1. INTRODUCTION

The workshop was held from 5 to 8 May 2000 in Zichron Yaakov, a charming village located in the foothills of the Carmel mountain range, south of Haifa. Sixteen scientists (see list at the end of volume) did participate at the invitation of CIESM.

Most regrettably, Prof. David Gutnick, Chair of the CIESM Committee of Marine Microbiology and Biotechnologies, and main convener of the workshop, was unable to attend – literally at the last minute – in order to treat an eye infection. In opening the meeting, the first words of Frédéric Briand, CIESM Director General, were to express the genuine concern of all participants and to relay their best wishes of a prompt recovery to David Gutnick, who had worked hard in collaboration with Prof. Tom Berman to ensure that the workshop – the first one to be held by CIESM in Israel – would be a success.

Prof. Briand went on to present the overall "philosophy" and aims of CIESM research workshops. He emphasized that their objective was not so much to present exhaustive reviews, but rather to venture at the edges of a given field. This would be particularly expected here, given the mounting recognition of the key role played by marine bacteria in biogeochemical and ecological processes. He strongly encouraged the participants to freely explore the new concepts and exciting perspectives offered by recent findings and new technologies in microbial ecology,

Prof. Tom Berman, workshop co-organizer, followed. He reminded everyone that the approach of the meeting was deliberately pluri-disciplinary : the presentations would cover various aspects of microbial ecology, diversity, culturing and dynamics before all these threads were brought together in general prospective discussions. Of particular interest were experimental approaches to microbial monitoring of organisms at the micro-scale, the ecology, activities and processes of marine bacteria in relation to ecosystem fonctioning.

2. "EMERGENCE" OF MICROBIAL FOOD CHAINS

The Mediterranean is a phosphorus-poor basin, with nutrient concentrations decreasing eastwards to extreme oligotrophy. It is also subject to a high level of solar irradiation. These features conspire to put the plankton under conditions of physiological stress. The Mediterranean differs from oceanic areas of extreme oligotrophy in that it also contains adjacent localised "hot spots" of intensive eutrophication (*e.g.* Western Adriatic, Gulf of Lions). As the environment becomes more oligotrophic, microorganisms are likely to assume a greater quantitative role, both as biomass components and in the flux of materials within the ecosystem.

Historically oceanographers viewed the structure of marine life as a simple grazing chain starting with phytoplankton leading to fish and other top predators, via metazoan zooplankton. In loose association with this was a microbial system, thought to be comprised of a small biomass of sluggishly growing micro-organisms. The effect of this latter component on the functioning of the planktonic system as a whole was regarded to be minor and was often disregarded. We now know that this perception is fundamentally wrong. The biomass, and particularly the metabolism, of the microbial community can far outweigh that of the metazoan organisms. Indeed, the largest continuous ecosystem on the planet, the North-central subtropical Pacific Gyre, is almost exclusively microbial.

Contemporary views of the microbial food web propose a profoundly different perspective of the planktonic ecosystem from that of 15 or 20 years ago. Current models, such as those of Fasham *et al.* (1999) and Nagata (2000), present micro-organisms as the main consumers of primary production, and thus as a major competitor of copepods, a trend accentuated in oligotrophic waters (see Fig. 1). In other words, while microbial processes result in rapid and extensive organic matter recycling, the "microbial loop" will often act as a sink, rather than a supplementary organic carbon source, for zooplankton production.

This acknowledgement of the multifaceted importance of microbial processes in marine ecosystem function requires a major revision of our understanding of the flows of organic material between marine organisms. The new paradigm implies that any shift in the scale of microbial processes will have a profound impact on ecosystem function and major consequential effects on the marine environment, including commercially important components of the food web such as fish.

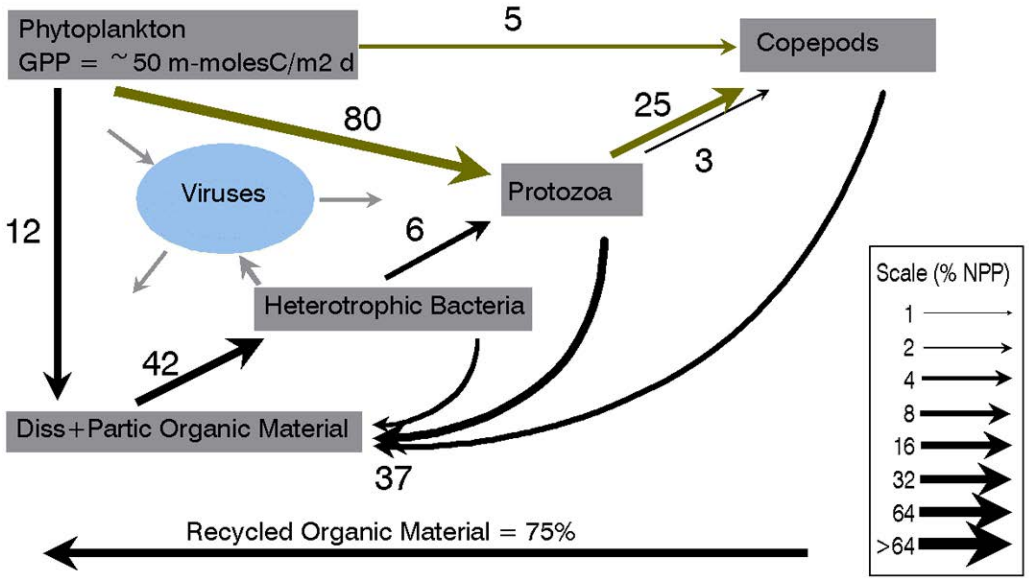

Fig. 1. Estimated flows in a oligotrophic planktonic food web - expressed as percentage of annual net planktonic primary production (from Nagata, 2000).

3. MICROSCALE PROCESSES

Our knowledge of the microbial food web, as illustrated in Figure 1, is limited to very broad statements of integrated activity over decimetre and large space scales. Both producer and consumer organisms on this scale can be regarded essentially as point sources. Thus, as has been experimentally observed, the environment itself is spatially heterogeneous on all scales. The overall processes measured on scales of decimetres to metres are the summation of reactions and interactions between individual organisms and consortia and the chemical milieu on the microscale, that is scales of from a few to several hundreds of microns. As a consequence, the control of processes observed on large scales is a result of events on the microscale. If we fail to take account of processes, controls and interactions on the microscale, we end up with a profoundly inadequate, even wrong conception of the marine environment.

As a simple example, consider the climatically active gas, dimethyl sulfide (DMS) which is produced by phytoplankton. This gas is not evolved by all algae, but particular phytoplankton classes and in some cases individual species are rich sources of DMS. Release of the gas is not passive and was originally thought to be a consequence of zooplankton grazing. We now know that, certainly in some species, it is a consequence of infection by host-specific viruses, a microscale process. Thus, we may argue that in the case of DMS, only when we can understand the processes, species and individuals involved at the microscale level, can we hope to effectively model the flux of the compound on the wider, ocean basin scale.

At present, we still have limited knowledge of the spatial distribution of organisms, their taxonomic and genetic diversity and abundance, their metabolic and ecological functions and the environmental controls that operate on the microscale. The matter is complicated by the fact that connection between the small and large scales is not one way. Whereas the consequences of microbial metabolism are transmitted from the small to the large scale; physical/chemical forcing *e.g.* photon flux, turbulence and nutrient input come down from the large scale. Large scale (meter, kilometre, and greater) province-wide biogeochemical features set the context and limits to the microscale events but do not determine these mechanisms: there are strong local control processes acting at the microscale. Thus, the actual response to major environmental changes will be determined at the microscale and not at the basin-wide level.

Since microbial processes dominate the ecological and biogeochemical dynamics of many marine environments, for instance the Eastern Mediterranean, it is imperative that we develop the necessary conceptual and technical bases to incorporate them into ecosystem models. In recent years, the thesis has been developed (*e.g.* Azam, 1998) that a mechanistic understanding of microbial interactions and activities at the micrometer scale is critical for integrating microbial loop processes into predictive ecosystem models. While it is axiomatic that microbes live and die in microscale "worlds", the significance of pelagic microscale ecology has been recognized only recently, due partly to the discovery of the microscale heterogeneity of the distribution of organic matter in the pelagic realm (Alldredge *et al.*, 1993; Azam *et al.*, 1993; Chin *et al.* , 1998). The response of microbes to such heterogeneity creates activity hot-spots which can critically influence microbially-mediated biogeochemical processes, both quantitatively and qualitatively.

Microscale patchiness of nutrients and the spatial distribution of bacteria have profound implications for the structure of the microbial food web, for carbon cycling and nutrient dynamics on a global scale. High concentrations of bacteria associated with particles have been documented causing intense and even unique biogeochemical transformation processes. Bacterial clustering around individual particles, including living algae, was initially proposed over a quarter of a century ago (Bell and Mitchell, 1972) but the evidence remains indirect or circumstantial. A more recent study (Mitchell *et al.*, 1996) shows unexpectedly high swimming speeds of marine bacteria (>100 µm s-1) which enable bacteria to behave chemotactically.

It should be stressed that the biogeochemical significance of microscale heterogeneity extends to all scales, from the micrometer to the ocean basin scale. It is therefore of importance not only to the students of microbial ecology but also to our discussion of microbial oceanography of the Mediterranean. This point is illustrated by the recently discovered control of pelagic bacteria on oceanic silicon cycle (Bidle and Azam, 1999): bacteria greatly accelerated diatom frustule dissolution by enzymatically hydrolysing the protective organic layer on the frustule. Here, the biogeochemical variable was not the bulk-phase bacterial activity but the activity expressed by the colonizing bacteria in the microenvironment of the diatom frustule.

Microbial processes on organic aggregates (*e.g.* marine snow) provide another example of the significance of microscale biogeochemistry for ecosystem structure and functioning. Alldredge and Cohen (1987) used an oxygen microelectrode to discover that the interior of the marine snow can represent suboxic and even anoxic microenvironments in an oxygenated bulk-phase seawater. A subsequent study showed that such reducing microzones could actually support sulfate reduction (Shanks and Reeder, 1993) and methanogenesis (Karl and Tilbrook, 1994). Intense bacterial ectohydrolytic activities on marine-snow leads to rapid POM-DOM fluxes (Smith *et al.*, 1992) and respiration (Ploug *et al.*, 1999). Furthermore, bacterial activities in the microscale lead to increased fluxes of nutrients (P, N, etc.) which cannot be measured by traditional analytical methods. These fluxes have pronounced implications not only for bacterial production but for primary production as well. Together these examples show that in the absence of a mechanistic understanding of microbial processes at the microscale we would miss the very existence of such potentially critical controls on ocean basin scale biogeochemistry.

The microscale environment of the pelagic realm is still essentially unexplored, not only biologically but also with respect to its chemistry and physics. The existing tools of oceanography are inadequate for this exploration. In view of the great challenge involved, it is important that we

sharply focus our effort on a limited number of fundamental questions of microbial ecology and biogeochemistry. The problem of microscale activities of bacteria has traditionally been framed in the context of attached and free bacteria (*e.g.* Berman, 1975). There has been a major shift recently in our concepts of the organic matter pool, blurring the distinction between the particulate and dissolved organic phases. It has been found that the "traditional" particles such as algal detritus and marine snow are only the proverbial tip of the iceberg of the particulate phase. Highly abundant transparent colloids and particles (Koike *et al.*, 1990; Wells and Goldberg, 1991, 1992; Alldredge *et al.*, 1993; Long and Azam, 1996) physically structure the pelagic realm into a hydrogel in which are embedded the traditional particles and microbes, forming an organic matter continuum (Azam *et al.*, 1993; Azam, 1998). This new concept of the organic matter continuum in the pelagic realm (see Fig. 2) requires a rethinking of our ideas on bacterial adaptations in the particulate and dissolved organic phases and the biogeochemical consequences of bacteria-organic matter coupling. Indeed, we require a new conceptual framework and new techniques to elucidate the ecology and biogeochemical activities of pelagic microbes within the hydrogel organic matter continuum. Equally importantly, we need to develop or adapt methods to characterize the physics and the chemistry of the organic matter continuum and their temporal dynamics. The requirement is to study microbial biology, physics and chemistry at the same scale of the environment. For the purpose of microbial processes we have chosen to assign a linear dimension of 50-200 µm to the pelagic microscale: this corresponds to the region where laminar flow prevails and encompasses most of the organisms of the microbial web.

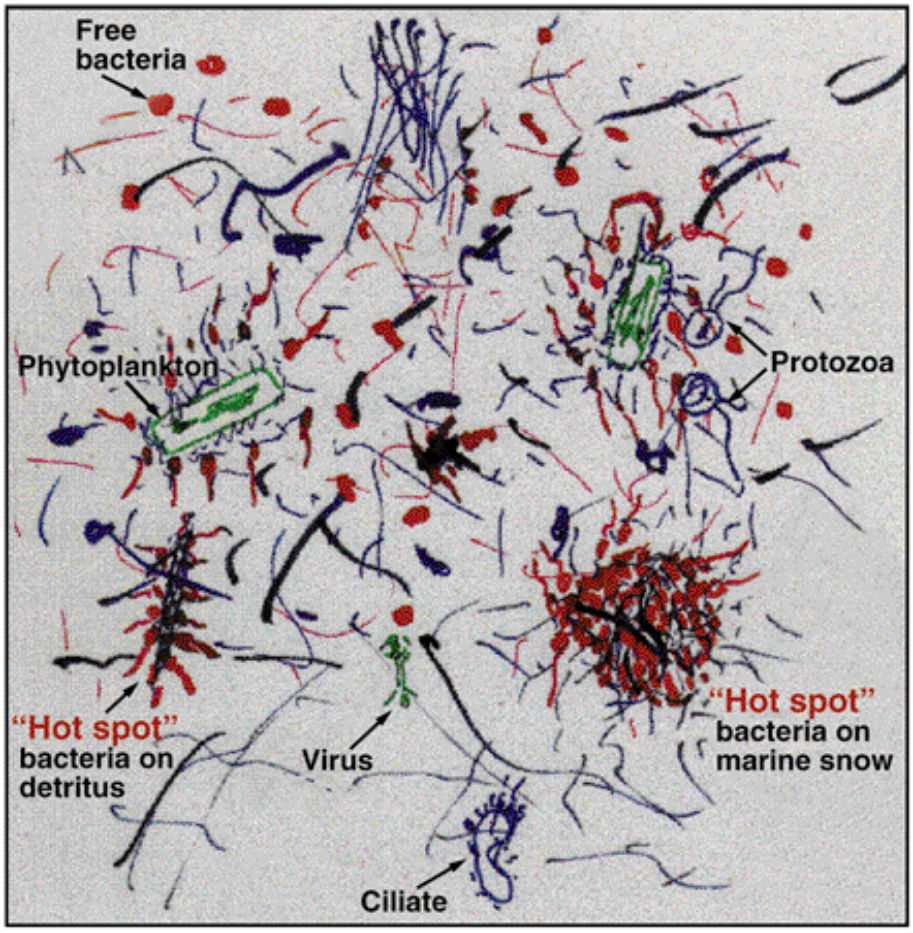

Fig. 2. The microbial loop: impressionist version. A bacteria-eye view of the ocean's euphotic layer. Seawater is an organic matter continuum, a gel of tangled polymers with embedded strings, sheets, and bundles of fibrils and particles, including living organisms, as "hotspots".

Specific goal/recommendations for research include:

a) bacterial diversity at the micrometer scale and the significance of microscale patchiness in the maintenance of diversity;

b) microscale interactions and behaviors of bacteria, protozoa and viruses within and towards the organic matter continuum;

c) microbial communication in the pelagic context (*e.g.* "Quorum Sensing", symbioses, consortial activities) and their significance for microbial ecology and biogeochemistry;

d) variation in bacterial metabolic states and growth efficiencies at the micrometer scale, to address whether the range of bacterial physiologies (from inactive to highly active) can be understood in terms of the characteristics of their microenvironments;

e) control of nutrient cycling by microscale microbial processes, to address how the microscale patchiness in the intensity of microbial activities (including the phytoplankton) may change the rates and even the direction of nutrient fluxes (*e.g.* whether bacteria act as net remineralizers or N, P, Fe or competitors for inorganic nutrients with phytoplankton (Tupas and Koike, 1991; Azam, 1998);

f) individual-based ecology of pelagic microbes, to mechanistically connect genotype, environmental phenotype and biogeochemical activities;

g) modelling of microbial communities and interactions. Individual-based modelling to constrain hypotheses on microscale community structure and activity;

h) methodology. Since the microscale ecology is essentially unstudied, new methods and tools will be required. A good example of the existing technologies is the microelectrodes (Alldredge and Cohen, 1987; Ploug *et al.*, 1999). Laser confocal microscopy holds promise since it can potentially provide 3-D images of distribution, phylogenetic identity and metabolic activities of microbes. Environmental Scanning Electron Microscopy and Atomic Force Microscopy can yield novel insights into the structure of the pelagic microenvironment (*e.g.* Chin *et al.*, 1998). Techniques which multiply interrogate individual bacteria with respect to phylogeny and physiology (*e.g.* STARFISH; Ouverney and Fuhrman, 1999; Lee *et al.*, 1999) offer much potential.

4. LINKING MICROBIAL DIVERSITY AND ECOSYSTEM FUNCTION

4.1. Why is the link needed ?

Since about 1980 the role of bacteria in primary processes in the marine environment has been broadly defined as the microbial loop, a conceptual model used thereafter as a basis for the incorporation of field measurements into quantitative models describing ecosystem function in marine waters. Our current level of understanding is limited to the roles of all encompassing groups of organisms such as bacteria, viruses, phytoplankton and protozoa. We understand the roles of individual types within these broad categories poorly. For example we do not know what types of bacteria are responsible for the conversion of exopolysaccharide to individual sugars, nor whether any bacteria specialise in sugar uptake in the sea.

Although protozoa and eukaryotic phytoplankton show enough morphological distinction for species to be identified and enumerated separately, this is not true for bacteria and viruses. Bacteria are major players in the processing of DOC in marine waters and many do not grow easily on general nutrient media, so culture-dependent methods of identification are of little use. Modern molecular techniques have made the identification of bacteria easier and realistic enumeration of different types possible. Furthermore, viruses have population densities about 10x greater than those of bacteria and are thought to have a major influence on bacterial populations in addition to grazing protozoa. However, we have almost no knowledge of marine virus diversity or how different viruses control specific groups of bacterial hosts. Clearly a major challenge in marine science is to advance understanding of how individual microbial types fit into the overall process models currently in use.

4.2. Spatial and temporal occurrence of marine microorganisms

It is now possible to address the composition of marine microbial assemblages by molecular techniques (Amann *et al.*, 1995). These studies have revealed the presence of new bacterial, archaeal and eukaryal lineages in the marine microbial plankton. The different techniques applied have

different potential and limitations. Environmental clone libraries have been fundamentally important for establishing exhaustive lists of microbial species present in the sea (Giovannoni *et al.*, 1990). However this approach is time consuming and is biased, in ways not yet fully understood, by the PCR amplification and cloning steps (Suzuki and Giovannoni, 1996). By applying fingerprinting techniques, such as DGGE (Muyzer *et al.*, 1997), T-RFLP (Liu *et al.*, 1997) or ARDRA (Acinas *et al.*, 1997), multiple samples can be compared simultaneously, and valuable information about temporal and spatial scales of variation of microbial assemblages have been obtained. These fingerprinting techniques are fast but have limited phylogenetic capacity ; they are also affected by PCR and cloning biases. Another approach has been the use of probes to hybridize with single cells (DeLong *et al.*, 1989) or extracts of community nucleic acids (Zheng *et al.*, 1996), which give direct estimates of the presence and abundance of particular phylotypes. These techniques are limited by sensitivity or by the range of probes that are available.

Once the limitations of the available techniques are recognized and taken into account, trends in the temporal and spatial distribution of microbial assemblages will clearly emerge. Molecular techniques have revealed that a significant gradient affecting bacterial and archaal composition is depth in the water column (Lee and Fuhrman, 1991; Acinas *et al.*, 1997). This finding is consistent with the phytoplankton distribution and the extreme differences in specific bacterial growth with depth. For example, several groups of Archaea are differently distributed in the photic and aphotic zones (Massana *et al.*, 1997). Also, different phylotypes in the SAR11 cluster, a widely distributed uncultivated group, appear at different depths (Field *et al.*, 1997). More examples exist of vertical stratification of other bacterial phylotypes (Gordon and Giovannoni, 1996, Giovannoni *et al.* 1996). Interestingly, the small spatial scales over which differences occur in the vertical axis do not generally apply horizontally. Thus, similar phylotypes appear in different oceans (Mullins *et al.*, 1995) and the same bacterial assemblages occur over large distances in the oceans (Acinas *et al.*, 1997; Murray *et al.*, 1998; Riemann *et al.*, 1999). At the same time, hydrographic features, such as the transition from coast to open sea, frontal systems, or latitudinal gradients also affect the composition of bacterial assemblages. Temporal differences have been investigated less intensively, although there is evidence for changes of bacterial assemblages over time, potentially following seasonal succession by other members of the community, such as the phytoplankton. Overall, there is still much to learn about the distribution of bacterial assemblages in marine systems.

An obvious goal to achieve a comprehensive understanding of microbial functioning in marine systems is to document quantitatively the dominant species and to establish how and why these species vary in time and space. In general, the methodologies used are insufficient for this purpose. The current assumption is that a few species dominate at a given time and place. Determination of the relative abundance of these species and of their relative participation in key microbial processes is very important. Knowledge of less abundant species is also relevant, since they can be involved in minor (in terms of carbon cycling), but nevertheless key, processes that are fundamental for ecosystem function, and because they represent a seed for future assemblages. There is a need for future technical improvements. Promising current advances in FISH, capillary electrophoresis and flow cytometry will undoubtedly lead to greatly improved understanding of the contribution of bacteria to marine processes (Cottrell and Kirchman, 2000).

4.3. Roles of marine microorganisms

Our understanding of the integrated functioning of microorganisms in nature is often methods-limited as researchers are still attempting to develop and evaluate the advantages and limitations of basic methods. Investigating the functional role of microorganisms in the field involves understanding (i) the physiology and ecology of these organisms and (ii) the factors regulating their abundance, such a grazing, viral infection, competition for nutrients and symbioses.

4.3.1. Population functions

The ability to bypass cultivation offers the advantage of a more complete description of bacterial assemblages but does not make the cultivation of microorganisms any less essential to microbial ecologists. There is not yet enough information available to confidently deduce the physiological characteristics of a cell from which a novel 16S rRNA sequence has been derived.

Neither can we yet predict the pattern of expression of an enzyme represented by a gene recovered from a novel organism. The establishment of cultures of the organism from which novel sequences are derived can provide basic biochemical, genomic, and physiological information that will greatly help endeavours to connect them with their role in the natural environment. The identification and sequencing of genes encoding for specific enzymes involved in key processes need to be developed. For instance, many studies have addressed the importance of polysacharides in the pool of organic matter in the Mediterranean as well as the enzymatic processes involved in their transformation. Yet studies addressing these important processes at the molecular level have never been undertaken, mainly because specific genes are not yet identified for study in an ecological context.

These investigations at the population level would also address life strategies by studying growth strategies (r and K strategies, motility, …), defense mechanisms against protozoa (communication, morphological adaptations, capsule release, …), horizontal gene transfer (conjugation, transduction, transformation) and interactions such as competition for nutrients between bacterial populations or between bacteria and phytoplankton species. However, studying these interactions requires that appropriate tools are available for studying the dynamics of specific populations within complex assemblages. Although the use of fluorescently labeled rRNA-directed oligonucleotides targeted at single cells to identify and enumerate bacteria or picophytoplanktonic cells has become a well established tool in microbial ecology, this technique remains limited when applied to oligotrophic waters because microorganisms contain too few ribosomes to permit sufficient probe binding (Lebaron *et al*., 1997). To date, only modest increases in fluorescent signal to background ratio have been achieved and new signal amplification strategies, such as polynucleotide labelling, are still under development. An additional limitation to the use of these techniques is the detection sensitivity of the instruments which do not allow the detection of rare groups within populations at natural densities. The recent development of new instruments such as solid-phase cytometry and capillary electrophoresis will probably help to improve these limits but this equipment remains expensive.

4.3.2. Fitting diversity and process studies

The use of process-specific probes in principle allows the identification of all microorganisms that can perform a particular function. However, low similarity of homologous sequences in different taxa and the existence of multiple enzymatic pathways performing the same function will make it difficult to assure that all potential contributors to a process are detected. One advantage of using gene encoding enzymes with specific functions for molecular ecological work is that they permit questions about physiological ecology to be addressed in addition to questions about the taxonomic community structure. For instance, mRNA extracted from natural communities allows the determination of whether the gene of interest is being expressed and how expression is regulated in the field. Successful investigations have already been reported for specific functions such as the nitrogen cycle. In situ functional investigations at the community level will probably be developed in the future with the advance of genomic studies.

Relationships between diversity and function can also be addressed at the cellular level by combining taxonomic and physiological probes. A variety of techniques have been used to quantify metabolically active bacteria in natural populations. In situ assays for cells containing nucleoid DNA and ribosomes, autoradiographic detection of cells incorporating radioactive substrates, redox dye detection of charged cell membranes, and cell enlargement assays for communities treated with DNA replication and division inhibitors have all been applied to natural microbial communities. The number of cells identified by these techniques varies widely. However, none of these methods alone is able to determine whether the most metabolically active cells comprise a plylogenetic subset of the microbial community.

The combination of methods to assess structure-function analyses in microbial ecology seems very promising but requires further development and application. In situ hybridization and microautoradiography can be combined to obtain cultivation-independent insights into the structure and function of bacterial communities (Lee *et al.*, 1999; Cottrell and Kirchman, 2000). Similarly, flow cytometry is a powerful tool for analysing large numbers of cells after labelling

with a fluorescent probe. When physiological probes are used, active cells can be detected, enumerated and sorted for further analyses with molecular techniques (Bernard *et al.*, 2000).

4.4. Culturing model marine microorganisms

Culturing microorganisms is essential for further in-depth physiological characterization and potential exploitation of microorganisms for a variety of biotechnological applications. Currently, only a rather limited number of microorganisms are in culture. Few bacteria that are known to be dominant in marine waters have cultured representatives. Most of the approaches used hitherto have not taken into account the variability of the substrate requirements these organisms use ; they might also poorly compete with others during enrichment or in plate culture. Thus new approaches should be developed to increase the number of microorganisms isolated and culturable.

Generally, isolation steps might be guided by specifically designed probes and PCR primers for isolation and enrichment. New or recently introduced approaches complementing traditional methods for isolating and culturing bacteria include the following :

- Use of antibiotics to inhibit growth of specific groups of species (Oremland and Capone, 1988).
- Use of growth stimulating substances.
- Dilution to extinction method (Button *et al.*, 1993).
- Fractionation approaches (such as size fraction by filtration, or based on surface charge (electrophoretic motility).
- Use of surfactant agents and ultrasonication to remove attached bacteria from particles for further isolation steps (Velji and Albright, 1986).

Specific growth conditions for culturing should include the following :

- Use of different nutrient concentrations and composition reflecting more closely the natural microenvironment of the bacterial species under study (Schut *et al.*, 1993).
- Variations of physical parameters (temperature, pressure, solar radiation).

Cultured microorganisms are a pre-requisite for studying the following in detail :

- Whole genome sequencing of environmentally important bacteria.
- Identification of genes involved in major transformation processes of organic matter.
- Further potential use for biotechnological applications of specific species or genes.
- To establish virus-host systems for transduction studies and development of probes for the identification of dominant marine bacteriophages.
- To isolate plasmids that are important in lateral gene transfer by conjugation.
- To investigate potential intra- and interspecific (synergistic and/or antagonistic) interactions.
- To establish microcosms with model microorganisms or communities to study intra- and interspecific responses to specific physical, chemical and biological conditions (degradation of specific components of DOM, virus-host interaction mechanisms, DNA repair, gene transfer, lytic vs lysogenic cycle of viruses (Weinbauer and Suttle, 1999).

5. CONCLUSION

The funding of scientific research is not exactly altruistic. In return society expects, amongst a number of things, the scientific community to provide predictions of how commercially and socially important features of the ecosystem will respond, for example, to climate change and massive anthropogenic pressure. How will this affect the grazing chain leading up to commercial fish change ? As we now realise that marine ecosystems are intimately dependent on the processes within the microbial web (bacteria, protozoa, viruses, fungi and other microorganisms) it is evident that we cannot understand how the components of the classical grazing chain (algae, zooplankton, fish) will vary by the study of its elements alone. We need to know how the microbial system, which competes with the macro-grazers for the products of primary production, will change. To date, these concepts have scarcely impacted on the design and conduct of microbiological research in Mediterranean waters and elsewhere.

We now have compelling evidence that the marine environment is not just a homogenous mixture of organisms and chemicals. Moreover, it is already abundantly clear that microorganism function is a major determinant of marine ecosystems. Our present comprehension of the details of marine life at the microscale is still rudimentary. Nevertheless, new approaches, instrumentation and techniques now available are enabling us to begin to fill in some of the gaps. New understanding holds promise not only of better ecosystem models but also of improved capability to make operationally meaningful predictions.

New approaches to integrating the microbial loop in the functioning of the Mediterranean ecosystem

Farooq Azam

Marine Biology Research Division, Scripps Institution of Oceanography, University of California, San Diego, USA

It is well recognized that microbial loop significantly influences the structure and functioning of the Mediterranean ecosystems. A large but variable fraction of the photosynthetically fixed carbon flows through the dissolved organic matter (DOM) into the heterotrophic bacteria. The variability in DOM ➠ bacteria flux has fundamental implications for the overall carbon flux patterns. It regulates carbon and energy flows to support tertiary production and thus the fisheries yield; it controls the pattern of depth-dissipation of organic matter and hence sedimentation, mesopelagic and bathypelagic energetics and ecosystem structure; and it affects the ocean-atmosphere carbon dioxide exchange. While we recognize the significance of the microbial loop in the ocean's ecology and biogeochemistry, we currently lack the ability to predict how the microbial loop influences ecosystem behavior. This situation critically constrains our ability to make objective decisions on environmental and economic issues such as ocean pollution, fisheries management and conservation of marine biodiversity.

While microbial oceanography has made remarkable progress during the last two decades our ability to predict ecosystem behavior is still very limited. Biological complexity and diversity have been implicitly considered too complex to be tractable, more so because the playing out of the biological activities and interactions must be understood in an environmental context. The organism'environment is an integral part of the inquiry. This requirement poses a particularly difficult challenge when considering the microbial loop organisms, since they live in as-yet-unexplored micrometer-scale "worlds". The common approach of studying the microbial loop processes in the relatively manageable average or bulk seawater environment leads to demonstrably limiting and often incorrect conclusions (Azam, 1998). Studies show how the microscale interactions of bacteria with diatom detritus influence the oceanic silicon and carbon cycles (Smith *et al.*, 1992; Blackburn *et al.*, 1998; Bidle and Azam, 1999). However, we do not yet know how to explore the relevant characteristics of the microbes' environments.*et al.*Microscale ecology is still in its infancy, a "boutique" discipline, perhaps reflecting the general sense that incorporating microscale interactions in ecosystem models is not yet tractable.

In my talk I will develop the argument that recent technological and conceptual breakthroughs, particularly in genomics, proteomics and bioinformatics, give cause for optimism. Genomics is a fundamentally new and powerful approach with much potential for understanding oceanic processes; indeed it may revolutionize oceanography. I do not propose that complete genome sequencing of marine microbes will, by itself, yield mechanistic knowledge for inte-

grating microbial loop processes into predictive ecosystem models. A combination of technologies to address biological complexity as well as environmental structure is needed. For instance, the development of sensors of microscale environmental characteristics and processes (including those based perhaps on genomic interrogation of the microenvironment) will be equally valuable. Important advances in measuring microscale and single cell activities have recently been made (*e.g.* Ouverny and Fuhrman, 1999; Mitchell *et al.*, 1996). Such combined new approaches will reveal the biotic diversity, consortial compositions and microenvironmental context for conceptualizing and integrating microbial loop processes into predictive models.

The long-term goal is a synthesis of genomics with ecosystem dynamics and biogeochemistry, leading to a fundamentally new understanding of how biological forces influence the ecosystem dynamics and response to perturbations. This unifying approach could lead to the development of a new type of predictive models of system behavior applicable to a broad range of marine environmental issues such as fisheries, toxic algal blooms, emerging diseases and pollution.

Diverse adaptation strategies but a sole pathway for hydrocarbon degradation

Franco Baldi

Department of Environmental Sciences, Cà Foscari University, Venezia, Italy

Marine microorganisms are considered to be the best agents for degradation of hydrocarbons originating from petroleum spills in oceans. Bacteria oxydise alkanes as energy and carbon sources. The role of bacterial species in alkanes degrading process has been extensively studied in *Pseudomonas oleovorans* and *P. putida.* In *P. oleovorans* the alk genes, responsible for *n*-alkane degradation, are localised on the large (over 300 Kbp) OCT-plasmid (Witholt *et al.*, 1990). The *alk* regulon is encoded by at least two distinctive regions separated by at least 50 Kbp: the 7.3 Kb *alk*BFGHJKL operon that encodes the enzymatic activities required for to the oxidation of alkanes and to the dehydrogenation of aliphatic alcohol, and the 4.9 Kb *alk*R region encoding two peptides, one of which (AlkS) is the positive regulator required for the expression of the structural genes of the *alk* system, whereas the second one (AlkT) has an enzymatic function. Moreover other genes involved in the n-alkane degradation, *alc*A and *ald*A, encoding for a soluble alcohol dehydrogenase and aldehyde dehydrogenase, respectively, are located on the chromosome. There are of course differences in base sequences of genes, of promotor region, regulation mechanism of operons belonging to different species and genera but alkanes are always degraded following this unique pathway : alkanes ➠ alchools ➠ aldheides ➠ fatty acids.

Degradation of hydrocarbons is not only a catalytic event because cells need to overcome a few problems in order to oxydize alkanes as carbon and energy sources: 1) to adhere on the oily surface; 2) to avoid phospholipid membrane dissolution due to alkane interactions; 3) to make alkanes available at nanodroplet size to enter membranes without causing physiological damage. Adaptation mechanisms are very different indeed. They are revealed by simple physiological tests such as oxygen consumption rates (Baldi *et al.*, 1999), and/or nutritional preferences in n-alkanes with low, medium and high number of carbon atoms (Fig. 1).

Such diversity is often the resultant of a cell adaptation. Some species produce emulsifying substances to make available *n*-alkanes by reducing significantly the superficial activity and forming micelles in aqueous solution. Bacterial species produce different types of emulsifying agents such the lipo-polysaccharides emulsan (Gutnick *et al.*, 1991). Other species produce other types of emulsyfing compounds and even particulate surfactants (Desai and Banat, 1997). Modifications on membrane fatty acids often occur by cis/trans modifications or fatty acids saturation. Capsular polysaccharides, glyco-proteins and pili can also be involved to render hydrocarbons more available due to cell-cell and cell-substrate adhesion.

Recently we found for the first time that a new “glucose-decorated” glyco-protein is induced on the outer membrane of *Acinetobacter venetianus* VE-C3 strain in the presence of fuel oil.

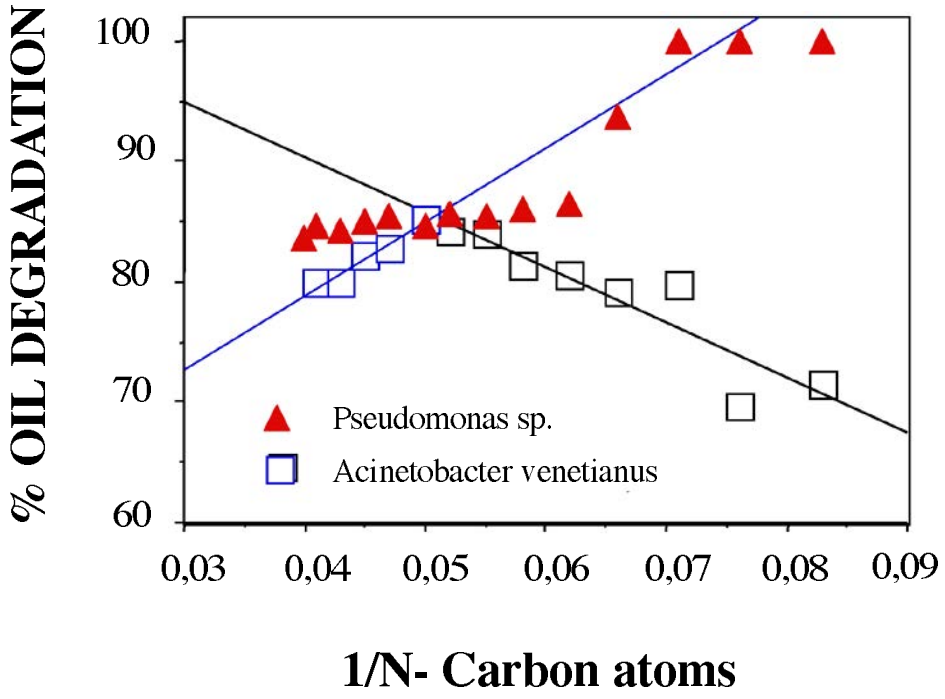

Figure 1. Percentage of oil (n-alkanes) degradation by two different species: Pseudomonas sp. (▲) and *A. venetianus* (❑) in relation to the reciprocal of carbon number atoms of n-alkanes.

Together with a constitutive "galactose-decorated" glyco-protein both formed a strong fibril network to protect themselves from oil rapid accumulation and consequent cell damage. So it is important for surviving cells to reduce the superficial tension of hydrocarbons and to break down the solvent to nano-droplet level around bacterial walls. In addition modifications of cell shape in *A. venetianus* VE-C3 from coccoid to different polyhedral shapes, often followed by cell lysis, occur as a result of rapid oil penetration.

So hydrocarbon degradation is not only due to the presence of catalytic proteins such as monooxygeneses and dehydrogeneses, but due to a more complex system (hydrocarbosome), which takes care of different factors to make hydrocarbons consumable and attenuate cell damage.

Metabolically active and non-active bacteria in aquatic environments

Tom Berman, Bina Kaplan, Sara Chava and Yehudit Weiner

Israel Oceanographic and Limnological Research,
Yigal Allon Kinneret Limnological Laboratory, Tiberias, Israel

With the introduction of fluorogenic stains (acridine orange and 4,6, diamidino 2 phenylindole, DAPI) combined with epifluorescent microscopy (Hobbie *et al.*, 1977; Porter and Feig, 1980), the enumeration of total bacterial cells in aquatic environments became routine. However, it is of considerable interest to know what proportion of the total bacterial population is metabolically active at any given period.

We have used three staining methods to estimate "active" bacteria in the waters of Lake Kinneret: CTC (5-cyano-2,3 ditolyl tetrazolium chloride, Rodriquez *et al.*, 1992), DAPI followed by propanol wash, Zweifel and Hagstrom (1995), and the Syto 9 Live/Dead stain developed by Molecular Probes, Eugene OR, U.S.A. Positive results from these methods purport to show actively respiring bacteria (CTC+), cells with intact nucleoids (NuCC+) and cells with intact membranes (MEM+), respectively. Concomitantly we also measured metabolic parameters (respiration, as O_2 uptake or electron transport system flux, ETS; leucine uptake and peptidase activity) in 3µm filtered lake water. Samples were taken over a period of two years from three depths at a central lake station.

Similarly to previous reports from both marine and freshwater environments (*e.g.* Choi *et al.*, 1999; Sherr *et al.*, 1999a), all three methods indicated relatively low percentages of "active" bacteria in the lake water. CTC+ ranged from 1.0 to 27.3% (average 5.1%), NuCC from 1.4 to 42.9% (avg. 8.3%) and MEM+ from 1.0 to 21.9 % (avg. 8.7%). No clear seasonal or spatial patterns were evident. Moreover, in samples taken directly from the lake, no significant correlations were found between the three methods used to determine actively metabolizing bacteria. By contrast, significant correlations were observed between the percentages of CTC+ and NuCC, CTC+ and MEM+, and NuCC and MEM+ bacteria in laboratory experiments that entailed the incubation of 3 µm filtered lake water.

Significant correlations were also found between metabolic parameters (peptidase and leucine uptake, ETS and O_2 uptake, ETS and peptidase, but not ETS and leucine uptake). From samples taken directly from the lake a significant correlation was noted between ETS and numbers of "active" (CTC+), but not of total (DAPI counts), bacteria.

A series of experiments was run with 3 µm filtered lake water to examine the response of indigenous bacterial populations to nutrient enrichment. Similarly to other reports (Sherr *et al.*, 1999a) no response was observed before 10-12 h incubation either in terms of increase in total

cell counts or the proportion of active bacteria. When outgrowth occurred, much higher percentages of apparently active bacteria within the total DAPI stained population were observed.

Our data confirm the hypothesis that, usually, only a small proportion (probably <<20%) of the total bacterial population in natural waters is active at any given time. This proportion may increase dramatically when there are localised inputs of nutrients, either on microscales (*e.g.* on or near organic particles) or on larger spatial scales (*e.g.* with upwelling events or with the crash of algal blooms). Each of the three staining methods used to determine "active" bacteria detects different aspects of cellular state and none are really satisfactory for determining what proportion of cells stained by DAPI in natural aquatic environments are totally dead, moribund, dormant or fully active.

The results of this study support the suggestion by Sherr *et al.,* (1999b), that CTC staining based on a protocol of short (2-3 h) incubations with 5 mM CTC detects only the more active of the "metabolically active" cells (in this case, active in terms of their electron transfer system). There are reservations about this approach (Ullrich *et al.,* 1999), nevertheless, as Sherr *et al.,* 1999b have pointed out the CTC method can be useful to gain some idea of the levels of highly active bacteria within natural populations and to follow changes in the active cell fraction subsequent to environmental perturbation (*e.g.* nutrient inputs, grazing, etc.). Moreover, the possibility of coupling CTC staining with flow cytometric analysis (Sieracki *et al.,* 1999) suggests that this method has useful potential for the detection of actively respiring bacteria in natural waters.

Obviously there are different criteria for characterizing a bacterial cell as "active" as shown by the lack of correlation between the 3 methods used in this study. Thus a kaleidoscope image emerges of various components of natural bacterial populations that are functioning at different levels of activity at any given moment, constantly modulating their physiological activities to the exigencies of the environment. The complications presented by having a more realistic picture of a heterogeneous bacterial population of bacteria not only in terms of genotype but also in terms of metabolic activity, will need to be incorporated into future models of aquatic ecosystems.

ACKNOWLEDGEMENT

This research was supported by Grant n° 95-0002 from the United States-Israel Binational Foundation (BSF), Jerusalem, Israel.

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Picoplankton and nanoplankton coupling in the euphotic zone of the Mediterranean Sea

Urania Christaki [1], Antonia Giannakourou [1], France Van Wambeke [2] and Gerald Gregori [3]

[1] NCMR Aghios Kosmas, 16604 Hellinikon, Greece
[2] Microbiologie Marine, CNRS, UPR 223, Campus de Luminy, Marseille, France
[3] COM, UMR 6535, Campus de Luminy, Marseille, France

A food web dominated by minute producers and consumers is a common feature of oligotrophic ecosystems (Wikner and Hagstrom 1988; Caron *et al.*, 1999; Christaki *et al.*, 1999; Sanders *et al.*, 2000). In the Mediterranean, this scenario is consistent with the one in which organic carbon and nutrients are remineralized and recycled efficiently within a complex microbial food web with little energy transfer to the higher trophic levels (Turley *et al.*, 2000). Therefore, the rates of production and consumption of these organisms are essential to the functioning of the ecosystem. The objective of the present study was to examine the distributions of the autotrophic and heterotrophic prokaryotes and their consumption by nanoflagellates in a longitudinal transect (9 stations, Fig. 1) from the Levantine Basin to the Balearic sea in June 1999 in the framework of the Transmediterranean cruise (TMC – MATER – Mass Transfer and Ecosystem Response).

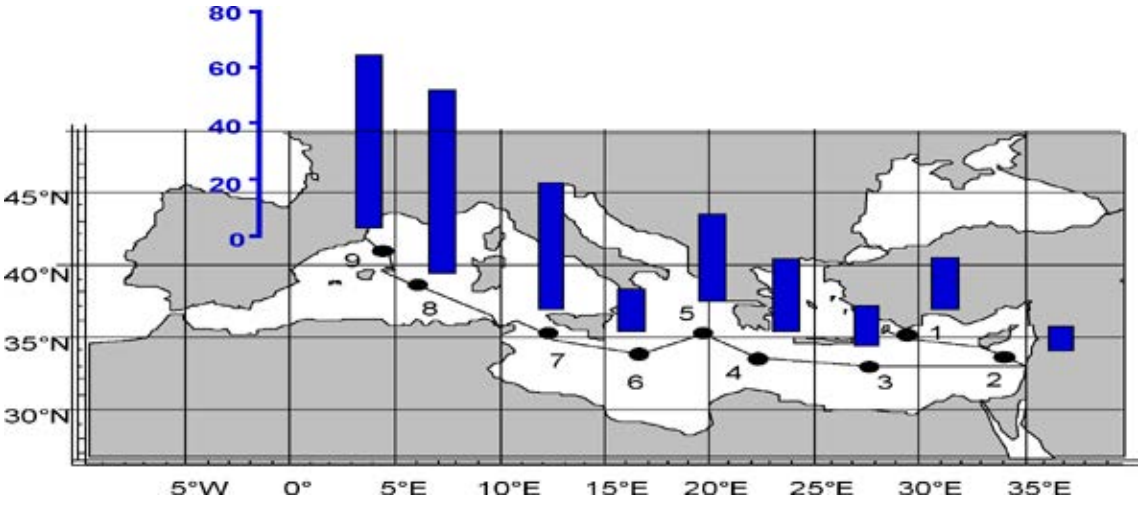

Fig. 1 Sampling stations and depth integrated (0-100 m) bacterial production (mg C m^{-2} d^{-1}).

The vertical and longitudinal concentration of heterotrophic bacteria varied little, being always of the order of 10^5 ml^{-1}. Synechococcus (SYN) and Prochlorococcus (PROC) showed a contrasting distribution. PROC were undetectable in surface waters, and their presence was recorded below 75 m in the zone, where SYN concentration decreased (Fig. 2).

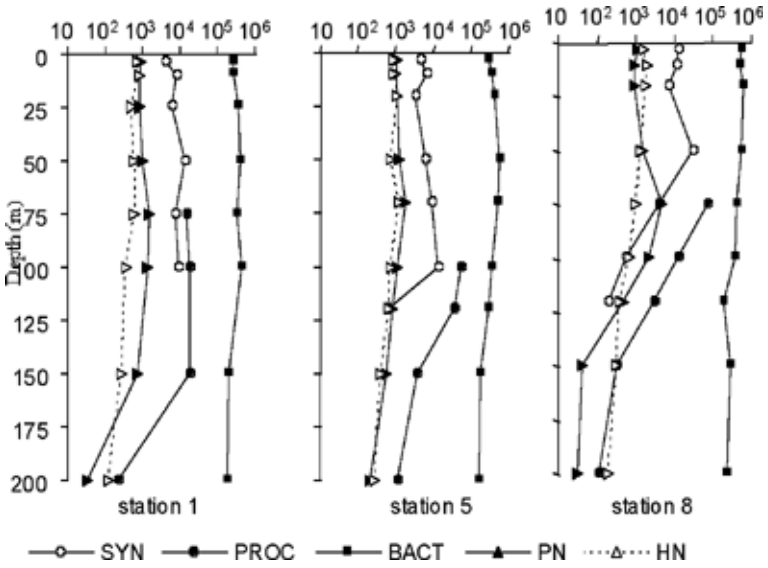

Fig. 2 Three representative vertical profiles of population abundances (No ml^{-1}) during the Transmediterranean cruise (TRC, June-July 1999).

Small protists (<3 µm) dominated the bacterivore assemblage and accounted for more than 90 % of the bacterial consumption. Experimentally estimated heterotrophic nanoflagellate bacterivory accounted for 65 ± 12 % (45 – 87 %) of bacterial production. HN consumption on *Synechococcus* (SYN) from food vacuole content analysis revealed that HN consumed from 0.5 to 45 % (mean 13 %) of SYN stock d^{-1}. However the HN food vacuole content was negatively correlated with the % of SYN in division confirming previously published results (Dolan and Simek, 1999) about the existence of a relationship between predation pressure of HN on SYN and SYN cell size. While HN clearance rate on SYN and BACT were of the same order of magnitude, ingestion rates for BACTwere 2 orders of magnitude greater than ingestion rates on SYN, suggesting that grazing impact was linked to the relative BACT – SYN abundance and that there was no apparent selection against single SYN cells. According to our calculations, by assuming that ingestion on PROC also falls under the rule of "encounter possibility", HN could ingest from 1.2 to 60% (mean 17%) of PROC stock d^{-1}. On average, HN obtained 30 % of their carbon content d^{-1} from heterotrophic bacteria and 6 % from SYN, and 10 % from PROC. However it should be underlined here that at depths where PROC were abundant, HN could obtain up to 100 % of their carbon d^{-1} from PROC. The tight coupling between microbial loop organisms observed in this study, suggests that most of the carbon fixed by procaryotes is likely to be mineralized within the loop and further supports the scenario of little energy transfer to higher trophic levels in the Mediterranean Sea.

Role of sulfate reducing bacteria in the oxidation of organic matter in the marine environment

Yehuda Cohen

Microbial and Molecular Ecology, Institute of Life Sciences, Hebrew University of Jerusalem, Israel

Being sulfate-rich, the marine environment has a high potential for mineralization of organic matter via sulfate reduction. Sulfate concentration in the sea is two orders of magnitude higher than that of dissolved oxygen and more than three orders of magnitude higher than nitrate. Traditionally though sulfate-reducing bacteria are considered strictly anaerobes, oxygen-sensitive and only few strains are facultative denitrifiers. Sulfate reducing bacteria were therefore traditionally been considered to play a role in mineralization of organic matter only in deep reduced marine sediments. Sulfate reducing bacteria were shown to occur in a variety of well oxygenated environments such as wastewater biofilms (Okabe *et al.,* 1999), and aerated activated sludge (Schramm *et al.,* 1999) and roots of sea grass (Kuesel *et al.,* 1999).

Analysis of sulfate reducing bacteria in marine cyanobacterial mats by 16S rRNA dot-blot hybridization (Minz *et al.,* 1999b) and dsr phylogeny (Minz *et al.,* 1999b) had shown that *Desulfomena* and *Desulfomena* groups of sulfate-reducing bacteria are ecologically important in the periodically highly oxygenated marine sediments. Recently we have shown (Sigalevich *et al.,* 2000a, b, c, d) that a marine sulfate reducing bacterium *Desulfomena oxyclinae* is a facultative aerobic heterotroph. This organism alone has a limited capacity to perform sulfate reduction activity by incomplete oxidation of lactate when exposed to low oxygen concentrations. Under these conditions the organism forms aggregates when grown in axenic chemostat. Yet, when grown in co-culture with the marine facultative aerobic hetrotophic bacterium Marinobacter sp. MB, *D. oxyclinae* grows even when exposed to 20% oxygen without forming aggregates. The ability of this sulfate reducing bacterium to grow at elevated oxygen concentration is probably due to the presence of superoxide dismutase produced by the Marinobacter sp. MB, but lacking in *D. oxyclinae*. The sulfate reducing bacterium can carry out sulfate reduction at low oxygen tension and then switches to aerobic respiration by the use oxygen for incomplete lactate oxidation to acetate. The ability to use oxygen is further demonstrated by the growth of this organism in co-culture even in the absence of sulfate.

These findings may explain the repeated observations of high numbers of sulfate reducing bacteria in aerated surfaces of marine sediments and even in marine snow particles freely floating in well-oxygenated seawater. Facultative sulfate reducing bacteria are probably well spread in the marine environment and play a more important role than presently recognized in the oxidation of organic matter in the marine environment. These organisms are especially important where organic matter is introduced into marine environments by human intervention. Lowering

of oxygen availability in microzones of high input of organic matter allows the performance of sulfate reduction by facultative sulfate reducing bacteria such as *D. oxyclinae*. The mechanism of sulfate reduction under microaerophilic conditions as aerobic respiration by sulfate reducing bacteria are presently studied in order to develop specific probes for these organism to enable the study of their role in pristine marine habitat in comparison to environments polluted by organic matter. This type of sulfate reducing bacteria may play a considerably larger role in the oxidation of organic matter in the marine environment compared to the well known strictly anaerobic sulfate reducing bacteria.

Diversity of bacteria in marine environments

John C. Fry

Cardiff School of Biosciences, Cardiff University, Cardiff, UK

Traditionally the diversity of marine bacteria has been assessed by identification with phenotypic tests and numerical taxonomic approaches from collections of isolates from plating on nutrient media and enrichment. The results of 7 such studies have shown (Table 1) a relatively small number of genera being found most frequently and these common types belonged mostly to the γ-Proteobactia, Cytophaga-Flexibacter-Bacteriodes (CFB) and gram-positive groups.

Table 1. Occurrence of Bacteria in marine samples from 7 traditional taxonomic studies on isolates (from Fry, 1987).

GeneraNumber of occurrences	
Pseudomonas, Vibrio	6
Flavobacterium	5
Aeromonas, Alcaligenes, Corynebacterium, Micrococcus	4
Moraxella, Achromobacter	3
Xanthomonas, Bacillus	2

However, it has been known for many years that plate counts of marine bacteria are much lower than direct total counts and it is accepted that <1% of marine bacteria are unculturable or uncultured. This problem was overcome when bacterial marine diversity was first investigated by 16S rDNA approaches (Giovannoni *et al.,* 1990). This was achieved by extracting community DNA, PCR amplification of 16S rRNAgenes, sequencing and identification of the source of these genes by phlyogenetic analysis (phylotypes). Culture-independent studies of this kind are now common and have found many bacteria that are only known from their 16S rRNAgene sequence and that do not match known cultured bacteria. Several of these phylotypes are common in marine samples and so are important (*e.g.* SAR11, Giovannoni *et al.,* 1990; JAP504, Rochelle *et al.,* 1994). Furthermore, 13 of the 36 currently known divisions in the domain Bacteria are only known from phylotypes (Hugenholtz *et al.,* 1998).

Remarkably little is known of the detailed roles of marine heterotrophic bacteria and this is unlikely to be rectified without pure cultures of the most abundant organisms. However, many marine bacteria that are grown on plates match neither existing cultured bacteria nor phylotypes (Suzuki *et al.,* 1997). It is widely accepted that the phylotypes identified from 16S rDNA molecular diversity studies will represent the most dominant bacteria in the oceans. Phyllotypes are placed in the major phylogenetic groupings by sequencing, probing clones with group specific probes or by direct *in situ* fluorescent hybridisation of marine bacteria concentrated by filtration (Glockner *et al.,* 1999). The major phylogenetic groups of Bacteria most commonly abundant in marine habitats are the CFB, α-Proteobacteria and γ- Proteobacteria (Table 2). These groups con-

Table 2. Occurrence of various phylogenetic groups of Bacteria in some marine habitats*

Habitat	Occurrence of Bacterial group (%)									Reference
	CFB	α-Prot	β-Prot	γ- Prot	δ-Prot	Plact	Verruc	G +ve	Other	
Coastal Pacific[a]	31	21		14		11			23	DeLong *et al.,* 1993
Oregon Coast[a]	0	38	15	40	0	0	0	3	4	Suzuki *et al.,* 1997
Four Seawaters[c]	22	7	6	8		0				Glockner *et al.,* 1999
Mediterranean	2	17	1	76	1	0	0	0	0	Acinas *et al.,* 1999
Coastal Arctic Sediment[b]	5	7		18	46	2	2	6	13	Ravenschlag *et al.,* 1999
Marine Nematode[a]	55			15	30				1	Poltz *et al.,* 1999
NE Atlantic	21	51	6	11	4	7	0	0	0	Davey *et al.,* 2000
Plymouth Coast	66	17	1	7	0	1	1	5	3	Cardiff, unpublished
Mean	**25**	**23**	**6**	**24**	**14**	**3**	**1**	**3**	**6**	

** Identities mainly achieved by sequencing 16S rDNA clones, but sometimes with the help of RFLP profilesa, and by probing clonesb and in one case by fluorescent in situ hybridisation of cellsc.*
CFB = Cytophaga-Flexibacter-Bacteriodes, Prot = Proteobacteria, Planct = Planctomyces, G +ve = gram positive Bacteria, Verruc = Verrucomicrobia.

tain many aerobic or facultative heterotrophs that are relatively easy to culture (*e.g.* >60% of Proteobacteria and >40% of CFBs are culturable; Hugenholtz *et al.*, 1998). Moreover, CFBs and γ-Proteobacteria were well represented in traditional taxonomic studies of marine bacteria (Table 1) However, many frequently occurring phyllotypes remain uncultured.

In Cardiff we have examined a collection of 180 16S rDNA clones from coastal water near Plymouth. This was dominated by CFB clones (Table 2; Plymouth Coast) and surprisingly 70% of these CFB clones were members of the AGG58 cluster (Fig. 1). This phyllotype was originally isolated by DeLong *et al.*, (1993) from near Santa Barbara, California (Table 1; Coastal Pacific), has not been isolated since and has no cultured close relatives. We have designed probes for the AGG58 cluster and are looking for these bacteria amongst colony isolates. These AGG58 specific probes work well as primers and so can be used to screen for these bacteria in colony and clone collections, enrichment cultures and marine samples. We must investigate diversity in more

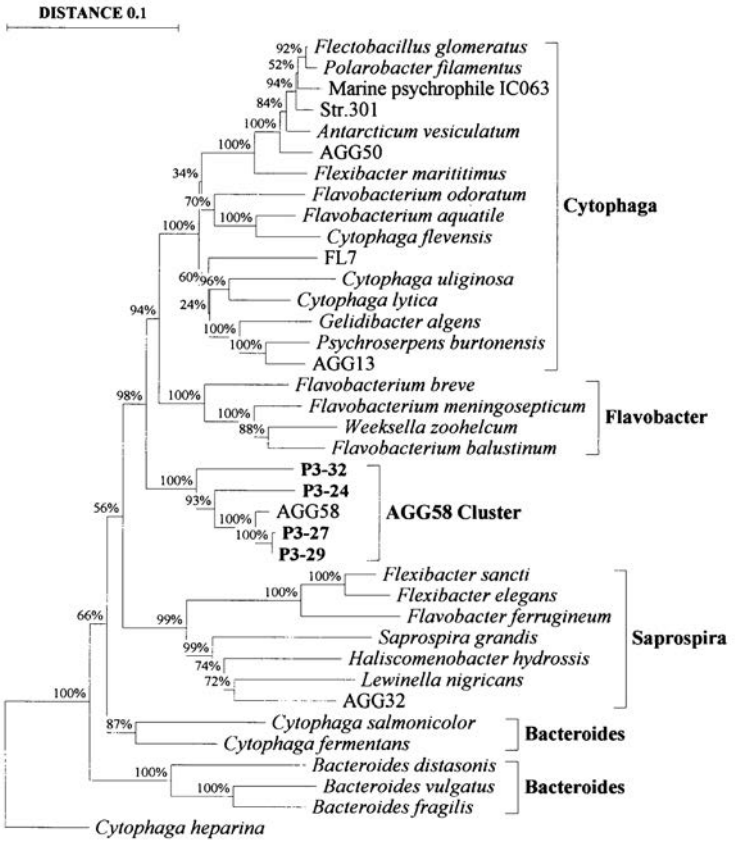

Fig. 1. Phylogenetic tree with 4 AGG58 16S rDNA clones (>1400 bp) from coastal water near Plymouth.

marine habitats by culture-independent methods to understand fully the true bacterial diversity in the sea. We must then try to culture the dominant bacteria to study their physiology and understand their role in the sea. Some α-Proteobacteria also known to be common marine phylotypes in the Roseobacter group have been isolated as colonies on simple nutrient media from coastal water (Gonzalez and Moran, 1997) and their physiology studied (Gonzalez *et al.*, 1999). Another way to study the physiology of dominant marine bacteria without culture is with autoradiography and FISH to determine the phylogenetic groups actively utilising organic compounds abundant in the sea (Cottrell and Kirchman, 2000).

Bacterial motility and its implications for nutrient cycling

Hans-Peter Grossart [1] and Farooq Azam [2]

[1] Institution of Chemistry and Biology of the Marine Environment, Univ. of Oldenburg, Germany
[2] Scripps Institution of Oceanography, University of California, San Diego, USA

Motility is a wide-spread phenomenon among bacterial isolates of different aquatic environments (Malmcrona-Friberg *et al.,* 1990; Mitchell *et al.,* 1996). It could play an important role in the ecology of pelagic bacteria and in the structure of microbial communities. The production and continuous release of dissolved organic matter (DOM) by algae, zooplankton, and from microscopic as well as macroscopic organic particles led to the hypothesis that bacteria cluster around these point sources of nutrients (Bell and Mitchell, 1972; Bidanda and Pomeroy, 1988; Azam and Smith, 1991). Efficient clustering in natural aquatic environments requires chemotactic behaviour, high speed, and considerable percentages of motile bacteria. Recent studies using darkfield microscopy showed that seawater bacteria behave indeed chemotactically and can reach velocities up to 500 µm s^{-1} after nutrient enrichment (Mitchell *et al.,* 1995; Mitchell *et al.,* 1996). Theoretical and conceptual models (Bowen *et al.,* 1993; Blackburn *et al.,* 1997) also suggest that constraints on behaviour and motility result in clustering.

To test whether bacteria “care” about the structure and patchy distribution of the organic matter field we have collected water samples off Scripps Pier (32° 53’N, 117° 15’W). In addition, nine different bacterial isolates off Scripps Pier were grown on ZoBell 2216E medium and washed in dialysis bags (~30-35 Angström, neoLab) filled with artificial seawater prior to use in attraction experiments. The distribution and percentage of motile bacteria was determined by darkfield microscopy which was callibrated with a Petroff-Hansen Chamber.

In contrast to earlier studies, seawater samples from Scripps Pier revealed a heterogeneous distribution of bacteria when counted successively in 200 nl-samples. Furthermore, bacterial numbers in these sub-samples were positively correlated with those of algae. In a 2 week old seawater culture highly enriched with *Cylindrotheca fusiformis* (10^5 cells ml^{-1}) in exponential growth bacterial numbers per algal cell were lower in comparison to non-enriched samples. The observation that bacteria show only short contact with living phytoplankton implies that marine bacteria are repelled by antibiotic production (Sieburth, 1964; Cole, 1982) of the algae.

However, bacterial motility strongly increased when incubating seawater for 20 hrs at in situ temperature. A similar increase in bacterial motility was found after the enrichment of samples with 0.15 µM glucose and peptone (final conc.). The increase in bacterial motility was even more pronounced after the addition of 1.5 µM glucose and peptone indicating that dissolved organic nutrients in seawater lead to increase bacterial motility (Fig. 1). This holds also true for seawater which was incubated for 24 h with *Cylindrotheca fusiformis*. Highest concentrations of bacteria

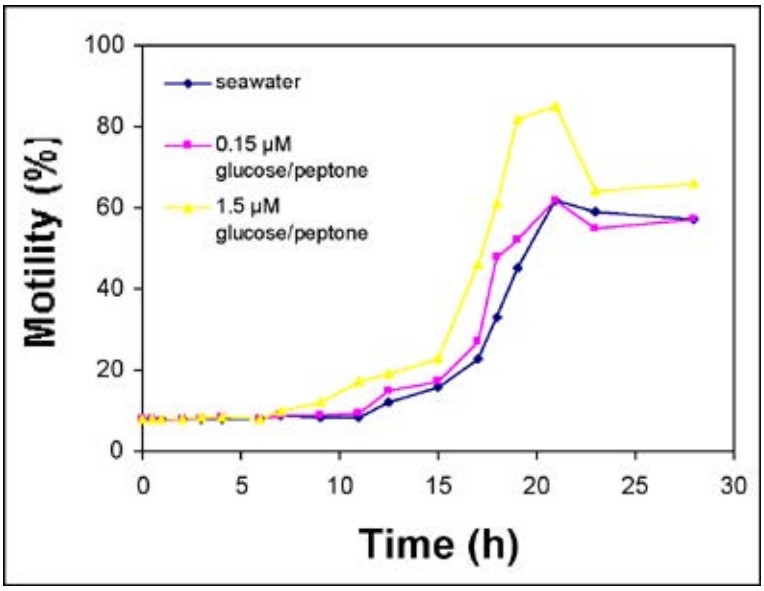

Fig. 1. Increase in bacterial motility (motility %) after addition of glucose/peptone (0.15 and 1.5 µM) to seawater of Scripps Pier on 17 January 1997. The standard deviation of 3 replicates was always <10%.

were always found in microzones around the algae demonstrating that a large fraction of seawater bacteria are motile and express positive chemotaxis towards nutrient point sources, *e.g.* phytoplankton.

All tested bacterial isolates of Scripps Pier showed positive chemotaxis towards capillaries filled with 0.1% peptone. In addition, all bacteria were attracted by capillaries filled with starch (10%), casein (1%), or broth medium (1%). Capillary tubes filled with diatoms in the stationary growth phase (*C. fusiformis* or *T. weissflogii*) led to a more diverse bacterial behaviour. In contrast, capillaries filled with axenic algal detritus achieved high attraction of all isolates tested suggesting rapid colonization and utilization of algal detritus by chemotactic marine bacteria. This is further supported by microscopic studies which show preferential attraction of bacteria by damaged or even dead algae (Grossart, 1999).

Pre-incubation of natural assemblages of marine bacteria with peptone for 12 hrs led to strongly increase bacterial motility. Motile bacteria were attracted by capillaries filled with *C. fusiformis* and *T. weissflogii* but they were not attracted by capillaries without any algae. The chemotactic behaviour of both, specific bacterial isolates and natural assemblages of seawater bacteria after nutrient enrichment, indicates the potential of motile bacteria to react efficiently to the patchy distribution of natural organic matter (Blackburn *et al.*, 1997).

This demonstrates that the heterogeneity of organic matter in natural waters causes “hot spots“ of bacterial abundance and activity (Azam, 1998). Clustering can be seen as the direct response of motile marine bacteria towards the presence of organic particles of various origin which release DOM into the surrounding. For example, protein rich particulate organic matter (POM) undergoing extensive hydrolysis, *e.g.* senescent algae, causes bacteria to form dense aggregations on a µm-scale (Azam *et al.*, 1998). Positive chemotactic behaviour enables bacteria to increase the utilization of nutrients and organic matter, which are enriched in these microzones (Blackburn *et al.*, 1998). The existence of bacterial clusters and their ecological consequences both on microscales and ocean basin scales demonstrate the necessity to incorporate bacterial processes in microzones into our imagination for reliable predictions and understanding of the marine ecosystem.

Marine bacterioplankton diversity and activity

Åke Hagström

Marine Sciences, Kalmar University, Kalmar, Sweden

The paramount importance of microbial life for the cycling of matter in the oceans makes finding the identity and phylogenetic diversity of the indigenous bacteria an obvious research goal. Presently, the phylogenetic tree of marine bacterioplankton is rapidly growing. This is largely a result of extensive cloning of bacterial 16S rRNA genes from the environment, which has revealed a large number of novel taxonomic groups (Acinas *et al.*, 1999; DeLong *et al.*, 1993; Fuhrman *et al.*, 1992; Fuhrman *et al.*, 1993; Giovannoni *et al.*, 1990; Mullins *et al.*, 1995). However, since a large majority of these novel taxa are not yet represented by bacteria in culture, the phenotypic expression of these bacteria remains unknown. Microbiologists early came to the conclusion that a majority of the bacteria in the sea were unculturable since only a small proportion of the bacteria counted by epifluorescence microscopy were able to form colonies on solid media (Jannasch and Jones, 1959; Kogure *et al.*, 1979). The remaining bacteria were believed to be unable to grow in culture using present techniques. This bias against cultured bacteria has been an essential element in the decision to study cloned bacterial DNA instead of isolates of marine bacteria (Amann *et al.*, 1995; Fuhrman and Campbell, 1998; Fuhrman *et al.*, 1992; Giovannoni *et al.*, 1990; Pace, 1996). Although another valid explanation to the discrepancy between total counts of bacteria and colony forming units might be low plating efficiency due to virus infection or nutrient stress (Nyström, 1998; Rehnstam *et al.*, 1993). In line with this reasoning several recent studies using various hybridization protocols have demonstrated that bacteria that are able to grow on solid media occupy a significantly higher fraction of the bacterial community than the number of colonies on the plates would suggest (Faude and Höfle, 1997; Fuhrman *et al.*, 1994; González and Moran, 1997; Moran *et al.*, 1995; Pinhassi *et al.*, 1999; Pinhassi *et al.*, 1997; Rehnstam *et al.*, 1993; Tuomi *et al.*, 1997; Weinbauer and Höfle, 1998a).

In a recent study the diversity of bacteria isolated on solid media from a number of different sea areas was investigated by means of 16S rRNAgene sequencing (Hagström *et al.*, 2000). The phylogenetic analyses revealed a considerable species diversity among the isolated bacteria, with sequence similarity values of the isolates ranging from 82 to 100% to previously reported sequences. Notably, half of the isolates showed a sequence similarity of <97% to previously reported sequences, with a high proportion (18%) having sequence similarities ranging from 82 to 93% These low similarity values indicate novelty at the genus level, and even at the level of new families (Devereux *et al.*, 1990). These observations are in agreement with other recent reports on the prevalence of novel bacteria among bacteria in culture (Bowman *et al.*, 1997; Kalmbach *et al.*, 1997; Pinhassi *et al.*, 1997; Rehnstam *et al.*, 1993; Suzuki *et al.*, 1997). The incidence of poorly characterized taxa was highest for members of the Flexibacter-Cytophaga-Bacteroides phylum, whereas the γ-Proteobacteria generally showed high similarity values to

known bacteria. A number of the isolated bacteria have previously been shown to occupy significant fractions of the bacterioplankton in different environments (Pinhassi *et al.*, 1999; Pinhassi and Hagström, 2000; Pinhassi *et al.*, 1997; Rehnstam *et al.*, 1993). It was therefore interesting to investigate the possible degree of similarity of the isolates to organisms detected through environmental cloning. This search showed that 13% of the isolates showed higher sequence similarities to cloned sequences than to cultured bacteria. Thus, it appears likely that increased efforts to cultivate bacteria from the environment will yield increasing insights into the indigenous bacterial diversity.

Based on a simple model of bacterial growth involving two types of bacteria, one opportunistic and one slow growing, the diel dynamics of marine bacterioplankton in the Bothnian Sea and the Mediterranean Sea will be discussed (Fig. 1). At both locations a marked diel variation was found in bacterial production, with highest values recorded during midday and lower values during night and early morning. The abundance of bacteria scored by the number of nucleoid containing cells (NUCC) also changed during the day, with minimum numbers during midday.

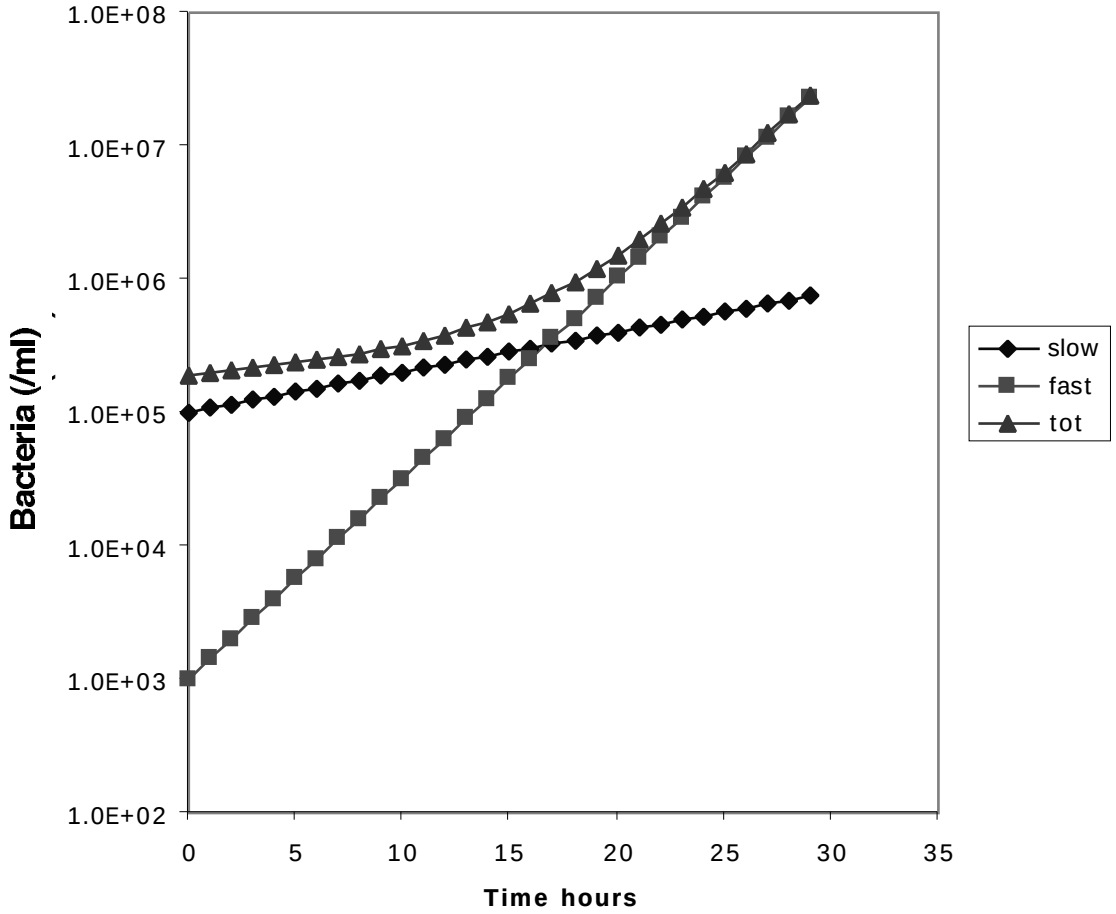

Fig. 1. Community response to opportunistic growth of bacteria.

The turnover rate based on thymidine incorporation and NUCC showed a significant positive correlation with phosphate concentrations both in the Bothnian Sea and in the Mediterranean. At the Mediterranean site nutrient addition experiments showed that phosphate was the least available commodity for the bacterial community, suggesting that changes in turnover were due to shifts in nutrient supply. Combined addition of carbon and phosphate further increased the turnover to values equal to the maximum found during the daily cycle. These results suggest that bacterioplankton have the ability to take advantage of a rapidly changing environment.

Heterotrophic bacterioplankton: phylogenetic diversity *versus* functional uniformity ?

Gerhard J. Herndl, Markus Moeseneder, Jesus M. Arrieta, and Christian Winter

Department of Biological Oceanography, Netherlands Institute for Sea Research, Den Burg, The Netherlands

The Mediterranean Sea exhibits some characteristic features which makes this marginal sea distinctly different from other seas. The most conspicuous features are the relatively warm deep water (Brasseur *et al.,* 1996; Mertens and Schott, 1998; Roether *et al.,* 1996) and the apparent phosphorus-limitation of the primary production (Lipizer *et al.,* 1997; Thingstad *et al.,* 1999; Thingstad and Rassoulzadegan, 1995). The relation between C:N:Pis referred to as Redfield ratio and is for phytoplankton 106:16:1 (molar ratio, Redfield *et al.,* 1963). Bacterioplankton are tightly coupled in their metabolism to phytoplankton activity. Heterotrophic bacterioplankton, however, exhibit a C:N:Pratio which is substantially lower (molar C:N:P=50:10:1; Fagerbakke *et al.,* 1996) than that of phytoplankton implying that bacterioplankton need more phosphorus than phytoplankton. Thus the P-limited conditions in the Mediterranean Sea are likely to influence heterotrophic bacterioplankton even more than phytoplankton. Under these P-limited conditions, dissolved organic carbon (DOC) which is usually rapidly taken up by bacterioplankton might accumulate (Zweifel *et al.,* 1993, 1995; Thingstad *et al.,* 1997). In experimental studies, it has been shown that under P-limited conditions phytoplankton release copious amounts of photosynthetically fixed carbon into the surrounding water where it is not taken up efficiently by bacterioplankton but accumulate (Obernosterer and Herndl, 1995). This accumulation of DOC due to P-limited bacterial growth results in repressed bacterioplankton ectoenzymatic activity (Obernosterer and Herndl, 1995) and might contribute, in its most extreme form, to the formation of mucilage as observed occasionally in the Mediterranean Sea (Herndl, 1992). More commonly the accumulated DOC is exported into deeper layers (Hansell and Carlson, 1998) after coagulation processes building up eventually colloidal and/or particulate organic matter.

The community of the heterotrophic bacterioplankton, mostly comprised of the group Bacteria, is highly structured in the Mediterranean Sea (Moeseneder *et al.,* 2000). The analysis of 132 T-RFLP (terminal-restriction fragment length polymorphism) electropherograms indicated a horizontal differentiation of attached and free-living bacterial communities in the eastern Mediterranean Sea (Moeseneder *et al.,* 2000). Generally, distinct differences in the community structure of attached and free-living Bacteria defined as operational taxonomic units (OTUs) were found in mesopelagic waters (>200 m depth) as compared to the upper mixed water column (≈ 10-200m). Attached and free-living bacterial communities differed considerably throughout the water column with only ≈ 30% of all OTUs being identical. About 50% of attached and free-living OTUs were found throughout the water column (Moeseneder *et al.,* 2000). The number of

station-specific OTUs were lower in free-living Bacteria, however, the number of OTUs unique for a distinct depth layer was significantly higher in the free-living than in attached Bacteria. Fingerprinting analysis using 16S rRNA indicated that only ≈ 14% of attached and ≈ 33% of the free-living OTUs were identical to the 16S rDNA fingerprints (Moeseneder *et al.,* 2000). Our data on the distribution of free-living versus attached Bacteria suggest that even in the absence of temperature as a major selecting factor for Bacteria in the deep water layers, there is a distinct deep-water community present. This free-living bacterial community appears to be as complex as the surface water bacterial community (Moeseneder *et al.*, 2000). Despite this variability in the bacterial community composition, the activity of the heterotrophic bacterioplankton as measured via thymidine and leucine incorporation appears to be rather uniform in the Mediterranean Sea in a given depth layer (Robarts *et al.,* 1996; Zohary and Robarts, 1992). On the other hand, bacterioplankton activity generally decreases with depth, while the number of OTUs remains fairly constant (Moeseneder *et al.,* 2000). Overall, the more uniform pattern in bacterial activity is in contrast to the diversity detected in the phylogenetic composition of the bacterial community.

Another example of phylogenetic differential response in contrast to the uniform response on the community level has been obtained for the sensitivity of bacteria to ultraviolet radiation (UVR). While individual bacterial isolates exhibit an enormous variability in their sensitivity to UVR and in their recover efficiency and mode (dark excision repair versus photoenzymatic repair) (Arrieta *et al.,* 2000), measurements on the community level indicate considerable uniformity (Herndl *et al.,* 1993; Kaiser and Herndl, 1997). This indicates that under changing environmental conditions other, previously less active members of the bacterial community become active and thereby maintaining a relatively constant overall bacterioplankton activity.

Another recently discovered hidden diversity in the bacterioplankton community is the diversity of ectoenzymes in the marine environment (Arrieta and Herndl, 2000). Bacterial ectoenzymes are responsible for cleaving DOC molecules larger than 600 Da to allow transport through the pores in the complex membrane of gram-negative bacteria (Priest 1984), which comprise the major fraction of heterotrophic bacteria in marine environments. We found that different bacterial isolates exhibit large variations in the expression of beta-glucosidase (Arrieta and Herndl, submitted). Fractionating the beta-glucosidase by capillary electrophoresis revealed that there is considerable diversity undectectable with the commonly used bulk measurements. During a spring phytoplankton bloom we detected up to 10 different bacterial-derived beta-glucosidases with Km values varying by less than one order of magnitude (Arrieta and Herndl, 2000). At the same time, the bacterioplankton community composition varied considerably (Arrieta, unpubl. data).

One possible reason for the high diversity of bacterioplankton in the ocean and the surprisingly constant overall activity of the bulk community is grazing control of the dominant, highly active members of the bacterioplankton community as well as viral lysis (Fuhrman and Noble, 1995; Weinbauer and Höfle, 1998b; Wommack *et al.,* 1999a, b). As soon as a specific member of the bacterioplankton community increases in abundance, the contact rate with the host-specific virus and with bacterivorous flagellates increases as well (Fuhrman, 1999). Thus, we hypothesize that viral lysis in concert with species-specific flagellate grazing on bacterioplankton maintains the relatively high bacterioplankton diversity and allows rapid replacement of a specific bacterial species by another species with an essentially identical function in the ecosystem. Thus, despite all the progress made over the last 2 decades in understanding the role of the microbial loop in the carbon and energy flux through marine systems, the complex regulatory mechanisms on the community level are still poorly understood. A combined approach is required using molecular techniques to characterize the phylogeny (richness of the species) and the abundance of individual bacterial species (evenness of the species distribution) in combination with bulk measurements on the bacterial community level. These approaches need to be combined with methods elucidating the magnitude of functional diversity in bacterioplankton communities to resolve the complex regulatory mechanisms in the microbial communities.

Bacterial carbon flux through microbial loop in the middle Adriatic Sea

Nada Krstulovic

Institute of Oceanography and Fisheries, Split, Croatia

Bacterial and heterotrophic nanoflagellates (HNF) abundance, production, growth and grazing rates have been studied in the middle Adriatic Sea since 1992 (Krstulovic, 1992; Krstulovic *et al.*, 1995, 1997; Solic and Krstulovic, 1994, 1995; Solic *et al.*, 1998). The role of substrate supply (expressed as a slope of the linear regression of bacterial abundance on bacterial production), bacterivory and temperature in controlling bacterial abundance were studied with respect to the spatial (trophic) and seasonal scales. The most significant relationship between bacterial abundance and production was established in the oligotrophic area, and during the winter-spring period suggesting that substrate supply could be the most important factor in bacterial control. On the other hand, inconsistent relationship between bacterial abundance and productivity in the eutrophic area and during the summer-autumn period suggests conditions in which substrate supply is above saturating level, or in which mortality factors such as bacterivory and viral lysis are very strong. Variability on the trophic and seasonal scales is shown by two ratios, bacterial production/bacterial biomass (P/B) and bacterial abundance/HNF abundance (B/HNF), which appeared to be good indicators of the control of bacterial dynamics. Variations of P/B ratio were more influenced by the trophic scale which explained 32% of the total variability in comparison to seasonal scale which explained 21%, whilst B/HNF ratio were more influenced by seasonal scale (71% of variability). Furthermore, both the scales interacted, and their interactions explained the significant part (31% and 15% of P/B and B/HNF ratios variability, respectively) of the observed variability in the spatial-temporal continuum. The degree of dependency of bacterial abundance on temperature varied over different trophic and temperature ranges. The control of bacterial abundance by temperature decreased from the oligotrophic to the eutrophic area, thus, control by temperature was negatively correlated with control by substrate supply. This suggests that in eutrophic area substrate set an upper limit for bacterial abundance, whilst other factors like temperature, predation pressure and/or viral lysis determine the level of realized abundance, particularly during summer. Furthermore, temperature was an important factor in controlling bacterial abundance during the nonsummer period (temperature < 20°C) when temperature and substrate supply acted synergistically. In contrast to trophic scale, on the seasonal scale the control of bacterial abundance by substrate supply was positively correlated with their control by temperature.

Evaluation of bacterial carbon flux through microbial loop was elaborated in the coastal area (Kastela Bay) particularly, where the protozoan grazing experiments were run using the size fractioning technique. The average production of bacteria (12.3 µgCd^{-1}) and HNF (4.8 µg C d^{-1}) suggests that these organisms fix a significant amount of organic carbon. Small HNF (<8 µm) were

the most important bacterial grazers, accounting, on average, for 80% of the total grazing on bacteria, whereas abundance and production of HNF were controlled by ciliate grazing. Bacterivorous protozoa stimulated bacterial growth and contributed to an enhanced turnover of bacterial biomass. About 20% of annual bacterial production was chanelled through the microbial loop. However, bacterial carbon flux through the microbial loop showed marked seasonal oscillations with considerably higher values recorded during the warmer part of the year (June to November). Thus, in that period the microbial loop could be an important link between primary production and higher trophic levels.

Diversity and role of microbes in the Mediterranean sea : new challenges in microbial ecology and biotechnologies

Philippe Lebaron

Observatoire Océanologique de Banyuls/mer, Univ. Pierre et Marie Curie, Banyuls/mer, France

Bacteria play a very important role in planktonic marine microbial food webs. They represent a significant part of plankton biomass and their activity has a large impact on ecosystem metabolism and function (Azam, 1998). They play a key role in the cycling of nutrients and, in coastal areas, in the transformation of anthropogenic compounds (Furhman, 1992). Almost all the water-carried wastes of continents enter coastal zones through estuaries. The Mediterranean sea is a semi-enclosed sea, with high levels and multiple sources of pollution. Most of this anthropogenic pressure affects coastal zones and may affect the functioning role of microorganisms communities. It may also result in the transfer of pollutants through the food webs.

Because of their wide diversity of metabolic properties, microorganisms play a vital role in marine ecosystems sustainability. However, the role of bacterial diversity in the functioning of natural communities and sustainability of ecosystems remains unknown. The effect of environmental changes on this diversity and on the relationships between taxonomic, functional and genetic diversity of bacterial communities is not yet clearly understood. For instance, it is well established that phosphorous limitation play a key role in the control of bacterial production in the Mediterranean sea and therefore, in the transformation of organic compounds (Thingstad and Rassoulzadegan, 1995; Fajon *et al.*, 1999). However, little is known on the effect of phosphorous limitation on the functioning role of natural communities.

Our understanding of the integrated functioning of microorganisms in nature is often methods-limited and researchers are still attempting to develop and evaluate the advantages and limitations of basic methods. The advent of molecular and cellular techniques, most notably the rRNA approach and the development of a wide variety of fluorescent probes targeting physiological functions to microbial ecology and evolution had a large impact on our perception of microbial diversity and on the methods to study it (Vives-Rego *et al.*, 2000; Pace, 1997). Microbial ecologists have long been and are still limited by methodological questions but recent developments allow new investigations in both fundamental and applied research (Lebaron *et al.*, 1999; Servais *et al.*, 1999; Urbach *et al.*, 1999).

Methods used to study microbial communities typically either measure net rates of biochemical processes or employ molecular analysis to assess the diversity of community members. Only few techniques link these methodological approaches by identifying community members responsible for biochemical transformations (Urbach *et al.*, 1999; Servais *et al.*, 1999). One widely used technique for assessing community productivity is the measurement of microbial incorporation of radiolabeled thymidine or leucine into newly synthesized DNA or protein,

respectively. Incorporation rates are used to estimate the number of cells produced and therefore to estimate carbon flux through the microbial compartments of complex ecosystems. In contrast, molecular techniques are used to identify the numerically dominant taxa resident in microbial communities. Phylogenetic analyses of 16S rRNA genes cloned from community DNAare commonly used to identify the microbes (Pace, 1997). Neither of these techniques can discriminate the relative contributions of different microbial taxa to community productivity.

A variety of techniques have been used to quantify metabolically active bacteria in natural populations. However, none is able to determine whether the metabolically most-active cells comprise a plylogenetic subset of the microbial community (for a review, see Vives-Rego *et al.*, 2000; Joux and Lebaron, 2000). In the past few years, flow cytometry has become a viable technique for counting natural planktonic bacteria. Flow cytometry allows multiparameter data acquisition and multivariate data analysis, high-speed analysis, and the ability to effect cell sorting (Lebaron *et al.*, 1999; Servais *et al.*, 1999).The availability of relatively simple and portable flow cytometers with lasers emitting in the blue zone of the spectrum, and of DNA stains that could be excited by these lasers simplified the protocols and allowed an avalanche of papers from many researchers using flow cytometry to count bacteria and to analyse the physiological state of individual cells.

Diversity studies have revealed an important diversity of unknown microorganisms including bacteria and picophytoplankton (Courties *et al.*, 1994; Pukall *et al.*, 1999a, b). Nevertheless, because of methodological limitations and of non-appropriate sampling strategies, microbial diversity is underestimated and diversity measurements are rarely related to the functioning and productivity of natural ecosystems. There is an urgent need to isolate these new and unknown organisms, to characterize their physiological, metabolic and ecological properties. This remains essential to define the biotechnological potentialities of these organisms for bioremediation and more generally, to better understand their role in the transformation of organic compounds. Some marine bacterial species are also known to produce extracellular biological activities of great interest for biotechnologies (Holmström and Kjelleberg, 1999).

Population ecology has long been limited by the lack of appropriate methodologies. However, studies of spatial and temporal variations among specific populations are increasing : they will enhance our understanding of the ecology of key species, their role of these species in the transformation of organic matter and how they are affected by environmental change.

In the field of public health, alternative methods to cultural techniques for the rapid and accurate detection and quantification of pathogenic microorganisms are emerging but still suffer from methodological limitations. These methods remain essential to develop biomonitoring systems for the management of marine coastal environments (Baudart *et al.*, 2000; Catala *et al.*, 1999).

Diversity of picoplankton assemblages in Mediterranean waters

Ramon Massana, Beatriz Díez, Michael Schauer and Carlos Pedrós-Alió

Institut de Ciències del Mar, CSIC, Barcelona, Spain

Picoplanktonic organisms are fundamental components of marine systems, being responsible for a significant fraction of total biomass and production (Fogg, 1995). The picoplankton is a heterogeneous assemblage, formed by bacteria (heterotrophs and photoautotrophs), archaea (of unknown metabolisms), and eukaryotes (small heterotrophic and autotrophic flagellates). The species composition of these assemblages is poorly characterized and its study has benefited from the introduction of molecular techniques in microbial ecology (Amann *et al.*, 1995). We are currently applying these techniques to study picoplanktonic diversity in Mediterranean waters.

Genetic libraries of SSU rDNA provided detailed lists of the species present in the plankton. An eukaryal library was performed from a surface sample (fraction 0.2 to 5 µm) in Alborán Sea (Western Mediterranean). Most clones affiliated with the algal groups Prasynophyceae, Dinophyceae, Bacilla-riophyceae and Cryptophyceae, and to a less extent with Eustigmatophyceae, Prymnesiophyceae, and Pelagophyceae (Fig. 1). Many clones (19% of the total) formed new lineages of Stramenopiles, and we postulated that they comprise the bulk of heterotrophic flagellates. These new lineages were also abundant in the North Atlantic Ocean and the Scotia Sea (Antarctica). Overall, the diversity of eukaryal picoplankton was high, with many different phylogenetic groups present. Archaeal libraries were performed from two samples in the Alborán Sea, at surface and at 450 m. Very different archaeal assemblages were found at both depths. As was found in another system (Massana *et al.*, 1997), marine euryarchaeotes dominate archaeal assemblages at surface, and marine crenarchaeotes dominate at depth.

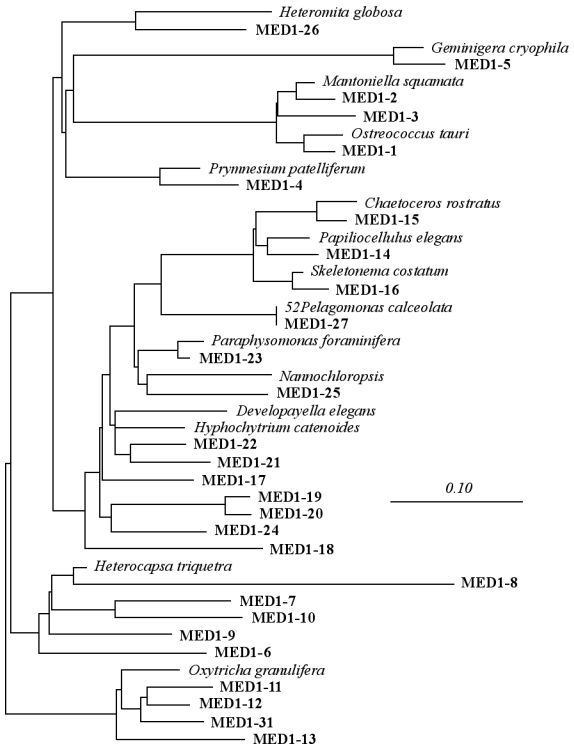

Fig. 1.

Archaeal diversity in both samples was low, and the phylotypes dominating each library appeared to have a cosmopolitan distribution.

We also studied changes of microbial species composition along environmental marine gradients by using a fingerprinting technique, DGGE (Muyzer *et al.*, 1997), which allows a direct comparison among assemblages. We compared bacterial assemblages from three coastal stations along the Catalan coast sampled at different seasons. We included a harbor sample, which always presented a distinct bacterial assemblage, as expected. The main differences among coastal bacterial assemblages were explained by the time of sampling, indicating the existence of bacterial seasonal succession (Fig. 2). For samples taken in the same season, there was evidence of spatial differences. Data on variability of picoeukaryotes by using DGGE will also be presented. The analysis of samples in a cruise in the Alborán Sea, when three stations were sampled four times during two weeks, showed a large temporal and spatial variability of picoeukaryotic assemblages.

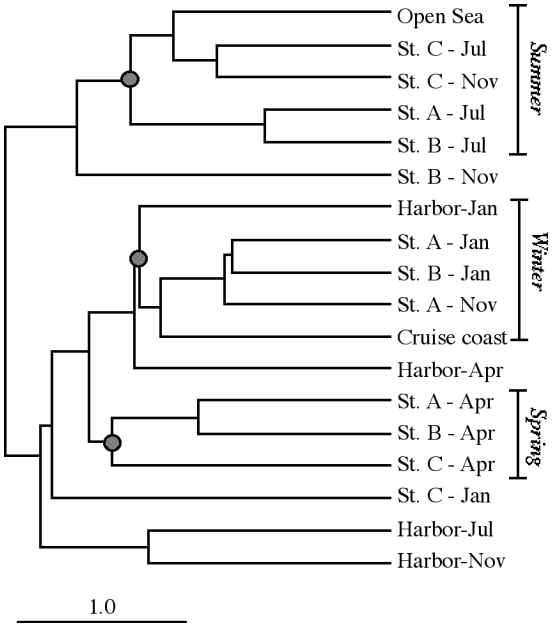

Fig. 2

In conclusion, there is a wealth of information about species composition of the different picoplanktonic groups that can be obtained with molecular tools. We have presented data from two different approaches, one devoted to investigate carefully the species composition, and the other to compare assemblages and study patterns of variation in marine systems. In general, the species composition of Mediterranean microbial assemblages does not seem to differ from that found in other marine systems.

Biodiversity of thermophilic bacilli isolated from shallow marine vents of Eolian Islands, Italy

T.L. Maugeri, D. Caccamo and C. Gugliandolo

Dipartimento di Biologia Animale ed Ecologia Marina, Università di Messina, Italy

Shallow marine hydrothermal vents are frequent off the coasts of the Aeolian Islands (Italiano and Nuccio, 1991). They provide easily accessible sampling locations for studying the relationship between microbial communities and natural fluids in extreme marine ecosystems. These fluids, ranging in temperature from ~ 40 to 95 °C, generally escape the subsurface through fractures in the rocks, but in several areas diffuse exhalations from the sediment can also be observed. Locally, the most active marine hydrothermal venting is observed off the Eastern coast of Panarea, where gases and thermal waters emanate from the sea floor at depths up to 150 m over an area covering ~ 4 km^2. The CO_2 output in this area was determined to be close to 107 litres per day. The submarine hydrothermal systems in the Aeolian Islands, in particular the thermal features in the Baia di Levante of Vulcano, are home to dozens of known chemosynthetic, thermophilic archaeal and bacterial isolates (Stetter, 1982; Fiala and Stetter, 1986; Keller *et al.*, 1995; Hafenbradl *et al.*, 1996). These organisms gain energy either heterotrophically by oxidizing organic compounds with sulfate, sulfite, thiosulfate, sulfur, or nitrate as common electron acceptors, or autotrophically by catalyzing, for example, the reduction of sulfur to sulfide, or the "knallgas" reaction. Microbiological investigations have been carried out at several shallow marine hydrothermal vents in the Baia di Levante (Gugliandolo and Maugeri, 1993). Chemolithotrophic sulfur utilizers were predominant in these samples and distributed in aqueous and sediment habitats according to their ecophysiological properties. Further studies at the same site showed the presence of a wide range of mesophilic heterotrophs that were well adapted to the local environmental conditions (Gugliandolo and Maugeri, 1998).

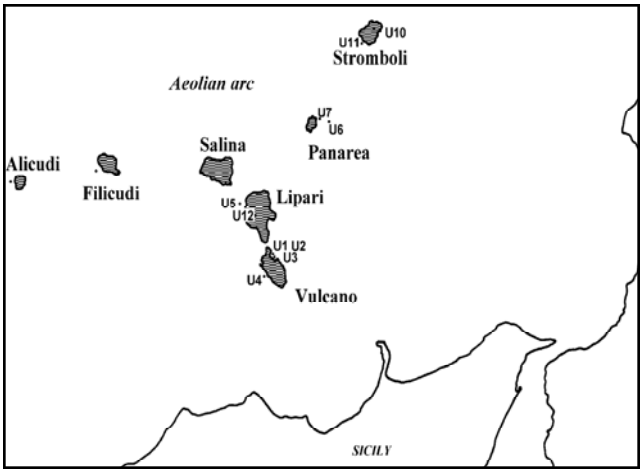

Fig. 1. Sampling site locations in the Eolian Islands.

Recently, in the frame of the MAST program entitled “Microorganisms in deep sea and marine hot springs as sources of potentially valuable chemicals”, we isolated 87 moderately thermophilic bacteria from water and sediment samples collected at different shallow sites of the marine hydrothermal system of Aeolian archipelago (Fig. 1). These isolates, all aerobic

spore-forming, were considered Bacillus–like strains. Our isolates showed high phenetic heterogeneity as regards numerous (136) characteristics. Numerical analysis was carried out for first species discrimination comparing the isolates with eight Bacillus reference strains. Most of isolates clustered at 65% SSM similarity with *B. thermodenitrificans*, in a large group including nine phenetically well distinguished clusters. All remaining isolates, but three linking *B. thermoleovorans*, appeared different from the reference strains (Fig. 2). The phenetic charac-

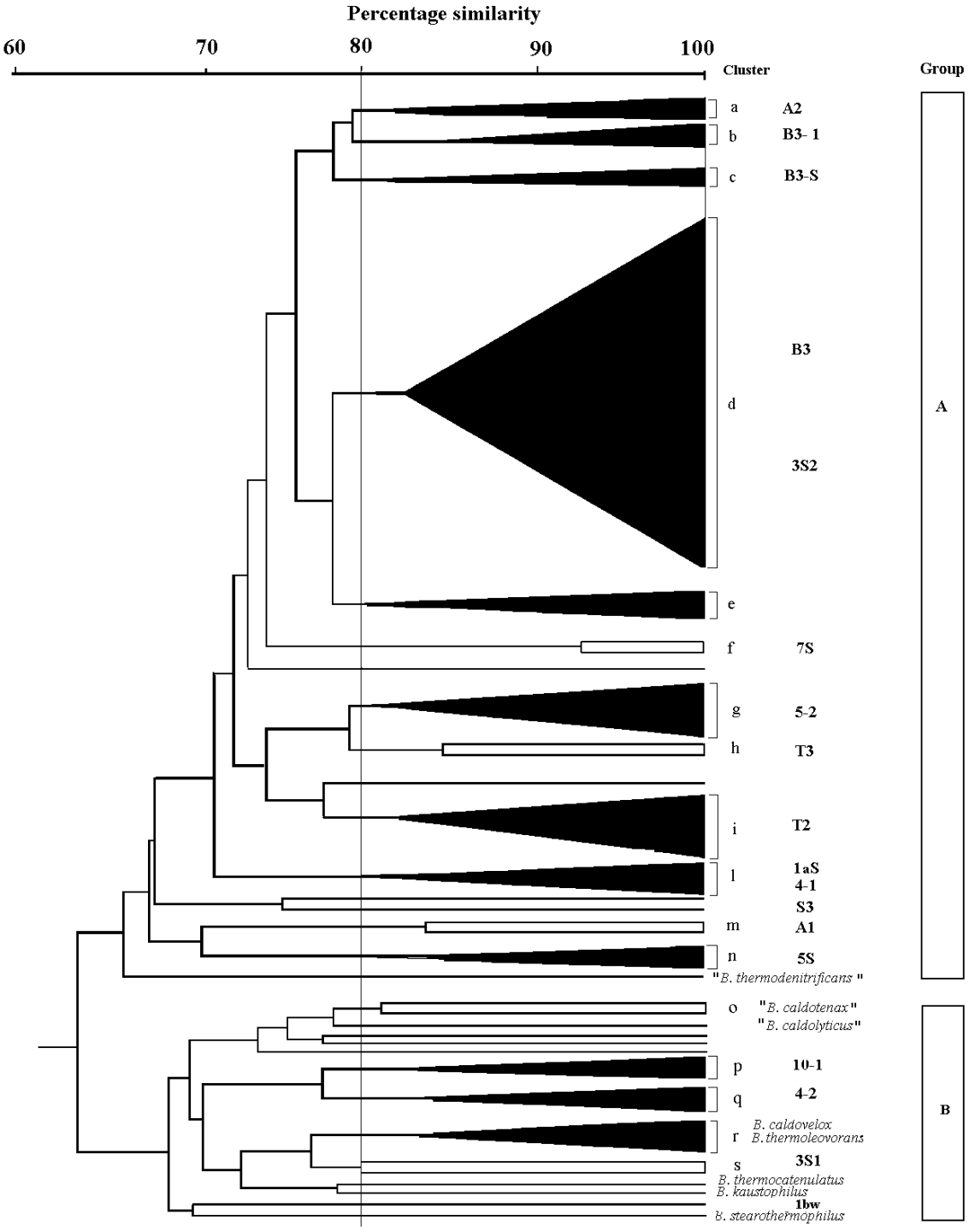

Fig. 2. Simplified dendrogram based on simple matching coefficient (SSM) showing phenotypic relationships among the 87 field strains and 8 reference Bacillus strains. Six subgroups (AI-AIV and BI-BII) were distinguished at 70-75% similarity and seventeen clusters (from a to s) were recovered at ≥80% similarity.

terisation of the group of 87 isolates, carried out in comparison with eight different Bacillus reference strains, points towards the high metabolic versatility of the isolates. Regarding nutritional characteristics, the isolates were quite similar to Bacillus spp. isolated previously from other terrestrial hot-springs or from man-made thermal sources (Sharp *et al.*, 1992). However, the isolates grew optimally only with the addition of 2% NaCl, a salt concentration higher than the optimum for most Bacillus reference strains. Some marine isolates were able to grow without added sodium chloride and these organisms should be considered more halotolerant rather than halophilic. After grouping the large set of 87 isolates into clusters of similar bacteria, 18 strains (shown in Fig. 2) were randomly selected among representatives of the different sites. In order to identify these marine isolates we carried out their genetic characterisation, using 16S rDNA sequence analysis, DNA/DNA reassociation studies and G+C mol% content determination. Comparison of metabolic properties of seven strains (A1, A2, B3, B3-1, B3s, S3 and T2) revealed that they resembled "*B. thermodenitrificans*", but were phenetically heterogeneous. Phylogenetic analyses of partial 16S rDNA sequences and DNA/DNA reassociation of one representative, strain A2, indicated that these isolates must be considered strains of this species which was the most dominant species among the ten thermophilic bacilli isolated from water and sediment at different locations of Porto di Levante (Vulcano Island).

These data revealed that the metabolic properties of "*B. thermodenitrificans*" are much broader than indicated in the original description of the species (White *et al.*, 1993). *B. thermodenitrificans* is more halotolerant than other described thermophilic Bacillus species (Sharp *et al.*, 1992) and it is therefore not surprising that the high salinity of thermal vents at Porto di Levante favours the presence of strains of this species. Three other halophilic strains (T3, 1as and 1bw) were also isolated from the same area. These strains, as well as the isolates 4-1 and 7s coming from other sites, remained unidentified and, based upon the results of DNA/DNA reassociation data lower than 70%, should be classified as three different new Bacillus species. Two isolates, strains 4-2 and 3s-2, from samples collected at La Roya and Punta Conigliara (Vulcano Island) were identified as *B. thermoleovorans*. Strain 5s, isolated from sediment of thermal vents at Inzolfata (Lipari Island), considered to be a strain of *B. pallidus*, was isolated together with a strain (5-2), found in water, which represents a new marine, strictly thermophilic Bacillus species. The presence of *B. thermoleovorans* and *B. pallidus*, usually isolated from terrestrial thermal sites, may be probably explained by frequent exchanges of thermophilic strains between nearby marine and terrestrial hot springs. It can be argued that halotolerant strains of these terrestrial species have a selective advantage in colonizing environments of moderate to high salinity (depending on the origin of thermal water and the degree of mixing with seawater).

Finally, two different new Bacillus sp., strains 10-1 and 3s-1, the latter identified as the new species, *B. vulcani* (Caccamo *et al.*, 2000), were found only at Zurro (Stromboli Island) and La Roya (Vulcano Island), respectively.

The spatial segregation of the habitats of these Bacillus species reveals that, probably due to biotic and abiotic differences in the environment, the particular taxonomic groups adjusted to and evolved within a particular niche or ecosystem.

This could also confirm the diversity of marine isolates in comparison with all thermophilic spore-formers, isolated from soil, compost, mud and air, described so far.

Spatial distribution of marine bacterioplankton species

Jarone Pinhassi, Anne Winding, Svend J. Binnerup, Åke Hagström

Kinneret Limnological Laboratory, IORL, Tiberias, Israel

Current efforts in marine microbiology are gradually revealing the distribution of extant bacteria. Several studies have demonstrated a pronounced vertical stratification in the species composition of aquatic bacterial communities (Lee and Fuhrman, 1991; Massana *et al.,* 1997; Ovreas *et al.,* 1997; Wright *et al.,* 1997). Insights into horizontal bacterial species distribution were recently presented by Riemann *et al.* (1999) in a study of bacterial community composition in the Arabian Sea. Using denaturing gradient gel electrophoresis (DGGE) of 16S rRNAgenes a dominance of the same 15 phylotypes, including cyanobacteria, alpha- and delta-Proteobacteria, was found in the surface mixed layer at stations as far as 1500 km apart. Here we report on the spatial distribution of bacterioplankton species across the Skagerrak-Kattegat frontal system.

Water samples in the Skagerrak and Kattegat were collected between 17 and 20 July 1997, in the surface (3 to 10 m), and chlorophyll maximum (10 to 25 m), at two stations on each side of the front. In the deeper waters of the Skagerrak additional samples were collected below the chlorophyll maximum (80 m). Each station was sampled at noon and the following morning. We performed DGGE analysis on PCR amplicons of community DNA samples at the different stations and depths. Similarity of the position of the 16S rDNAfragments on the DGGE gel formed the basis of boot strapping of the data before creation of a consensus tree based on Dice similarity coefficient and the neighbor joining method. This resulted in a clustering of data according to depth and side of the front (i.e. water origin). Results obtained by hybridization of whole-genome DNA, from bacteria isolated in the area, towards community DNA samples were in agreement with the DGGE data demonstrating differences in species distribution with depth and side of the front. The abundance of 28 different species (alpha-, gamma-Proteobacteria, and Cytophaga relatives) was determined at the different sampling stations, and together they accounted for 3 to 59% of the total bacterial counts and 21 to 100% of the counts obtained by the general bacterial probe EUB-338 *(in situ* hybridization). 12 species showed abundances higher than 1.0×10^4 cells ml^{-1}, with a number of alpha-Proteobacteria reaching above 1.0×10^5 cells ml^{-1}. Thus, in the Skagerrak *Agrobacterium* sp. and *Hyphomonas oceanitis* were dominant at the chlorophyll maximum and surface, respectively, while in the Kattegat *Erythrobacter citreus* and *Roseobacter* sp. dominated at the chlorophyll maximum and surface, respectively (Fig. 1).

Our results on depth distribution of different bacteria obtained by DGGE analysis and the species density protocol are consistent with previous reports indicating that depth is an important determinant of bacterioplankton species composition (Lee and Fuhrman, 1991; Gordon and Giovannoni, 1996; Riemann *et al.,* 1999). Considering the similarity of the surface water bacterial community over thousands of kilometers in the Arabian Sea reported by Riemann *et al.* (1999), our finding of differences in bacterial community composition at closely located sam-

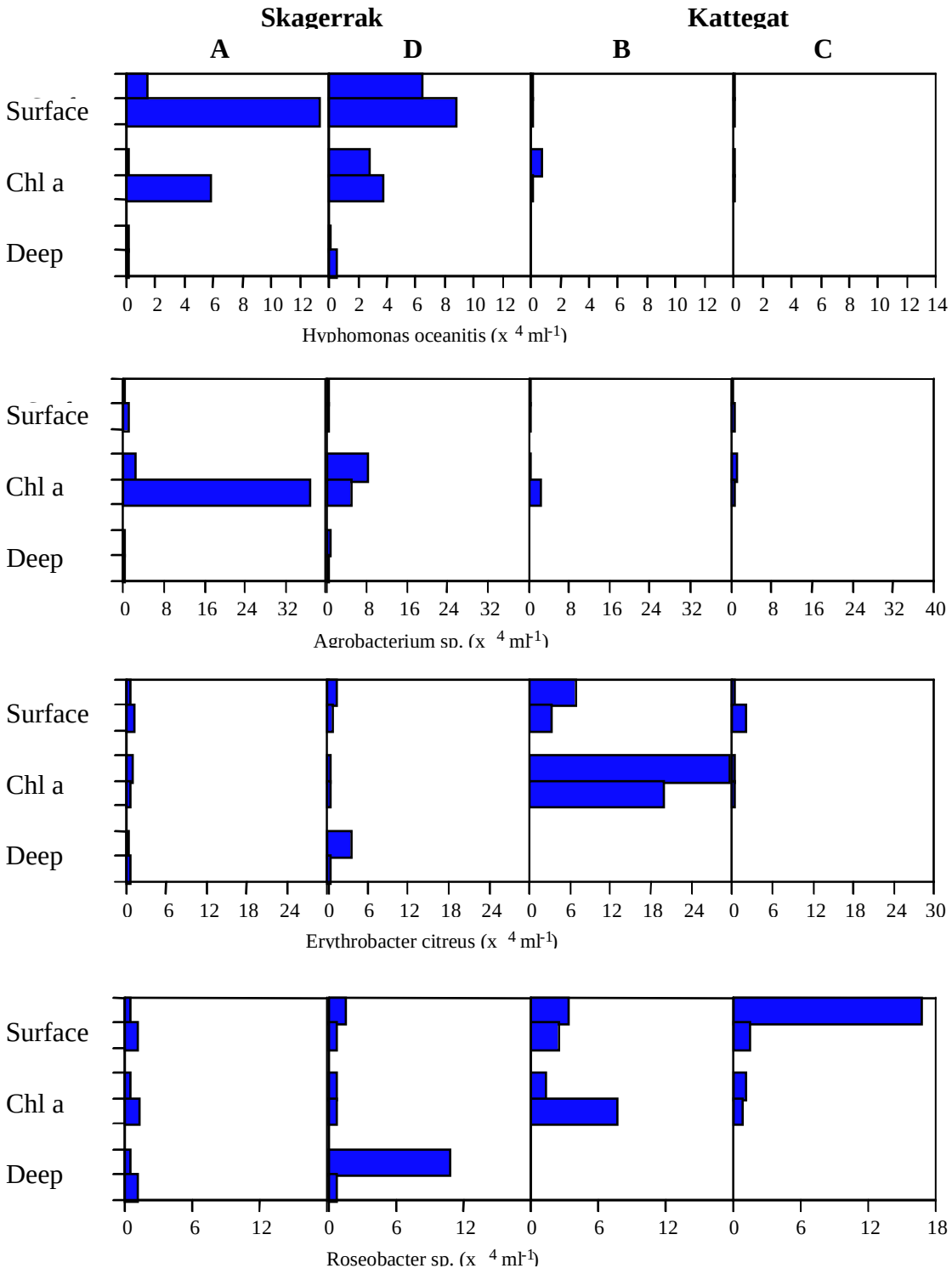

Fig. 1. Abundance of four different populations of marine bacteria across the Skagerrak-Kattegat frontal system.

pling stations (tens of kilometers) may seem surprising. However, considering the different origin of the water bodies at the Skagerrak-Kattegat front these seemingly disparate results could be explained. Thus, our results suggest that differences in bacterial community structure can be found over short distances when and where different water masses meet, *e.g.* at frontal systems and upwelling areas.

Bioremediation of marine pollutants by marine cyanobacterial mats - An interdisciplinary approach *

J. Rullkotter [1], Y. Cohen [2], J. Safi [3]

[1] *International Coordinator and German Principal Investigator*
[2] *Israeli Principal Investigator*
[3] *Palestinian Principal Investigator*

SUMMARY

According to observations in nature and initial laboratory culture experiments, cyanobacterial mats are able to degrade organic-carbon-bearing pollutants (crude oil, aromatic-hydrocarbon-rich mixtures, polychlorinated biphenyls). Due to the biological complexity of these syntrophic microbial communities little is known so far about the mechanisms of degradation. In the proposed German-Israeli-Palestinian Research Project, modern molecular biological sensors and molecular and isotopic analytical techniques as well as the development of new methods and techniques shall be used to gain a better understanding of the structure of the microbial populations in the mats and the reactions involved in the degradation of pollutants. This improved knowledge is a prerequisite for the development of a technical plant in which cyanobacterial mats can be used for the bioremediation of marine pollutant and where the life conditions of the mats can be optimized for the degradation of specific xenobiotics.

OBJECTIVES

The Middle East is an important oil-producing region. The Red Sea and the Eastern Mediterranean Sea serve as a major rout for international oil transport from the Middle East to Europe. Presently, significant oil pollution problems exit along the Red Sea and the Eastern Mediterranean coasts of Gaza Strip, Palestinian National Authority and Israel. Intense agricultural practice and industrial development are additional sources of pollution in the area. Presently, cumulative environmental pollution in the Gaza Strip by untreated domestic and industrial sewage; heavy use of pesticides in extensive agriculture and repetitive oil spills constitutes a major hazard to the well-being of the inhabitants of this densely populated region.

The aim of this research is to study various aspects of the above-mentioned phenomena by an interdisciplinary approach including organic chemistry and stable isotope research, geochemistry of carbon nitrogen and sulfur cycling, molecular and physiological studies of the microbial community and the characterization of the microorganisms responsible of bioremediation of model compounds typical of crude oil constituents and finally the isolation of microbial strains involved in bioremediation and their study in co-cultures under defined growth conditions in continuous culture.

** A trilateral German - Israeli - Palestinian research project.*

THE OBJECTIVES OF THE PROPOSED RESEARCH CAN BE SUMMARIZED UNDER THE FOLLOWING TOPICS:

- understand the biological community structure of marine cyanobacterial mats with particular emphasis on sulfate-reducers and methanogens;
- develop novel techniques for the micro-scale analysis of microbial community structure and function in relation to their immediate environment and the geochemical fluxes at the mat (sediment)-water interface;
- investigate the capability of the cyanobacterial mats to degrade different types of petroleum (including sulfer-rich oils) and other organic contaminants;
- study the (stepwise) degradation of the involved substance (*e.g.*, saturated and aromatic hydrocarbons, organo-sulfur compounds and other hetero-components in crude oils, chlorinated xenobiotics) and determine the metabolic products and their fate (*e.g.*, total vs. partial degradation, incorporation into microbial biomass, low-molecular- weight products other than CO2);
- study the degradation, conditions (oxic *vs.* anoxic) and possible effects metabolic products on the activity of community members (*e.g.* sulfate reducers, methanogens);
- determine the optimal conditions for maintaining the activity and maximum capacity of the cyanobacterial mats (*e.g.*, light/dark nitrogen limitation, addition of organo-clay complexes) to provide scientific support for the development of pilot plant for bioremediation of selected pollutants;
- provide the scientific background for the development of a pilot plant for bioremediation of polluted (sea) water.

THE STATE OF THE ENVIRONMENT IN GAZA STRIP

Gaza Strip is a semi-arid region of roughly 365 km^2, which lies on the Mediterranean Sea. On this narrow strip, almost one million of the Palestinian people live and work. The ground water is used for irrigation, as well as for industrial and domestic purposes. A “catastrophic” water shortage, water pollution with high salinity and micro-pollutants, lack of sewage and solid waste treatment, maritime pollution, overcrowding, poverty and uncontrolled use of pesticides are the most pressing environmental problems in Gaza Strip. Internationally suspended, banned and cancelled pesticides which considered mutagenic and carcinogenic are still used in the agricultural environment. Mediterranean Sea is used for the disposal of sewage water, solid wastes, agricultural and industrial waste water. There is absence of environmental policy, awareness, education and legislation. In general these environmental problems have multiplied the Palestinians human environment problems, socio-economic problems and increasing health hazards. Therefore, the environment in Gaza Strip requires a more thoughtful and comprehensive policy and planning of awareness and conservation. There is need for regional and international support and co-operation in the fields of building the infrastructure of Gaza Strip environment and the environmental policy, planning, strategy, legislation, information, awareness, education, monitoring, impact assessment, protection and pollution control.

The central role of protozoa in the marine food chain

Peter. J. leB. Williams

School of Ocean Sciences, University of Wales, Bangor, U.K.

Early notions of the structure of the marine plankton community envisaged it to comprise a simple linear food chain linking the algae with the higher organisms (Steele, 1974). This simple structure was replaced in the late-1970s to early 1980s by a more complex network consisting of the classical algal metazoan in association with a microbial network – the so-called "microbial loop" (Pomeroy, 1974, Azam *et al.,* 1983). A number of renditions of this viewed the microbial part of the network as a means whereby detritus (used in the broad sense to include dissolved as well as particulate organic material) was recycled, enriched and supplemented the classical food web (*e.g.* Williams, 1981). As part would be decomposed en route to satisfy the energy needs of the microbial community, the microbial loop seen to act as both a sink of carbon and a food link to the metazoa. Our present understanding of the number of steps leading up to the metazoa and the growth yields of the organisms involved, leads to the conclusion that the yields would be small (<10%) and so the microbial network would be a weak link. Whatever the balance of these processes, the views were that the loop itself proved added material to the metazoan food chain (*e.g.* Anderson and Williams (1998).

These ideas were the essence of the debate in the 1980s. What is new, and represents a significant shift in emphasis, is the growing awareness through the 1990s of the role of the protozoa. We are now beginning to recognise the they crop a major part, if not the majority, of phytoplankton production (Landry *et al.,* 1993; Burkill *et al.,* 1993).

Many, if not all, of the early renditions of the microbial loop contained a large measure of intuition and guess work and thus their outcomes very predictable. More recently substantially more objective analyses of the marine foodwebs have been published (Fasham *et al.,* 1999; Nagata, 2000) and they can be used to explore the contemporary view of the planktonic food web. I have simplified the structure of these original models to bring them to a common format and they are shown in Figure 1. It should be noted that neither were steady state models – they accumulate organic material in a number of compartments, so the flows shown should not be expected to balance.

A number of features are common to both models. The flow through the bacterial component – the classical basis of the microbial loop is similar in both cases – about 45%. As a consequence, the amount received by the copepods originating from the bacteria, is essentially the same (3%). Although the overall flux of organic detritus is very similar in the two models, the sources are very different. In the Fasham *et al.* model, the main source is direct release by the algae, whereas in the Nagata model the sources are more varied - the major component being the protozoa. Consequently the general flows are materially different, in as much as in the Nagata model much of the material entering the microbial network (75%) is material cycling a second time through a

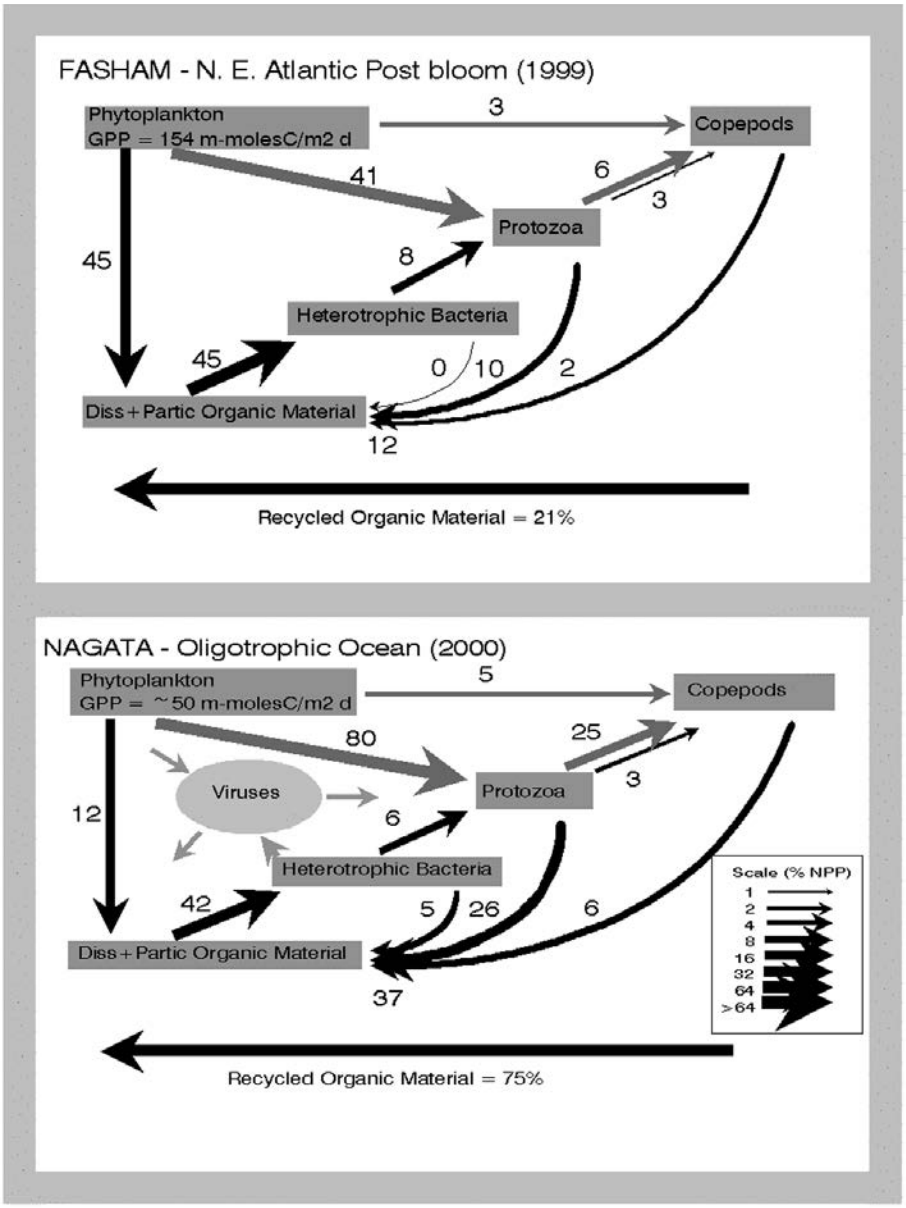

Fig. 1. Carbon flows in the marine planktonic food web simplified from Fasham *et al.* (1999) and Nagata (2000). The rates are expressed as a percentage of net primary production.

heterotrophic compartment, where as in the Fasham model this recycled material accounts for a minor fraction (21%), the major fraction being "first pass" material from the algae. This is schematised in Figure 2.

The striking feature of the two models are the flows from the algae to the copepods and the protozoa. The direct flow in both cases to the copepods is a few percent, by far the major part of primary production flows to the microbial network. In the Fasham model, roughly equal amounts (~40%) flow to the bacteria (as DOC exudates) and to the protozoa, with a tiny proportion going to the copepods directly. Even after the substantial growth yield demands of the micro-organisms are met, in the Fasham model the microbial network is providing three times more organic material that obtained from direct grazing by the copepods upon the algae. The Nagata model in this respect is even more dramatic, in that the supply from the microbial network is 5-6 times that obtained by direct grazing. Thus, in this respect the microbial net work may be seen as a major food link to the metazoa, but this overlooks the reality that the micro-organisms consume all but

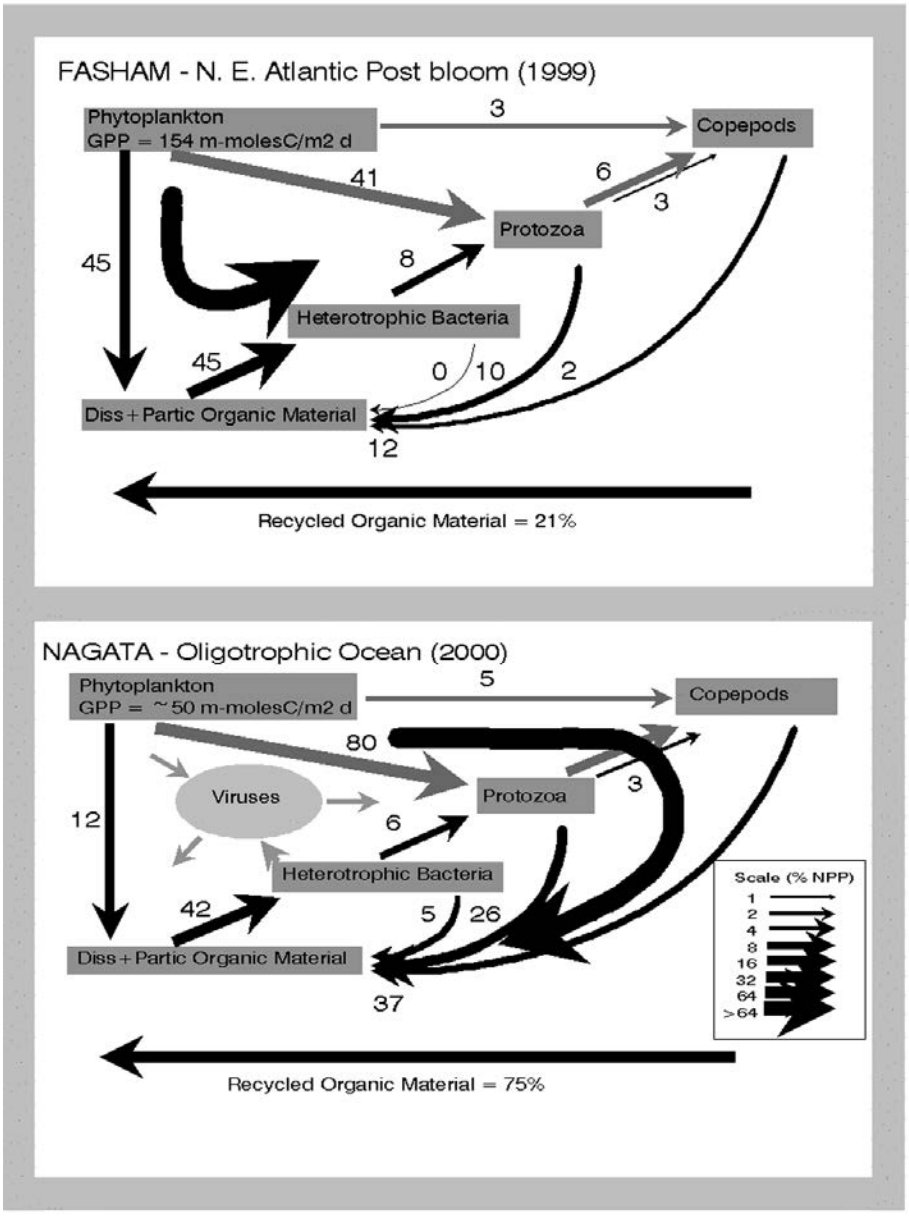

Fig. 2. As Fig. 1, with broad arrows indicating the major flow of carbon to the bacteria component of the microbial network.

a small percent of primary production and appear to very effectively outcompete the metazoa. This is a long way from the conclusion of Steele (Steele, 1974, p. 5) that "*these herbivores* (he was considering the marine copepods*) are highly efficient at transferring energy through the food chain from the plants to the primary carnivores*".

It would now seem more appropriate to view the protozoa as the nub of the marine planktonic food web.

Experimental study of microbial P-limitation in the Eastern Mediterranean

Tamar Zohary [1] and Richard D. Robarts [2]

[1] *Israel Oceanographic and Limnological Research Ltd., the Yigal Allon Kinneret Limnological Laboratory, Tiberias, Israel*

[2] *National Hydrology Research Institute, Environment Canada, Saskatoon, Saskatchewan, Canada*

In this study we experimentally tested the hypothesis that phosphorus (P) was the primary nutrient limiting phytoplankton and bacterial growth in the Eastern Mediterranean Sea, and examined the spatial variability in P-limitation during winter. A suite of complementary measurements were employed, using water sampled during January 1995 from nine pelagic stations east of the straits of Sicily. Ambient concentrations of inorganic P (P_i) in the upper 50 m of the water column in seven of the stations were 20-40 nM. The upper limit of bioavailable P ranged from 6 to 18 nM, suggesting severe P shortage. Orthophosphate turnover time ranged from 2 to 7 h in those P_i-depleted waters. In nutrient enrichment bioassays using subsurface water from the Ionian and Levantine basins, P-addition caused significant increases in bacterial activity, bacterial numbers and chlorophyll *a* (Chl *a*) relative to unenriched controls. The addition of NH4 + Fe + EDTA did not have these effects. In a similar bioassay using Cretan water microbial growth was obtained even in the unernriched controls, suggesting that other factors (*e.g.*, grazing, light) were influential. Higher ambient P_i concentrations were encountered in the Cretan Sea (90 nM), and in the core of the Rhodes gyre (210 nM), where our sampling coincided with a convective mixing event. In those stations, P-sufficiency was indicated. We concluded that in the pelagic waters of the Eastern Mediterranean Sea in winter, Pwas the primary limiting nutrient when other factors (such as light or grazing) did not control microbial biomass or activity. In ultra-oligotrophic waters, a delicate and dynamic balance differentiates between times when the microbial populations are nutrient-limited and other times when growth becomes limited by other factors. We caution that the interpretation of data obtained using conventional methods that were developed and tested in more enriched systems may not be valid in ultraoligotrophic systems.