., 2007) but were also found to be abundant in the particulate fraction of seawater samples from the Western Mediterranean Sea (Acinas *et al*., 1999). In addition it has been reported that bacteria affiliated with opportunistic genera like *Alteromonas* were abundant in clone libraries derived from the Eastern Mediterranean Sea (Zaballos *et al*., 2006). Data regarding the relative contribution of *Alteromonas* sp. to total bacterioplankton in the eastern Mediterranean Sea are still scarce and it remains debatable whether these copiotrophs can contribute substantially to the bacterioplankton community (Zaballos *et al*., 2006) or constitute only a minor fraction of heterotrophic bacteria (Feingersch *et al*., 2010) in the Eastern Mediterranean Sea.

Although the "initial" composition of the bacterial community *in situ* of station H03 has not been determined, DGGE analyses of stations H02, H10, Ier01 and Rho02 indicate that not yet cultured bacteria were dominant. In contrast, bacteria grown in dialysis cultures were affiliated with typical cultured representatives of copiotrophic marine bacterioplankton.

Growth experiments were performed using dialysis bags to prevent grazing activity. It is known that the absence of grazers significantly influences bacterial community composition (Matz and Jürgens, 2003; Allers *et al*., 2007; Weinbauer *et al*., 2007). It has been suggested that the *Alteromonadales* and *Vibrionales* are especially sensitive to grazing by heterotrophic nanoflagellates (Beardsley *et al*., 2003). This might be an alternative explanation for the high abundance of bacteria affiliated with these genera in clone libraries derived from three dialysis cultures. It could be demonstrated that among actively growing marine bacteria the *Vibrionales* and *Alteromonadales* constitute a major group while these orders were not abundant in the corresponding *in situ* communities (Hamasaki *et al*., 2007; Taniguchi and Hamasaki, 2008). These findings support the hypothesis that bacteria that are not very abundant could nevertheless play a significant role in biogeochemical cycling of nutrients.

## 5.4 THE ACCUMULATION OF NATURAL BACTERIOPLANKTON IN CAPILLARIES IS CAUSED BY CHEMOTAXIS

While bacterial cells close to nutrient gradients accumulate rapidly within seconds (Stocker *et al*., 2008), the mean rates with which bacterial populations drift up chemical gradients are in the range of a few $\mu m \cdot s^{-1}$ such that bacterial cells enter capillaries only very slowly (Berg and Brown, 1972). In order to recover sufficiently high numbers of chemotactic cells for phylogenetic analyses, capillary exposure times therefore had to be increased to 24 h. However, since bacteria experience substantially higher nutrient concentrations in the capillaries, the increased cell numbers observed at the end of the incubation theoretically could be the result of growth rather than chemotaxis. However, this possibility can be refuted based on five independent observations.

Firstly, the maximum bacterial growth rates observed in the present study at increased nutrient concentrations and at 15° C were 0.08 $h^{-1}$, corresponding to a doubling time of 8.7 h. After 24 hours of incubation, the cell numbers per capillary detected in assays containing phosphate amounted to 26,803 and 4,035 at stations H02 and Ier01, respectively, while total cell numbers in the corresponding controls were 1,691 and 543. The control capillaries contained solely filtered seawater without additional attractants. Therefore, bacteria in the controls must have entered the control capillaries by chance. If a similar number of cells had entered the capillaries containing phosphate and subsequently had grown exponentially, maximum cell numbers of 5,127 and 1,646 would be expected for samples from H02 and Ier01, respectively (eq. 6). These numbers are 5 times lower than the numbers of cells actually observed in our experiments. This calculation does not even account for the 20 h lag period typically observed for bacterial growth in the samples.

Secondly, a direct microscopic examination of the localization of bacterial cells within the capillaries revealed that motile cells had accumulated close to the opening of the capillary. This accumulation is typical for the chemotactic response since a narrow gradient develops across the opening of capillaries in this type of chemotaxis assay and most cells do not continue swimming further into the capillary as the diffusion gradients become too shallow deeper inside the capillary (Overmann, 2005). Growth of the cells, on the other hand, would have led to a more even distribution of the cells inside the capillary.

Thirdly, the DOC present in Eastern Mediterranean surface waters (maximum 100 µM DOC) (Thingstad, 2005) would support the formation of only 2,186 bacterial cells in the 5

µl-capillaries loaded with inorganic phosphate, based on a percentage of 7% of bioavailable DOC, a heterotrophic bacterial growth efficiency of 14% (as determined in the ultraoligotrophic Sargasso Sea; Carlson and Ducklow, 1996) and a mean biovolume of marine *Gammaproteobacteria* of 0.118 $\mu m^3$ (Straza *et al*., 2009) (corresponding to a cellular carbon content of 26.9 $fgC \cdot cell^{-1}$; Norland, 1993). The number of cells which accumulated in the capillaries thus surpassed the numbers expected based on just bacterial growth up to 12 fold.

Fourthly, different attractants resulted in the accumulation of phylogenetically distinct bacteria. In particular, *Thalassospira* sp. was exclusively detected when using phosphate as an attractant but were absent in capillaries containing organic carbon substrates.

Fifthly, a *Thalassospira* strain was isolated from the same Eastern Mediterranean water samples used for the chemotaxis assays and was shown to exhibit a pronounced chemotaxis toward phosphate (5.7). Based on these facts, the accumulation of bacteria in the capillaries observed over a time period of 24 hours can be attributed mainly to chemotactic behavior.

Moreover, bacteria in seawater samples (inside the Meplats bottles) are highly unlikely to be affected by the small quantity of phosphate diffusing out of the capillary tip during the chemotaxis experiments. According to the fluorescein control experiment, phosphate was lost from only the first 3 mm of each capillary and would result in an increase by 18.4 nM phosphate in the bacterioplankton sample. Based on our growth rate measurements, a bacterial doubling time of 39 days would be expected at such a low phosphate concentration.

## 5.5 DIFFERENT BACTERIAL TAXA EXHIBIT A DISTINCT CHEMOTACTIC RESPONSE

In the present study, all chemotactically active bacteria were found to be affiliated with genera that comprise already cultivated and motile members. Phylogenetic analysis demonstrated a distinct chemotactic response for the different genera of planktonic bacteria. *Thalassospira* sp. exhibited a rapid response toward inorganic phosphate which was highly specific since members of this genus were never found in microcapillaries containing mixtures of organic carbon compounds. *Alteromonas* sp. showed a rapid response toward phosphate and also reacted, albeit more slowly, toward organic carbon. In contrast, members of the genus *Pseudoalteromonas* showed a pronounced preference toward organic carbon substrates and *Vibrio* sp. never responded toward inorganic phosphate. Furthermore, our data indicate that regional differences exist in the chemotactic response toward organic carbon compounds with *Pseudoalteromonas* dominating at station Ier01 while *Vibrio* sp., and to a lesser extent *Alteromonas* sp. accumulated at the more western station H02.

Although being members of well described bacterial genera, most of the chemotactically accumulating bacteria represent previously unknown phylotypes based on the detailed 16S rRNA gene sequence comparisons. Thus, only 1 of the 6 sequence types of *Thalassospira* sp., 1 of the 45 *Alteromonas* phylotypes, and 5 of the 37 different *Pseudoalteromonas* phylotypes were identical to sequences present in the GenBank database.

This result was unexpected considering that particularly the genus *Alteromonas* in the Mediterranean has been subject to intensive study (Pukall *et al*., 1999; Sass *et al*., 2001; Schafer *et al*., 2001; Pinhassi and Berman, 2003; Allers *et al*., 2007). In combination with the fact that a considerable number of these novel phylotypes were recovered repeatedly and also detected in different chemotaxis assays, the obtained results suggest that the chemotactically active bacteria represent distinct, novel subpopulations of *Thalassospira*, *Alteromonas* and *Pseudoalteromonas* spp. that had not been detected previously.

Because of their close sequence similarity, some of the 16S rRNA gene sequences recovered from the chemotaxis capillaries may actually originate from different *rrn* operons of the same bacterium. The complete genome sequence of *Alteromonas macloedii* DSM17117 (acc. no. NC 011138) comprises five different *rrn* operons. The corresponding 16S rRNA genes differ by 0.4 to 1.1%. Very similar values for inter operon heterogeneity

have been reported for other bacterial genomes (Acinas *et al*., 2004). Moreover, it is known that PCR-based clone libraries are prone to artifacts leading to a possible overestimation of sequence diversity (Acinas *et al*., 2004; Acinas *et al*., 2005). Most of the sequence dissimilarities seem to be introduced by amplification errors of the Taq-polymerase which are more pronounced when applying a high number of amplification cycles (Acinas *et al*., 2004; Acinas *et al*., 2005). Due to the very limited amount of template available for DNA extraction and subsequent PCR reactions, 35 cycles where necessary to obtain sufficient material. As it has been suggested that sequences should be grouped into 99% similarity groups to account for these artifacts (Acinas *et al*., 2005), the 1% cutoff appears reasonable to assess the diversity of chemotactically active bacteria recovered in the present study. Considering this cutoff value, at least 13 and 5 different and novel types of marine *Alteromonas* sp. and *Pseudoaltermonas* sp., respectively, exhibited chemotactic capabilities.

The pronounced and differential chemotactic accumulation of *Thalassospira*, *Alteromonas, Pseudoalteromonas* and *Vibrio* spp. from the ultraoligotrophic Eastern Mediterranean as determined in the present work has implications for our understanding of the ecological niche of these typical but less abundant marine planktonic genera and their potential role in the marine cycles of carbon and phosphorus.

## 5.6 CHEMOTAXIS AS A STRATEGY OF NATURAL BACTERIOPLANKTON FOR COPING WITH NUTRIENT LIMITATION

*Alteromonadales* and *Vibrionales* represent copiotrophs and r-strategists that follow a feast and famine life strategy and attain comparably high specific growth rates at higher nutrient concentrations. *Alteromonas* sp. and *Vibrio* sp. contain large genomes that code for a high number of global regulatory systems and are thought to be adapted to a rapid utilization of nutrient pulses (Giovannoni *et al*., 2005; Lauro *et al*., 2009). Correspondingly, nutrient amendments of marine mesocosms led to a strong dominance of *Alteromonas* sp. and *Vibrio* sp. (Pukall *et al*., 1999; Eilers *et al*., 2000a). These bacteria were also shown to be capable of surviving extended periods of starvation (Eilers *et al*., 2000b). Whereas generation times of 29 h have been observed for the abundant marine oligotroph *Pelagibacter ubique* (Rappé *et al*., 2002), much shorter values of 9 h are reached by seawater cultures of *Alteromonas* sp. from the North Sea (Eilers *et al*., 2000a). The latter values are very similar to that of our substrate amended dialysis cultures (≥8.7 h), indicating that the *Alteromonas* phylotypes in the Eastern Mediterranean also represent *r*-strategists despite their ultraoligotrophic environment.

Regardless of their copiotrophic life strategy, marine members of the *Alteromonadales* and *Vibrionales* are widespread throughout oligotrophic oceanic provinces like the Northern Pacific Subtropical Gyre where they occur at low abundances (Taniguchi and Hamasaki, 2008). They also constitute a small fraction of bacterioplankton in the more nutrient-rich North Sea where *Alteromonas*, *Pseudoalteromonas* and *Vibrio* represent <1% of total bacterial cell numbers (Eilers *et al*., 2000b). Correspondingly, *Alteromonadales* were detected at a low abundance of ~2% (Feingersch *et al*., 2010) in a metagenomic library prepared from near surface waters of the Eastern Mediterranean and dominated the particulate fraction of marine bacterioplankton communities in the Western Mediterranean Sea (Acinas *et al*., 1999) . *A. macleodii* constitutes up to 8% of the free-living bacterioplankton in the Western Mediterranean Sea (García-Martínez *et al*., 2002) and for an even higher fraction in the Ionian Sea (Zaballos *et al*., 2006). The widespread and continuous presence of *Alteromonas*, *Pseudoalteromonas* and *Vibrio* in ultraoligotrophic waters and the active state of the cells imply that these bacterial genera find appropriate ecological niches for growth within their oligotrophic marine environment.

Typically, *Alteromonas* sp. are found within the phycosphere of key phytoplankton species from the North Sea (Sapp *et al*., 2007) and are specifically associated with particles

> 8 µm in the western Mediterranean Sea (Acinas *et al*., 1999; García-Martínez *et al*., 2002). Accordingly, it has been speculated that the distinct association of *Alteromonas* sp. with particles is due to the protection from bacteriovores and the higher nutrient supply provided by aggregates (Acinas *et al*., 1999).

The results of the present study indicate that marine populations of *Alteromonas* sp. actively and rapidly accumulate on, or in the vicinity of, aggregates by means of chemotactically reacting toward organic carbon compounds, in particular amino acids, that are liberated during degradation of the aggregates (Smith *et al*., 1992). This conclusion is commensurate with the chemotactic response of laboratory cultures of *Pseudoalteromonas haloplanktis* toward various amino acids (Barbara and Mitchell, 2003) and the rapid accumulation of these cells in nutrient plumes of algal exudates maintained in the laboratory (Stocker *et al*., 2008).

However, Mediterranean surface waters have been documented to contain very low concentrations of phosphate, nitrate and ammonium and are characterized by limitation of bacterioplankton growth by inorganic phosphate (Thingstad *et al*., 1998; Lebaron *et al*., 2001). The previous study indicates that chemotaxis represents an additional mechanism of adaptation toward this pronounced phosphate limitation and is employed by a previously unrecognized component of marine ultraoligotrophic bacterioplankton. The fraction of *Thalassospira* 16S rRNA genes in clone libraries from phosphate-containing capillaries reached 25% whereas natural abundances of *Thalassospira* in the Eastern Mediterranean Sea were determined to range between 0.0004 and 1.2% . Based on these data, chemotaxis enabled *Thalassospira* cells to attain a relative enrichment by a factor of up to 60,000. Obviously, *Thalassospira* is specifically adapted to chemotactically accumulate at point sources of phosphate. Chemotaxis increases the exposure of bacterial cells to their substrates up to four fold (Stocker *et al*., 2008). As a major implication of the present study the chemotactic behavior discovered for natural bacterioplankton likely constitutes an alternative strategy of motile bacteria to enhance their phosphate acquisition in an ultraoligotrophic and inhomogeneous planktonic environment.

*Thalassospira* sp. has never been detected in marine bacterioplankton assemblages so far but has only been recovered from marine enrichment cultures. The closest studied relative to the chemotactically active bacteria from the Mediterranean is *Thalassospira lucentensis*, a strictly aerobic and halophilic, motile, facultatively oligotrophic bacterium growing chemoheterotrophically on carbohydrates, organic acids or amino acids as carbon

source and ammonium or nitrate as nitrogen source (López-López *et al*., 2002). Isolates of *Thalassospira* sp. have subsequently been reported from West Pacific Ocean sediments, surface water of a waste-oil pool in China (Liu *et al*., 2007) and from petroleum-contaminated seawater in Japan (Kasai *et al*., 2002; Kodama *et al*., 2008). Future research will reveal whether *Thalassospira* is a widely distributed and typical component of oligotrophic marine bacterioplankton that has been overlooked so far, and whether these bacteria actually colonize phosphate-liberating marine snow themselves or just remain in and exploit the nutrient plumes caused by exoenzymatic activity of other, particle-attached bacteria (Kiørboe and Jackson, 2001), or by the egestion of food vacuoles by protists (Blackburn *et al*., 1998; Azam and Malfatti, 2007).

In future it will be important to address the question why copiotrophic bacteria are usually not abundant in natural bacterioplankton communities if they are able to effectively exploit nutrient sources like fecal pellets or cell lysates. There are several possible explanations. It could be shown that organisms belonging to the *Alteromonadales* and *Vibrionales* are selectively grazed upon by heterotrophic nanoflagellates (Beardsley *et al*., 2003). Another top-down factor controlling bacterial abundance is viral lysis which might kill specific bacteria. It could be demonstrated that viruses are a major factor accounting for bacterial mortality in an oligotrophic, north-western Mediterranean coastal site (Boras *et al*., 2009). Therefore, it is possible that bacteria exhibiting a high-growth rate close to nutrient-rich particles are preferable lysed by viruses.

## 5.7 RESPONSE OF THALASSOSPIRA TOWARD PHOSPHATE STARVATION

In the present work, cultures of *Thalassospira* sp. EM, a novel isolate obtained from the Eastern Mediterranean Sea, were starved for organic carbon or inorganic phosphate. The cultures were compared in order to identify specific responses of this bacterium toward the phosphate-limiting conditions that prevail in its natural marine habitat.

The pronounced elongation of the cells and the 2 fold increase in cell volume in the *Thalassospira* sp. EM cultures exposed to phosphate limitation is in line with morphological changes reported for other copiotrophic marine bacteria under these conditions. Starvation of *Vibrio angustum* strain S14 for phosphorus also leads to markedly enlarged cells (Kjelleberg *et al*., 1993) whereas a starvation of bacteria for carbon substrates typically induces a reductive division and dwarfing due to degradation of endogenous cell material, eventually resulting in the formation of small coccoid cells (Kjelleberg *et al*., 1993; Llorens *et al*., 2010). Likewise, phosphate starvation of *Thiobacillus ferrooxidans* induces a filamentation of the cells (Seeger and Jerez, 1993). By comparison, obligately or facultatively oligotrophic bacteria undergo no or only a limited reduction of their cell volume during starvation (Janssen *et al*., 1997).

It has previously been observed that low nutrient conditions lead to high swimming velocities in natural assemblages of marine bacteria (Mitchell *et al*., 1995). In the cyanobacterium *Synechococcus*, phosphate stress induces genes encoding cell-surface proteins required for swimming motility (Tetu *et al*., 2009). During prolonged starvation of *Thalassospira* sp. EM mean swimming speeds increased by about 20 $\mu m \cdot s^{-1}$ compared to the onset of starvation. However, mean swimming speeds of *Thalassospira* sp. EM did not differ between cultures starved for organic carbon or inorganic phosphate and thus does not seem to be differentially regulated by the limiting nutrient. In cultures of *Vibrio angustum* S14, motility is almost completely lost during the first 24 hours of starvation for organic carbon (Malmcrona-Friberg *et al*., 1990) and starvation also strongly decreases the fraction of motile cells in cultures of *Silicibacter* sp. strain TM1040 (Miller *et al*., 2004). The loss of motility in *Thalassospira* sp. EM cultures was much less pronounced. Even after 10 days of starvation the fraction of motile cells still amounted to 43% in phosphate depleted cultures. Maintaining motility ensures that the cells remain capable of tracing point sources of phosphorus even after prolonged periods of phosphate shortage.

Natural bacterial communities throughout the Eastern basin of the Mediterranean Sea exhibited high alkaline phosphatase activities. The activity of this enzyme has been

shown to be inversely correlated with inorganic phosphate concentrations *in situ* (Van Wambeke *et al*., 2002). Induction of alkaline phosphatase (in particular PhoA or PhoX) is a typical response of planktonic microorganisms to phosphate limitation (Stihl *et al*., 2001) and has also been observed in other marine *Alphaproteobacteria* of the *Roseobacter* clade (Sebastian and Ammerman, 2009). The induction of extracellular alkaline phosphatase allows the cells to scavenge phosphate from organic sources, is repressed by inorganic phosphate and hence represents a strategy to cope with low availability inorganic phosphate. In a similar fashion, acid phosphatase in *Thiobacillus ferrooxidans* is induced by phosphate limitation (Seeger and Jerez, 1993). It was therefore unexpected that starvation of *Thalassospira* cells for organic carbon produced high levels of alkaline and acid phosphatase activity similar to those of phosphate-starved cells. Under both conditions, alkaline and acid phosphatase exhibited the highest activity among all 19 exoenzymes tested. Similar to *Thalassospira* sp. EM, extracellular phosphatase activity is not regulated by inorganic phosphate in other bacteria (e.g., *Mycobacterium bovis*; (Braibant, 2001)).

Taken together, the results of the present study suggest that the specific adaptative response of *Thalassospira* sp. EM to phosphate limiting conditions involves other mechanisms than an increased motility or increased hydrolysis of organophosphate esters.

## 5.8 CHEMOTAXIS TOWARD PHOSPHATE AS AN ADAPTIVE STRATEGY

Phosphate concentrations in the Eastern Mediterranean Sea are in the nanomolar range (Zohary and Robarts, 1998). Recent metagenomic analyses indicate that certain members of the bacterioplankton community of the Eastern Mediterranean Sea have adapted to this limitation by employing high affinity phosphate uptake systems as well as by utilizing organophosphonates (Feingersch *et al*., 2010). The majority of the corresponding functional genes were affiliated with the *Alphaproteobacteria* including members of the SAR11 group. The latter represent oligotrophic bacteria that are known to harbor a disproportionately large number of substrate binding proteins for phosphate and phosphonate (Sowell *et al*., 2009), do not grow at high-nutrient concentrations and typically are non-motile like, e.g. *Pelagibacter ubique* which also has long generation times of 29 h (Rappé *et al*., 2002). By comparison, the marine copiotrophs *Alteromonas*, *Pseudoalteromonas* and *Vibrio* typically occur at low abundance, yet are widespread and continuously present in ultraoligotrophic oceans where they have also been demonstrated to occur in an active metabolic state (Taniguchi and Hamasaki, 2008). Obviously, even marine copiotrophic bacteria find appropriate ecological niches within such oligotrophic marine environments.

All *Thalassospira* strains isolated so far are adapted to higher nutrient concentrations and grow well in complex media (López-López *et al*., 2002; Liu *et al*., 2007; Kodama *et al*., 2008). Based on its consistent presence throughout the Eastern Mediterranean Sea, the comparatively short doubling times of 6.2 hours, and its pronounced changes in cell morphology during starvation determined in the present study, *Thalassospira* sp. represents a previously unrecognized but widely distributed genus of marine copiotrophs. The obtained data indicate that chemotaxis represents a mechanism that permits members of this genus to populate the Eastern Mediterranean Sea despite the pronounced phosphate limitation in this environment.

Chemotaxis toward inorganic phosphate is absent in *E. coli* and *Salmonella typhimurium* and to date has been demonstrated to occur in the two facultatively pathogenic bacteria *Pseudomonas aeruginosa* (Kato *et al*., 1992) and *Enterobacter cloacea* (Kusaka *et al*., 1997). Among the *Archaea*, only *Halobacterium salinarum* has been shown to exhibit phosphate chemotaxis (Wende *et al*., 2009). Our finding of phosphate chemotaxis in a marine planktonic *Alphaproteobacterium* suggests that this chemotactic behavior occurs in typical environmental bacteria and may also be present in other bacterial lineages.

Besides the type species *T. lucentensis*, subgroup 1 of the genus *Thalassospira* mostly comprises isolates from various algal cultures that are likely to be adapted to an exploitation of nutrients in the algal phycosphere. In addition, subgroup 1 encompasses novel phylotypes that were shown to exhibit phosphate-directed chemotaxis using cultivation-independent methods. Most members of *Thalassospira* subgroup 2 originate from hydrocarbon affected environments but this group also encompasses the isolate of the present study. The previous isolation of a close relative of *Thalassospira* strains EM, *T. profundimaris* WP0211$^T$, from West Pacific deep-sea sediment suggests a broader distribution also of this subgroup. The present comparative study, together with culture-independent evidence for additional *Thalassospira* phylotypes, thus demonstrates that chemotaxis toward phosphate occurs across the entire phylogenetic breadth of the genus *Thalassospira* and may therefore be a characteristic of this genus.

In other marine bacteria, the chemotactic response may either decrease significantly within only a few hours of starvation (Malmcrona-Friberg *et al*., 1990) or, alternatively, may be increased by prior starvation or during the starvation phase (Miller *et al*., 2004). Our results show that *Thalassospira* sp. EM follows the second pattern. In *Vibrio angustum* S14, the genes that are up- regulated during starvation for phosphate differ from those induced by carbon starvation. Correspondingly, the proteins of the phosphate starvation stimulon are distinctly different from that of carbon-starved cells (Kjelleberg *et al*., 1993). In the Gram-negative *Enterobacter cloaceae*, a genetic knock-out of phosphate chemotaxis does not affect the chemotaxis toward peptone (Kusaka *et al*., 1997). Phosphate-directed chemotaxis of *Pseudomonas aeruginosa* PAO1 is induced by phosphate starvation (Ohtake *et al*., 1998) and involves two novel chemoreceptors (Wu *et al*., 2000). The observation that P-starvation but not C-starvation elicits the chemotactic response of cells toward inorganic phosphate suggests that the chemotaxis genes also in *Thalassospira* sp. EM are differentially regulated and form part of a specific P-starvation response. In *Pseudomonas aeruginosa* PAO1, both phosphate chemotaxis and alkaline phosphatase expression are controlled by the same negative regulator *phoU* (Kato *et al*., 1994). In contrast, our results suggest that the regulation of phosphate chemotaxis in *Thalassospira* sp. EM is independent of the expression of alkaline phosphatase. Detailed genetic analysis of the regulation of chemotaxis genes in *Thalassospira* sp. has to await the availability of the genome sequence of a representative of this genus.

In phosphate-depleted ocean surface waters dissolved organic phosphate constitutes an additional source of phosphorus for marine organisms (Dyhrman *et al*., 2007). In the marine environment, phosphonates represent a considerable fraction of 25% of organic phosphorus compounds (Kolowith *et al*., 2001). Recent metagenomic approaches revealed that phosphonate utilization genes are widespread and abundant in phosphate-depleted ocean surface waters and present in diverse bacteria (Martinez *et al*., 2010). Besides organic carbon substrates, 2-aminoethylphosphonate elicited a chemotactic response of *Thalassospira* sp. EM that proved to be independent of the phosphate supply of the cultures. This seems to be the first report of bacterial chemotactic behavior toward a phosphonate. Besides the hydrolysis of organophosphate esters and the chemotaxis toward phosphate, chemotactic accumulation and subsequent utilization of phosphonates may thus represent an additional adaptation of *Thalassospira* sp. EM to phosphate-depleted ocean surface waters. Like *Trichodesmium* IMS101 which is able to use multiple organic and inorganic phosphorus sources simultaneously (Beversdorf *et al*., 2010) *Thalassospira* sp. EM might also take advantage of multiple phosphorus sources in its phosphate-depleted environment.

## 5.9 ECOLOGICAL RELEVANCE OF PHOSPHATE-DIRECTED CHEMOTAXIS

Modeling has revealed that chemotaxis permits motile bacteria to take advantage of point sources of organic carbon substrates and also to use plumes of dissolved organic matter formed in the wake of sinking organic particles (Blackburn *et al*., 1998). Ultimately, chemotaxis toward organic carbon compounds accelerates their turnover in the natural environment since it significantly increases the exposure of bacterial cells to their substrates (Stocker *et al*., 2008). Based on the results of the present study, *Thalassospira* sp. is expected to accumulate at microscale phosphate sources which would enable the cells to scavenge inorganic phosphate more efficiently under the ultraoligotrophic phosphate-limiting conditions that prevail in the Mediterranean Sea (Zohary and Robarts, 1998). Chemotaxis toward point sources of phosphate thus is likely to constitute an alternative strategy of motile bacteria to enhance their phosphate acquisition in an ultraoligotrophic and inhomogeneous planktonic environment.

Compared to *Pseudomonas aeruginosa* PAO1 or the eukaryotic algae *Chattonella antiqua* (Ikegami *et al*., 1995), the threshold concentrations of phosphate-directed chemotaxis were 10 to 50 fold lower in *Thalassospira* spp. While natural assemblages of marine bacteria exhibit very high swimming speeds of >230 μm · $s^{-1}$ (Mitchell *et al*., 1995), marine copiotrophic bacteria like *Pseudoalteromonas, Alteromonas* or *Vibrio* sp. only reach mean velocities of 11 to 38 μm · $s^{-1}$ (Johansen *et al*., 2002) that are lower than the values determined for *Thalassospira* sp. EM. The higher sensitivity of its chemotaxis together with its comparatively high swimming speed provides *Thalassospira* with a selective advantage for rapid accumulation at phosphate sources in a heterogeneous environment.

## 5.10 CONCLUSION

Bacteria inhabiting the Eastern Mediterranean Sea are confronted with low nutrient-concentrations (Krom *et al*., 2004). Especially concentrations of inorganic phosphate are in the nanomolar range and thus likely limit bacterial growth (Zohary and Robarts, 1998; Krom *et al*., 2004). Potential adaptive strategies of heterotrophic bacterioplankton to cope with nutrient scarcity in their natural habitat were addressed in the present thesis. Affinities of alkaline phosphatases were high throughout the eastern basin of the Mediterranean Sea and correlated with changes in the composition of the bacterial community due to the concentration process employed, which was mandatory because of low overall bacterial biomass and activity. Bacterial growth was shown to be stimulated by inorganic phosphate and a mixture of organic carbon substrate but not by nitrogen compounds, indicating a limitation of bacterial growth by phosphate as reported previously (Krom *et al*., 2004; Thingstad *et al*., 2005). The observed chemotactic response of bacteria present *in situ* in the Eastern Mediterranean Sea is likely to accelerate cycling of phosphorus and indirectly suggests the existence of phosphate hot-spots analogously to the well established hot-spots of organic carbon (Azam and Malfatti, 2007). A marine copiotrophic bacterium, *Thalassospira* sp. EM, was isolated from the phosphate-depleted Eastern Mediterranean Sea and was shown to react chemotactically toward carbon substrates, inorganic phosphate and phosphonate. This further supports the hypothesis that chemotaxis constitutes an adaptive strategy bacteria exhibit in response to nutrient scarcity. Since phosphorus limitation of bacterioplankton may also occur in other oligotrophic open ocean environments like the Sargasso Sea (Cotner *et al*., 1997), the Caribbean Sea (Martiny *et al*., 2009), the subtropical North Pacific (Karl, 2002), or the Red Sea (Fuller *et al*., 2005), bacterial chemotaxis toward inorganic phosphate may also affect nutrient cycling and productivity in other oceanic provinces.

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# I List of Abbreviations

| | |
|---|---|
| AMC | 7-amino-4-methylcoumarin |
| ASW | artificial seawater medium |
| $ASW_{GluNP}$ | ASW with glucose, ammonium and phosphate |
| $ASW_{GluNPYE}$ | ASW with glucose, ammonium , phosphate and yeast extract |
| $ASW_{GluNYE}$ | ASW with ammonium and yeast extract |
| ATP | adenosine triphosphate |
| AW | Atlantic Water |
| BGE | bacterial growth efficiency |
| bp | base pairs |
| °C | degree Celsius |
| CFU | colony forming units |
| Chl | Chlorophyll a |
| COG | Clusters of orthologous groups |
| DAPI | 4',6-diamidino-2-phenylindol |
| DCM | deep chlorophyll maximum |
| ddH2O | double-distilled water |
| DGGE | denaturing gradient gel electrophoresis |
| DNA | desoxyribonucleic acid |
| DOC | dissolved organic carbon |
| DOM | dissolved organic matter |
| DON | dissolved organic nitrogen |
| DSMZ | Deutsche Sammlung für Mikroorganismen und Zellkulturen |
| HEPES | 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid |
| kb | thousand base pairs |
| LIW | Levantine Intermediate Water |
| MCP | methyl accepting protein |
| MUF | 4-methylumbelliferone |
| n.d. | not determined |
| PCR | polymerase chain reaction |
| POM | particulate organic matter |
| rrn | ribosomal ribonucleic acid |
| rRNA | ribosomal ribonucleic acid |
| spp. | several species |
| v/v | volume / volume |
| w/v | weight / volume |

# II Supplement

Sequences not deposited in GenBank

A. Exoenzymes/Diversity

>Figure 4-5/Table 4-2 (diversity) band 1
GACGCAGCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTCGTAAAGCTCTTTTATGGGGGAAGATGATGAC
GGTACCCCATGAATAAGCACCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGTGCGAGCGTTGT
TCGGAATTACTGGGCGTAAAGGGCGTGCAGGCGGATTGGCAAGCCGGAGGTGAAAGCCCGGGGCTCAACC
CCGGAGGGTCTTTCGGAACTGCCAGTCTTGAGAGGGTCAGGGGCCAGCGGAATTCCTGGTGTAGAGGTGA
AATTCGTAGAGATCAGGAGGAACACCGGCGGCGAAAGCGGCTGGCTGGGGCCACTCTGACGCTGAGGCGC
GAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGGGCACTAAGCGTC
CGACGGGTTCGACCCTGTTGGGTGCTGCAGTTAACGCGTTAAGTGCCCCGCCTGGGGAGTACGGTCGCAAG
ACTAAAACTCAAAGAA

>Figure 4-5/Table 4-2 (diversity) band 2
CTGACGCAGCCACGCCGCGTGCGGGATGAAGGCTCTAGGGTTGTAAACCGCTTTAGGCAGGGAAGAAAAT
GACGGTACCTGCAGAATAAGGTCCGGCCAACTACGTGCCAGCAGCCGCGGTAACACGTAGGGACCGAGCGT
TGTCCGGATTTATTGGGCGTAAAGAGCTCGTAGGCGGTTCGGTAAGTCAGGTGTGAAAGCTCGAGGCTCA
ACCTCGAGATGCCACCTGATACTGCTGTGACTTGAGTCCGGTAGAGGAGTGTGGAATTCCTGGTGTAGCGG
TGAAATGCGCAGATATCAGGAGGAACACCAATTGCGAAGGCAGCACTCTGGGCCGGTACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGTTGGGCACTAGGT
GTGGGATCGAACTAACCGATTCCGTGCCGTAGCTAACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCA
AGGCTAAAACTCAAAGGAATTGACGGA

>Figure 4-5/Table 4-2 (diversity) band 3
AGCCTGANCAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTTATCCGGGACGATA
ATGACGGTACCGGAGGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCTAGC
GTTGCTCGGAATCACTGGGCGTAAAGG
GCGCGTAGGCGGCGTTTTAAGTCGGGGGTGAAAGCCTGTGGCACAACCACAGAATGGCCTTCGATACTGG
GACGCTTGAGTATGGTAGAGGTTGGTGGAACTGCGAGTGTAGAGGTGAAATTCGTAGATATTCGCAAGAA
CACCGGTGGCGAAGGCGGCCAACTGGACCATTACTGACGCTGAGGCGCGAAAGCGTGGGGAGCAAACAGG
ATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAATGCCAGCTGTTGGGGTGCTTGCACCGCAGTAGCG
CAGCTAACGCTTTGAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAAGGAAATTGACGG

>Figure 4-5/Table 4-2 (diversity) band 4
GACGGAGCACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTTATCAAGGAAGAAGATCTG
ACGGTACTTGATGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTT
ATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCCTTAAAGTCTGCTGTTAAAACGTGGAGCTTAAC
TCCATCATGGCAGTGGAAACTGTTGGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTG
AAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATMACTGACGCTCATGGA
CGAAAGCCAGGGGAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTAGGTGT
CGGGGGAATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACGCACGCAAGT
GTGAAACTCAAAGGAATTTGACGGA

>Figure 4-5/Table 4-2 (diversity) band 5
GACGGAGCACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTTATCAAGGAAGAAGATCTG
ACGGTACTTGATGAATAAGCCACGGCTAATTCCGGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGCGTT
ATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCCTTCAAGTCTGCTGTTAAAAAGTGGAGCTTAAC
TCCATCATGGCAGTGGAAACTGTTGGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTG
AAATGCGTAGATATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATCACTGACGCTCATGGAC
GAAAGCCAGGGGAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTAGGTGTC

GGGGGAATCGACCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACGCACGCAAGTG
TGAAACTCAAAGGAATTTGACGGA

>Figure 4-5/Table 4-2 (diversity) band 6
CCTGACGGAGCACGCCGCGTGAGGGATGAAGGCCTCTGGGCTGTAAACCTCTTTTATCAAGGAAGAAGATC
TGACGGTACTTGATGAATAAGCCACGGCTAATTCCGTGCCAGCAGCCGCGGTAATACGGGAGTGGCAAGC
GTTATCCGGAATTATTGGGCGTAAAGCGTCCGCAGGCGGCCCTTCAAGTCTGCTGTTAAAAMGTGGAGCT
TAACTCCATCATGGC
AGTGGAAACTGTTGGGCTTGAGTGTGGTAGGGGCAGAGGGAATTCCCGGTGTAGCGGTGAAATGCGTAGA
TATCGGGAAGAACACCAGTGGCGAAGGCGCTCTGCTGGGCCATCACTGACGCTCATGGACGAAAGCCAGGG
GAGCGAAAGGGATTAGATACCCCTGTAGTCCTGGCCGTAAACGATGAACACTAGGTGTCGGGGGAATCGA
CCCCCTCGGTGTCGTAGCCAACGCGTTAAGTGTTCCGCCTGGGGAGTACGCACGCAAGTGTGAAACTCAAA
GGAATTGACGG

>Figure 4-5/Table 4-2 (diversity) band 7
CCATGCCGCGTGAGTGATGAAGGCCTTAGGGTCGTAAAGCTCTTTCNCCAGGGATGATGATGACAGTACC
TGGTAAAGAAACCCCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCGGA
ATTACTGGGCGTAAAGCGCACGTAGGCGGACCAGAAAGTTGGGGGTGAAATCCCGGGGCTCAACCCCGGA
ACTGCCTCAAAAACTCCTGGTCTANAGTTCGAGAGAGGTGAGTGGAATTCCNAGTGTAGAGGTGAAATTC
GTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGTGCGAAAG
TGTGGGGAGCAAACAGGATTANATACCCTGGTAGTCCACACCGTAAACGATGAATGCCGTACGTCGGGTA
GCCTGCTATTCNGTGTCACACCTAACGGATTAAGCATTCCGCCTGGGGAGTACGGTCGCAAGATTAAAACT
CAAA

>Figure 4-5/Table 4-2 (diversity) band 8
CCTGACGCAGCCATGCCGCGTGAGTGATGAAGGTCTTAGGATTGTAAAATTCTTTCACCGGGGACGATAA
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TTGCTCGGAATTACTGGGCGTAAAGGGAGCGTAGGCGGACATTTAAGTCGGGGGTGAAATCCCGGGGCTC
AACCTCGGAATTGCCTTTGATACTGGGTGTCTTGAGTATGAGAGAGGNGTGTGGAACTCCNAGTGTAGAG
GTGAAATTCGTAGATATTCGGAAGAACACCNGTGGCGAAGGCGACACACTGGCTCATTACTGACGCTGAG
GCTCGAAAGCGTGGGGAGCAAACAGGCTTAGATACCCTGGTAGTCCACGCCGTAAACGATGATTGCTAGT
TGTCGGGATGCATGCATTTCNGTGACGCANCTAACGCATTAANCAATCCGCCTGGGGAGTACGGTCGCAA
GATTAAAACTCAAAGGAATTGACGG

>Figure 4-5/Table 4-2 (diversity) band 9
CAGCCATGCCGCGTGAGTGATGAAGGCCTTAGGGTCGTAAAGCTCTTTCGCCAGAGATGATAATGACAGT
ATCTGGTAAAGAAACCCCGGCTAACTCCGTGCCAACAGCCGCGGTAATACGGAGGGGGTTAGCGTTGTTCG
GAATTACTGGGCGTAAAGCGCGCGTAGGCGGACCAGAAAGTTAGGGGTGAAATCCCGGGGCTCAACCCCG
GAACTGCCTCTAAAACTCCTGATCTAGAGTTCGAGAGAGGTGAGTGGAATTCCGAGTGTAGAGGTGAAAT
TCGTAGATATTCGGAGGAACACCAGTGGCGAAGGCGGCTCACTGGCTCGATACTGACGCTGAGGTGCGAA
AGTGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACACCGTAAACGATGAATGCCANNCGTCNGG
TAGCATGCTATTCNGTGACACACCTAACNGATTAAGCATTCCNCCTGGNGAGTACGGNCGCAAGATTAAN
ACTCA

>Figure 4-5/Table 4-2 (diversity) band 10
CATGCAGCGTGTGTGAGAAGGCCTGAGGGTTGTAAAGCACTTTCAGTAGGGAAGATAATGACGGTACCTA
CAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAAT
TACTGGGCGTAAAGCGTACGTAGGATGTGATGCAAGTCGGATGTGAAATCCCCGGGCTTAACCTGGGAAC
TGCATTCGAAACTACATTACTAGAGTGTAGTAGAGGTAAGCGGAATTTCGGGTGTAGCGGTGAAATGCGT
AGATATCCGAAAGAACATCAGTGGCGAAGGCGGCTTACTGGACTAACACTGACTCTGAGGTACGAAAGCG
TGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGTCAACTAGACGTTGGGTTCC
TTGAGAACTTAGTGTCGTAGCTAACGCGTTAAGTTGACCGCCTGGGGAGTACGGCCGCAAGGTTGAAACT
CAAAGGAA

>Figure 4-5/Table 4-2 (diversity) band 11
CTTTCTNNGGTGATGATGATGACGGTACCCGTAGAATAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGT
AATACGGAGGTGGCGANCGTTNTTCGGAATTATTGGGCGTAAAGGGCTCGCAGGCTGCTTGAACAGTTAG
ACGTGAAATCCCCGGGCTCAACTTGGGAACTGCGTTTAATACTGTCAAACTAGAGACTGATAGAGGAAAG
TGGAACTCCCAGTGTAGAGGTGAAATTCGTAGATATTCGGAAGAACACCNNTGGCGAAAGCGACTTTCTG
GCTATTTTCTA

>Figure 4-5/Table 4-2 (diversity) band 12
TGATCAGCATGCCGCGTGTGTGATGAAGGCCTTAGGGTTGTAAAGCTCTTTCAACGGTGAAGATGATGAC
GGTANCCGTAGAAGAAGCCCCGGCTAACTTCGTGCCAGCAGCCGCGGTAATACGAAGGGGGCGAGCGTTA
TTCGGAATTATTGGGCGTAAAGGGCTCGCAGGCTGCTTGAACAGTTAGACGTGAAATCCCCGGGCTCAACC
TGGGAACTGCGTTTAATACTNTCAAGCTAGAGAAATAGAGAGGAAAGTGGAACTCCCAGTGTAGAGGTGA
AATTCGTAGATATTGGGAAGAACACCAGTGGCGAAAGCGACTTTCTGGCTATTTTCTGACGCTGAGGAGC
GAAAGCGTGGGGAGCAAACAGGATTAAATACCCTGGTAGTCCACGCCGTAAACGATGTGTGCTAGATGTT
GGAAGGTTACCT

>Figure 4-5/Table 4-2 (diversity) band 13
CTATGCCGCGTGCAGGATGACGGCCCTACGGGTTGTAAACTGCTTTTATACAGGAAGAAATACCCCGACGT
GTCGGGGCTTGACGGTACTGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGAG
GATNCGAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGTAGGCGGTTTTTTAAGTCAGAGGTGAAAT
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TGACGCTCAGGGACGAAAGCGTGGGTAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GGATACTAGCTGTATGTCTACCTCAGGTAGATGTGTGGCCAAGCGAAAGTGATAAGTATCCCACCTGGGG
AGTACGATCGCAAGATTGAAACTCAAAGGAATTGACGG

>Figure 4-5/Table 4-2 (diversity) band 14
CCAGCTATGCCGCGTGCAGGATGANGGCCNTANGGNTNGNAANCTSCTTTTATACAGGAAGAAAAACTCC
GACGTGTCGGAGCTTGACGGTACTGTAAGAATAAGGATCGGCTAACTCCGTGCCAGCAGCCGCGGTAATAC
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TGGNGAGTACGATCGNAAGATTGAAACTCAAAGGAATTGACGG

>Figure 4-5/Table 4-2 (diversity) band 15
GTCTGATCCAGCTATGCCGCGTGCAGGATGACGGCCCTATGGGTTGTAAACTGCTTTTATACAGGAAGAA
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CCCACCTGGGGAGTACGATCGCAAGATTGAAACTCAAAGGAATTGACGG

>Figure 4-5/Table 4-2 (diversity) band 16
GCCATGCCGCGTGCAGGAGANTGCCCTATGGGTTGTAAACTGCTTTTATACAGGAAGAAACTTATCCACG
TGTGGGTAACTGACGGTACTGTAAGAATAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTAATACGGA
GGGTGCAAGCGTTATCCGGAATCATTGGGTTTAAAGGGTCCGCAGGCGGTCAATTAAGTCAGAGGTGAAA
TCCTACAGCTTAACTGTAGAACTGCCTTTGATACTGGTTGACTTGAGTCATATGGAAGTAGATAGAATGT
GTAGTGTAGCGGTGAAATGCATAGATATTACACAGAATACCGATTGCGAAGGCAGTCTACTAGGTATGTA
CTGACGCTCATGGACGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGA

TGGACACTAGTTGTTGGAATTATTCAGTGACTAAGCGAAAGTGATAAGTGTCCCACCTGGGGAGTACGAT
CGCAAGATTGAAACTCAAAGGAATTGACGGA

B. Enrichment cultures

>Figure 4-18 (enrichment) band 1
CCTGATCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGATGCGAAGATGAT
GACGGTAACATCAGAAGAAGCCCCGGCTAATTTCGTGCCAGCAGCCGCGGTAATACGAAAGGGGCTAGCG
TTGTTCGGATTTACTGGGCGTAAAGGGCACGCAGGCGGTCTTGCCAGTCAGGGGTGAAAGCCCGAGGCTCA
ACCTCGGAACTGCCTCTGATACTGTAAGACTTGAGACTAGGAGAGGGTGGTGGAATTCCCAGTGTAGAGG
TGAAATTCGTAGATATTGGGAGGAACACCAGAGGCGAAGGCGGCCACCTGGACTAGATCTGACGCTCAGG
TGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGTT
GTCGGGACTTCGGTTTCGGTGACGCAGCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGATT
AAAACTCAAAGGAAATTGACGGA

>Figure 4-18 (enrichment) band2
ACCTGATCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGATGCGAAGATGA
TGACGGTAACATCAGAAGAAGCCCCGGCTAATTTCGTGCCAGCAGCCGCGGTAATACGAAAGGGGCTAGC
GTTGTTCGGATTTACTGGGCGTAAAGGGCACGCAGGCGGTCTTGCCAGTCAGGGGTGAAAGCCCGAGGCTC
AACCTCGGAACTGCCTCTGATACTGTAAGACTTGAGACTAGGAGAGGGTGGTGGAATTCCCAGTGTAGAG
GTGAAATTCGTAGATATTGGGAGGAACACCAGAGGCGAAGGCGGCCACCTGGACTAGATCTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGT
TGTCGGACTTCGGTTTCGGTGACGCAGCTAACNCATTAA

>Figure 4-18 (enrichment) band 3
ACCTGATCAGCCATGCCGCGTGAGTGAAGAAGGCCTTCGGGTTGTAAAGCTCTTTCAGATGCGAAGATGA
TGACGGTAACATCAGAAGAAGCCCCGGCTAATTTCGTGCCAGCAGCCGCGGTAATACGAAAGGGGCTAGC
GTTGTTCGGATTTACTGGGCGTAAAGGGCACGCAGGCGGTCTTGCCAGTCAGGGGTGAAAGCCCGAGGCTC
AACCTCGGAACTGCCTCTGATACTGTAAGACTTGAGACTAGGAGAGGGTGGTGGAATTCCCAGTGTAGAG
GTGAAATTCGTAGATATTGGGAGGAACACCAGAGGCGAAGGCGGCCACCTGGACTAGATCTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAGT
TGTCGGGACTTCGGTTTCGGTGACGCANCTAACGCATTAAGCACTCCGCCTGGGGAGTACGGTCGCAAGAT
TAAAACTCAAAGGAATTGACGGGG

>Figure 4-18 (enrichment) band 4
ACCTGATCGGCACTGCCGCGTGAGTGATGAGGGCCTTNGGGTTGTAAAGCTCTTTCAATNGGGAAGATGA
NGACNGTACTTGCAGAAGAANCCCCGGCTAATTTCGTGCCAGCAGCCGCGGTAATACGAAAGGGGCTAGC
GTTGTTCGGATTTACTGGGCGTAAAGGGCACGCAGGCGGTCTTGCCAGTCAGGGGTGAAAGCCCGAGGCTC
AACCTCGGAACTGCCTCTGATACTGTAAGACTTGAGACTAGGAGAGGGTGGTGGAATTCCCAGTGT

## III Publications

Publications originating from this thesis

**Hütz, A., K. Schubert, M. Mayer, and J. Overmann.** 2011. Chemotactic response of bacterioplankton in the ultraoligotrophic Eastern Mediterranean Sea. Manuscript

**Hütz, A., K. Schubert, and J. Overmann.** 2011. *Thalassospira* sp. isolated from the oligotrophic Eastern Mediterranean Sea exhibits chemotaxis toward inorganic phosphate during starvation. Appl Environ Microbiol **77:**4412-4421.

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