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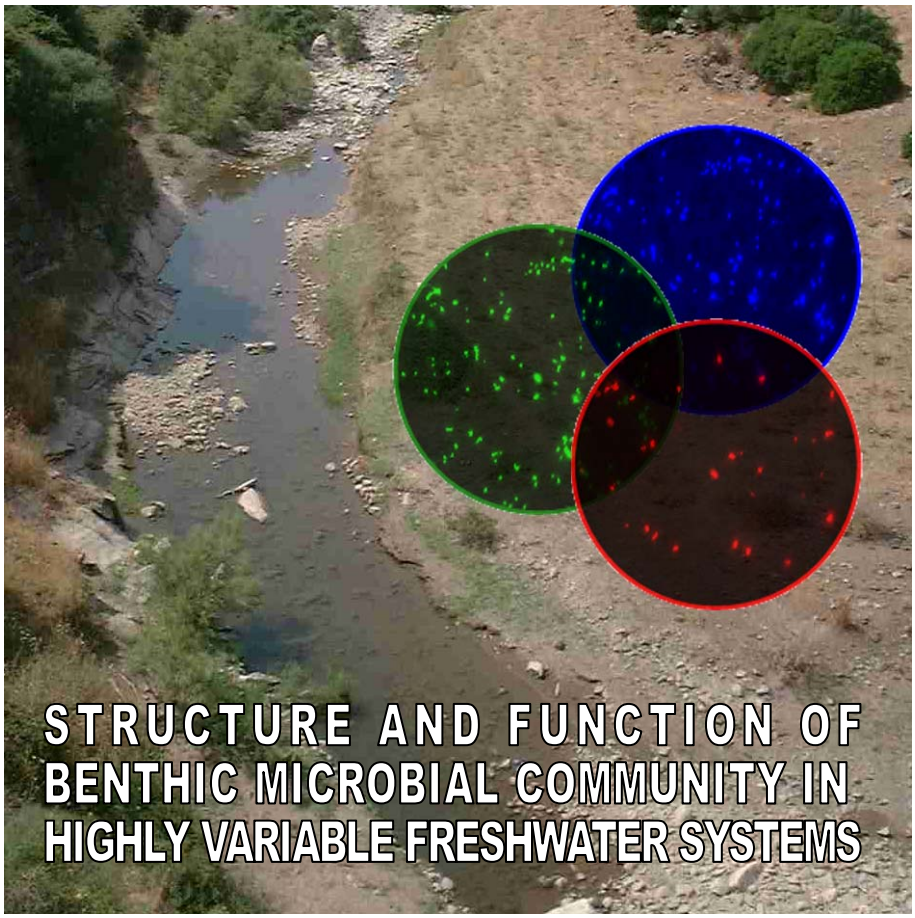

Doctorate Thesis

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UNIVERSITÀ DI ROMA "TOR VERGATA"

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STRUCTURE AND FUNCTION OF BENTHIC MICROBIAL COMMUNITY IN HIGHLY VARIABLE FRESHWATER SYSTEMS

Corso di dottorato in **Biologia Evoluzionistica ed Ecologia**

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La presente tesi è stata svolta dal Dr. Stefano Amalfitano nel periodo Novembre 2003 - Maggio 2007, presso l'Istituto di Ricerca sulle Acque del Consiglio Nazionale delle Ricerche, nell'ambito delle attività di ricerca svolte dal gruppo di Ecologia Microbica.

Il lavoro sperimentale e la stesura della tesi sono stati supervisionati dal Dr. Alberto Puddu in collaborazione con il Dr. Stefano Fazi.

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Foto in copertina: Tratto del fiume Mulargia (Sardegna) durante il periodo estivo

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SINTESI

Il lavoro sperimentale che ho condotto nell'ambito del Dottorato di Ricerca ha riguardato lo studio delle comunità batteriche associate ai sedimenti fluviali. L'obiettivo generale dell'attività di ricerca, finanziata ed inserita nell'ambito del Progetto Europeo "TempQsim" (EVK1-CT2002-00112), è stato quello di studiare le dinamiche strutturali e funzionali delle comunità microbiche bentoniche ed il loro ruolo nel ciclo del carbonio in corsi d'acqua a regime temporaneo. Questi sistemi sono particolarmente diffusi nel bacino del Mediterraneo e, con l'evoluzione climatica prevista, andranno ad interessare aree sempre più estese. In particolare lo scopo di questo lavoro è stato quello di analizzare come le caratteristiche microbiologiche dei sedimenti fluviali siano influenzate dagli stress idrici. A tal proposito sono state studiate le risposte fisiologiche delle popolazioni batteriche bentoniche durante l'essiccamento e la re-idratazione del sedimento; infine è stata seguita la colonizzazione microbica della colonna d'acqua quando, al termine del periodo di siccità, riprende lo scorrimento superficiale.

La struttura della comunità microbica è stata descritta attraverso l'utilizzo di tecniche biomolecolari, mentre l'attività batterica è stata stimata tramite tecniche radioisotopiche e di immunofluorescenza. Come descritto nel **Capitolo 2**, tutti i metodi utilizzati hanno richiesto una fase di ottimizzazione, avvalendosi anche di stage formativi all'estero. In questo capitolo sono anche brevemente descritte le caratteristiche dei siti di campionamento ad idrodinamismo costante (fiumi Albegna, Ente, Fiora in Toscana; fiume Cremera nel Lazio) ed intermittente (fiume Mulargia e Tagliamento in Italia; fiume Krathis in Grecia; fiume Pardiela in Portogallo).

Dal punto di vista metodologico, è stata inizialmente testata l'efficienza sia di metodi di estrazione delle cellule batteriche sia di tecniche di ibridazione *in situ* (Fluorescence In Situ Hybridization – FISH; Catalyzed Reporter Depositino-FISH – CARD-FISH) in relazione alle differenti caratteristiche chimico-fisiche del sedimento (**Capitoli 3.1** e **3.2**). In seguito, la CARD-FISH è stata ottimizzata al fine di aumentarne l'efficienza analitica per lo studio di specifici gruppi filogenetici associati ai sedimenti secchi (**Capitolo 3.3**).

Le indagini di campo sono state effettuate sui sedimenti del Fiume Mulargia, campionati nel sito di chiusura del bacino idrografico (**Capitolo 3.4**). Per studiare in dettaglio l'effetto dello stress idrico sulle comunità batteriche, le indagini di laboratorio sono state eseguite in microcosmi artificiali in cui il prosciugarsi del sedimento (fino al completo essiccamento) e la sua successiva re-idratazione sono stati riprodotti in condizioni ambientali semplificate. I sedimenti utilizzati sono stati prelevati non solo dal fiume Mulargia, ma anche dagli altri tre fiumi temporanei, siti di studio di altri partner europei all'interno del progetto TempQsim: l'EAWAG di Zurigo (fiume Tagliamento), la Technical University di Creta (fiume Krathis) e il Marine Environment and Technology Center di Lisbona (fiume Pardiela) (**Capitolo 3.5**). Infine, sempre in microcosmi, sono state seguite le dinamiche di colonizzazione della colonna d'acqua da parte della comunità microbica presente nei sedimenti secchi (**Capitolo 3.6**).

Il lavoro sperimentale condotto in collaborazione con i colleghi dell'IRSA-CNR ha prodotto una serie di pubblicazioni, relazioni in convegni nazionali ed internazionali e rapporti tecnici di cui sono coautore (**Annex I)**. In appendice sono anche riportati i periodi di ricerca all'estero, svolti in istituti europei durante il Dottorato (**Annex II)**.

SINTESI DEI RISULTATI

Ottimizzazione delle tecniche di analisi

Capitolo 3.1 Messa a punto della procedura di estrazione delle cellule batteriche dal sedimento

Il lavoro sperimentale ha consentito di ottimizzare una procedura di estrazione e separazione delle cellule batteriche dal sedimento. L'eliminazione di segnali di interferenza ha permesso una più attendibile analisi quantitativa tramite la microscopia, rendendo anche possibile l'utilizzo della citometria a flusso su campioni di sedimento purificato.

Capitolo 3.2 Tecniche di ibridazione in situ per lo studio di comunità batteriche associate a diversi substrati bentonici

La ricerca ha permesso l'ottimizzazione di specifiche tecniche di ibridazione *in situ* ed ha riguardato lo studio delle comunità batteriche associate a tipi diversi di substrato bentonico (sedimento, detrito fogliare, biofilm) in diversi torrenti dell'Italia centrale. I risultati hanno mostrato come la CARD-FISH sia un metodo più efficiente della FISH per l'identificazione dei batteri nei sedimenti con basso contenuto di sostanza organica. Questi batteri, infatti, sono metabolicamente meno attivi con un minore contenuto di RNA ribosomiale a cui si legano le sonde fluorescenti per la FISH. L'amplificazione del segnale fluorescente per via enzimatica, prevista nella CARD-FISH, permette invece la visualizzazione anche delle cellule meno attive. Lo studio ha inoltre dimostrato una stretta relazione tra il tipo di substrato bentonico e le caratteristiche strutturali delle comunità batteriche, con la prevalenza dei *Proteobacteria* nel sedimento povero di sostanza organica e del gruppo dei *Cytophaga-Flavobacterium* nel detrito di origine fogliare e nei biofilm con più alto contenuto di sostanza organica.

Capitolo 3.3 Tecniche di ibridazione in situ per lo studio di comunità batteriche associate a sedimenti di fiumi temporanei

A seguito dell'indagine descritta in precedenza, l'efficienza della FISH è stata confrontata con quella della CARD-FISH in sedimenti di fiumi temporanei, caratterizzati da un diverso grado di umidità. I risultati hanno evidenziato come, con un appropriato metodo di permeabilizzazione, la CARD-FISH permetta la visualizzazione di un maggior numero di cellule batteriche nei sedimenti secchi in cui le cellule sopravvivono in uno stato di bassa attività metabolica. Tale tecnica è stata quindi utilizzata per confrontare comunità batteriche associate a sedimenti con diverso grado di umidità. Inoltre il metodo proposto ha permesso una migliore visualizzazione degli *Actinobacteria*, Gram-positivi, che da precedenti studi

risultavano difficilmente visualizzabili tramite le tecniche di ibridazione *in situ* attualmente disponibili.

Indagini di campo e di laboratorio

Capitolo 3.4 Caratterizzazione stagionale delle comunità batteriche bentoniche nell'ambito dell'attività di monitoraggio ambientale sul fiume Mulargia

Nell'ambito dell'attività di monitoraggio ambientale condotta per oltre un anno sul fiume Mulargia è stato effettuato uno studio specifico focalizzato sulle variazioni delle caratteristiche strutturali e funzionali delle comunità batteriche presenti nel sedimento. I risultati della ricerca, innovativi per quanto riguarda l'analisi della composizione e dell'attività metabolica in sedimenti fluviali sottoposti a stress idrico, hanno mostrato una sostanziale capacità di adattamento delle comunità alle diverse condizioni stagionali. Tuttavia nel periodo di campionamento non si sono mai osservati livelli estremi di siccità. Il sedimento ha mantenuto un regolare livello di umidità per effetto delle acque iporreiche, consentendo al popolamento batterico di mantenere una costante attività metabolica.

Capitolo 3.5 Effetti dello stress idrico sulle dinamiche strutturali e funzionali della comunità batterica

Obiettivo di questo studio è stato quello di analizzare le risposte fisiologiche della comunità batterica al progressivo disseccamento del sedimento fluviale in condizioni di laboratorio. A tale scopo sono stati allestiti dei microcosmi artificiali con sedimenti provenienti dai quattro fiumi temporanei. Durante l'essiccamento, avvenuto in condizioni simili a quelle naturali, sono state seguite le dinamiche strutturali e funzionali della comunità batterica. Le comunità dei quattro fiumi, nonostante alcune iniziali differenze strutturali, hanno reagito in modo sostanzialmente simile allo stress idrico, con una progressiva riduzione delle attività metaboliche che cessano completamente nei sedimenti secchi. In parallelo, si è registrata una diminuzione del 74% dell'abbondanza e del 78% della biomassa batterica. Un test aggiuntivo è stato condotto per valutare i tassi di riattivazione metabolica delle comunità presenti nei sedimenti secchi dopo re-idratazione. Le comunità hanno dimostrato un rapido recupero dell'attività metabolica raggiungendo, dopo circa otto giorni dalla re-idratazione, i più alti valori di produzione di carbonio, simili a quelli registrati nei sedimenti naturali.

Capitolo 3.6 Colonizzazione della colonna d'acqua da parte della comunità microbica presente nel sedimento secco

Questo studio è stato condotto al fine di analizzare le modalità con cui la comunità batterica presente nel sedimento secco colonizza la colonna d'acqua, quando, al termine del periodo di siccità, riprende lo scorrimento superficiale. Simulando l'inondazione del sedimento naturale, si è osservata una rapida colonizzazione della fase acquosa da parte del gruppo dei *Firmicutes*, in grado di approfittare della sostanza organica disciolta che dal sedimento secco si diffonde rapidamente nella colonna d'acqua. In seguito la comunità si stabilizza anche per effetto della predazione da parte di nanoflagellati, la cui influenza aumenta progressivamente equilibrando la distribuzione tra i diversi gruppi batterici.

ABSTRACT

In the semi-arid Mediterranean regions, where the length and frequency of dry periods are likely to increase as a consequence of climate change, extended reaches of rivers and streams show a recurrent dry phases of varying duration and spatial extent. Since freshwater systems are particularly important in linking terrestrial and marine environments, the effects of water stress on the ecological processes of temporary rivers are essential to better understand the sediment transformations during dry periods and the interaction among re-suspended materials and the overlying water after a flood event.

My Doctorate thesis research, supported by the European TempQsim project (EVK1-CT2002-00112), was aimed to investigate the dynamics of microbial communities associated to sediments in temporary rivers. In particular, I have been investigating the effects of water stress on the structure and function of benthic bacterial communities and their role in the carbon cycle of rivers characterized by a seasonal hydrologic regime.

Bacterial abundance and phylogenetic composition were assessed by molecular techniques, while bacterial activity was estimated by measuring the incorporation rates of radioactive tracers and by immunofluorescence. From a methodological point of view, all applied techniques required a specific optimization phase in order to increase their analytical efficiency. In particular, a detachment procedure to extract and purify bacterial cells from freshwater sediments was optimized by the combined use of different chemical and physical treatments, followed by high-speed density gradient centrifugation using the medium Nycodenz. Moreover, the high purity of treated sediment samples allowed for the successful utilization of flow citometry and the straightforward applicability of fluorescent dyes and probes on the extracted cells. This procedure was initially applied to analyze the benthic bacterial communities in rivers with stable water flow conditions (Rivers Albegna, Ente, Fiora – Tuscany, Italy; River Cremera – Lazio, Italy).

In addition, the efficiency of specific *in situ* hybridization techniques (Fluorescence *In Situ* Hybridization with fluorescently monolabeled probes – FISH; Fluorescence *In Situ* Hybridization with signal amplification by Catalyzed Reporter Deposition – CARD-FISH) was tested with regard to the different physicochemical characteristics

of selected sediments (i.e. sediment organic matter and moisture content). In this respect, CARD-FISH protocol was improved to better estimate the occurrence of specific phylogenetic clusters in dry sediments.

For the field study, the River Mulargia (Sardinia, Italy) was selected across the sites monitored by other TempQsim partners. Sediments were regularly collected at the river outlet section. A seasonal in-depth study was performed for benthic bacterial composition and activity analyses. Owing to the stable moisture content of Mulargia sediments during the annual sampling survey, additional tests were performed in artificial microcosms to experimentally describe benthic bacterial responses to drying and rewetting processes, by simulating desiccation and re-inundation of sediments collected from four European temporary rivers (River Mulargia and Tagliamento - Italy; River Krathis - Greece; River Pardiela - Portugal). In a further laboratory experiment, the composition and activity of the bacterial community that primary colonized the water phase was followed in microcosms set up with Mulargia sediments. This study could contribute to better understand the ecological role of those benthic microbes that reside in a state of low-activity in dry sediments and promptly colonize the new incoming water at the end of the dry period.

In synthesis, my Doctorate research contributed to a better understanding of the functional role of bacterial community in river sediments. The results could help to elucidate the mechanisms involved in the sediment transformation processes during drought periods, which are attended to increase in length and frequency as a possible effect of climatic changes.

1 INTRODUCTION

1.1 Habitat heterogeneity and carbon fluxes in river systems

Rivers are highly diverse, dynamic, and complex ecosystems that experience cycles of expansion, contraction, and fragmentation along the longitudinal, lateral, and vertical dimensions (Ward et al., 2002). The spatial and temporal heterogeneity of riverine landscapes is expected to reflect on ecosystem functioning, depending on the nature of boundary between landscape components with different process rates and the patterns of connection between components (Meyer, 2005).

Connecting land and sea, riverine systems are vital links in carbon and nutrient biogeochemical fluxes among atmosphere, land and marine ecosystems (Raymond, 2005). In particular, rivers integrate terrestrial and marine reservoirs of carbon resources and their transformation processes (Figure 1.1). Carbon inputs originate mainly when rainfall hits continental surfaces and either dissolves geological carbon, generating dissolved inorganic carbon (i.e. weathering of carbonate and silicate rocks), either carries it to rivers in particulate (i.e. erosion of soil and leaf litter) and dissolved organic form (i.e. solubilization of soil organic matter). In addition, part of the riverine carbon comes from atmospheric CO_2, fixed by autotrophs in lakes and reservoirs (Aumont et al., 2001), and therefore represents mobile greenhouse carbon that either cycles back to the atmosphere by respiration or enters the sedimentary storage compartment that is not in contact with the atmosphere (Raymond, 2005).

Owing to the heterogeneity of riverine environments (geomorphology, soil properties and composition, rainfall patterns and vegetation coverage, land use and anthropogenic impact), river carbon fluxes are highly variable. Globally, the reported values range around 0.4 Pg C yr^{-1} for both inorganic and organic carbon (evenly divided between the particulate and dissolved phases) (Richey, 2004). Although the total amount of C cycling through rivers corresponds to a small fraction of the total C stored in the terrestrial system (~2000 Pg C), oceans (~40000 Pg C) and atmosphere (~750 Pg C) (Grace, 2004), it is significant if compared to the net oceanic CO_2 uptake (~2 Pg C yr^{-1}) (Caldeira and Akai, 2005).

The mineralization of terrestrially derived organic matter is an important regulator of organic C fate, since organic materials, that

escape decomposition in the terrestrial environments, must become susceptible to decomposition in linked aquatic environments (Cole & Caraco, 2001). Within this regard, any environmental perturbation that affects decomposition rates will influence the quality of the exported organic matter and the net exchange of CO_2 among atmosphere, land and water systems (Algesten et al., 2003).

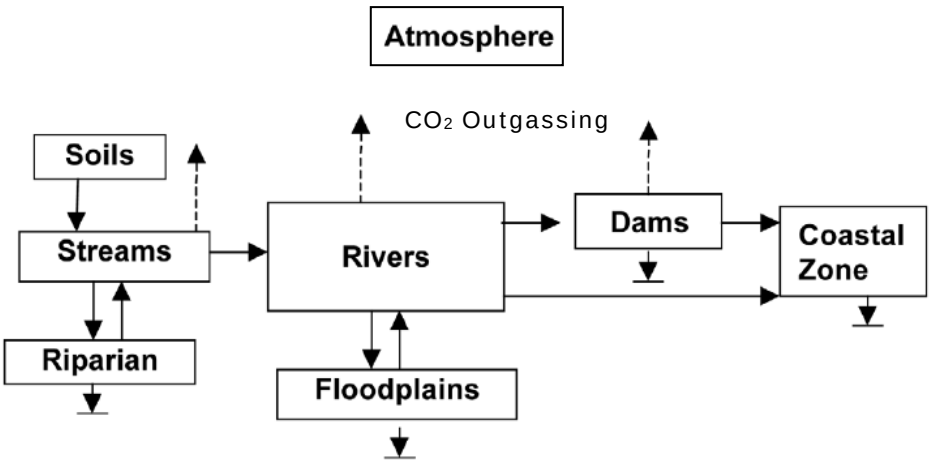


Figure 1.1. Major pathways in the flux of atmospheric CO_2 to and through fluvial systems. Atmospheric CO_2 fixed into streams and their riparian zones (as dissolved CO_2 or organic matter) is stored, released back to the atmosphere, or transported to larger river systems. Finally, the remaining fluvial carbon is exported to the recipient water bodies (modified from Richey, 2004).

In the semi-arid Mediterranean regions, the seasonal and episodic nature of water flow has significant consequences for river C cycling. During summer, extensive evapo-transpiration, water abstraction, high irrigation demand and over-exploitation of groundwater drastically affect river flow, with extended reaches becoming completely dry (Dahm et al., 2003). In these regions, the dominant types of rivers are considered as temporary and experience a recurrent dry phase of varying duration and spatial extent (Uys and O'Keeffe, 1997). Often even extended reaches of large rivers are exposed to dry periods (Mariotti et al., 2002). The dynamic transformation processes in sediments during the period of no surface runoff and the interaction among re-suspended materials and the overlying water after a flood

event are conceptually fundamental to investigate how hydrological variability affects ecological processes in temporary rivers (Figure 1.2).

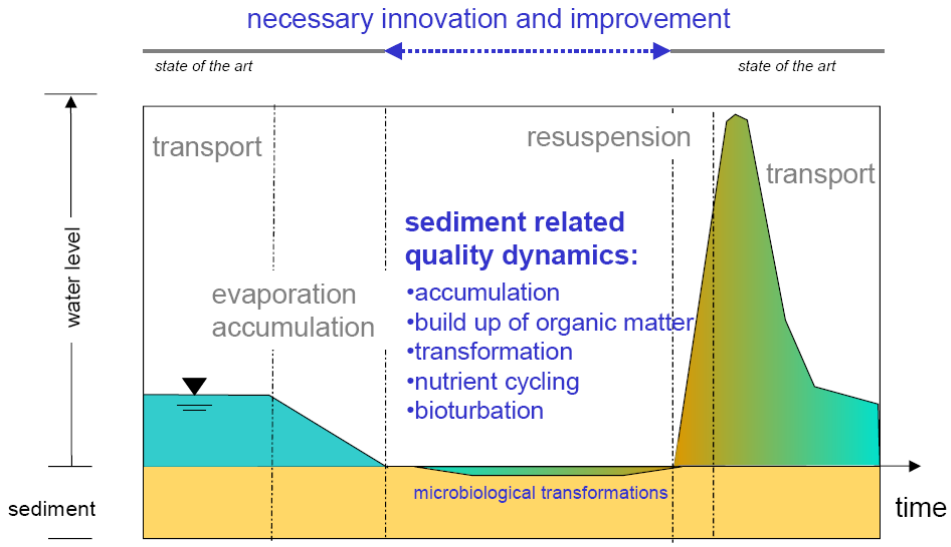


Figure 1.2. Conceptual flush response in temporary rivers (modified from TempQsim-Consortium, 2006).

The first water flush entering in contact with sediments after a prolonged drought period could come out extremely modified in terms of quality with respect to the riverine base flow, thus inducing modifications in the quality of receiving water bodies (e.g. lakes, reservoirs, coastal waters). The majority of the temporary rivers are found in catchments where there are little to no data regarding their hydrologic and biogeochemical regime. Most ecological studies are predominantly focused on streams with permanent flow and available data are disproportionately scarce for temporary waters, although they have the same functional value as permanent rivers. Within the latest climatic scenario where average temperatures, duration and frequency of dry periods and consequently hydrological variability are likely to increase as a consequence of climate change (Gregory et al., 1997; IPCC, 2007), increasing water scarcity requires a closer consideration of temporary rivers.

The TempQsim European project, which supported my doctorate fellowship, has been among the first to make specific improvements to water management for particular application to temporary streams. The

project was aimed to provide tools to improve the integrated management of water resources in Mediterranean river catchments through quantifying the water and sediments quality dynamics and the development of water quality models (Figure 1.3).

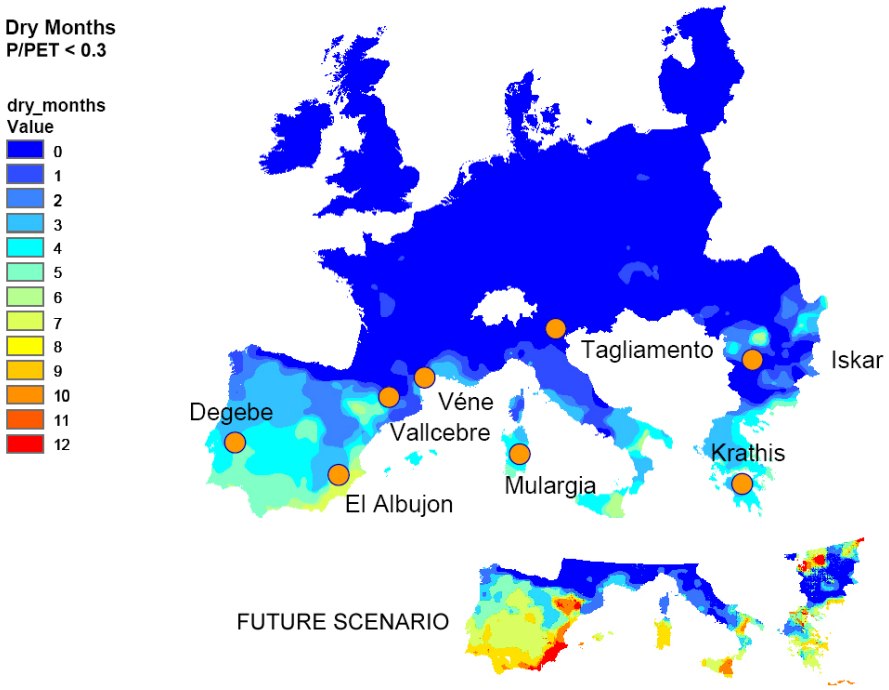


Figure 1.3. Generalised distribution of catchments included in TempQsim EU project and a tentative prediction of future climatic scenario. The ratio P (precipitation)/PET (potential evapotranspiration) = 0.3 approximately delineates the duration of dry channel conditions (modified from TempQsim-Consortium, 2006).

1.2 The role of heterotrophic microbes for the ecological functioning of benthic compartment

Riverbed substrates are extremely patchy in terms either of space ("hot spots" with high processes rates) or of time ("hot moments" with irregular periods in which significant biogeochemical events may occur) (McClain et al., 2003). In river benthic compartment, primary production is often negligible and thus biogeochemical fluxes mainly cycle through the detritus-based food web (Fleituch et al., 2001; Findlay et al., 2002; Logue et al., 2004). The microbial heterotrophic activity significantly affects the transformation and mineralization processes associated to sediments. Bacterial metabolism may alter the chemical composition of allochthonous and autochthonous particulate organic matter and its nutritional value, resulting in an increasing C:N ratio from particulate to dissolved organic matter. The organic matter cycling efficiency depends on the specific hydrolyzing capacity of appropriate microbial enzymes, which allowed carbon and nutrient incorporation into bacteria and, successively, toward upper trophic levels (flagellate, ciliates and metazoan), with a significant influence on biogeochemical fluxes (Figure 1.4).

While the organic matter is mineralized, a rapid structuring of a heterotrophic community occurs. Benthic bacteria and fungi are crucial to the overall riverine ecological functioning and play a crucial role as trophic resource for the upper trophic levels (Findaly et al., 2002; Leff, 1994; Lyautey et al., 2003). Microbial abundance and metabolic activities highly vary among detritus types: fungi prevail on leaf litter, whereas bacteria dominate heterotrophic biofilms and fine particulate organic matter (Findlay et al., 2002; Gulis and Suberkropp, 2003). However, there have been little comparisons of microbial *in situ* abundances among different organic matter types, and only few studies describe differences in the composition and activity of these assemblages (Spring et al., 2000; Logue et al., 2004).

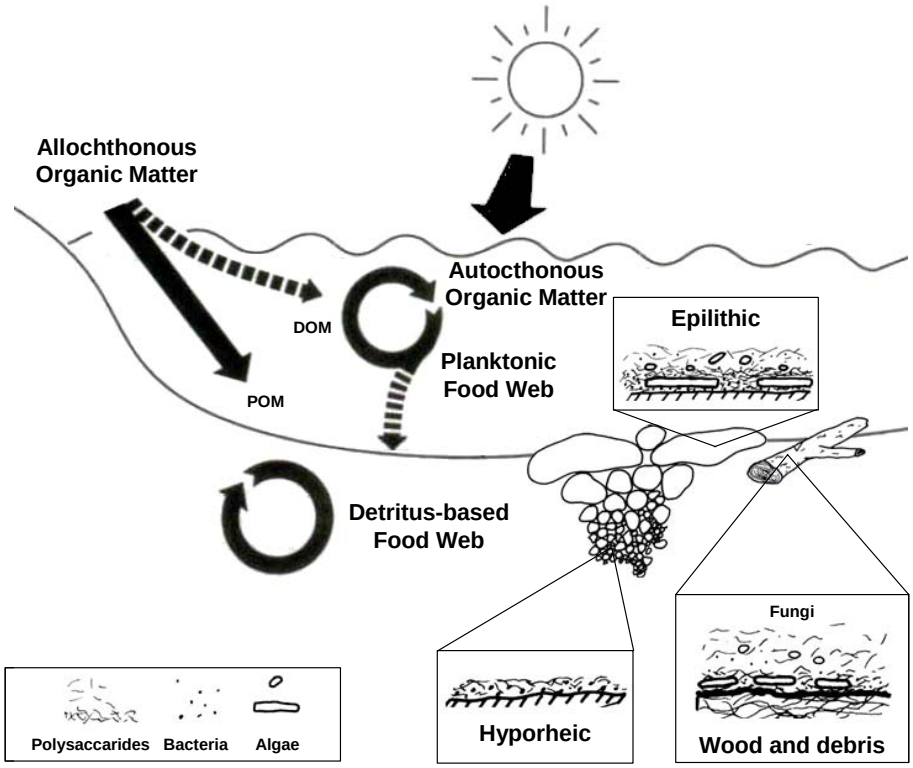


Figure 1.4. Schematic representation of riverine particulate (POM) and dissolved (DOM) organic matter cycling.

In natural benthic bacterial communities, a considerable fraction of bacteria was found to be metabolically inactive, or even non-living (Haglund, 2004). However, when calculating bacterial cell-specific properties (e.g. specific growth rate) through bulk measurements of abundance and activity, all cells are typically considered equally active, thus masking the intrinsic variations in cell-specific properties. The majority of the studies on the active bacterial fraction focused on pelagic environments. Active bacterial fractions in planktonic habitats range between a few percent and almost 100% (e.g. Del Giorgio and Scarborough, 1995). In sediments, the total bacterial activity is generally low, considering the high bacterial abundance, which implies long doubling times or a small active fraction. Some studies report fractions of active bacteria in sediment, ranging from 4% to 67% (Fischer and Pusch, 1999; Luna et al., 2002). Several processes in

attached and pelagic communities regulate the active fraction of bacteria: (i) the growth rate of active bacteria (production of new cells); (ii) the rate of inactivation and death; (iii) the loss rates from the active and inactive pools (Del Giorgio and Scarborough, 1995). These processes are regulated by several mechanisms including temperature, grazing, nutrient and carbon supply. At least on a seasonal scale, temperature may explain up to 80% of the variation in the fraction of active pelagic bacteria (Jugnia et al., 2000). This dependence on temperature disappears if shorter time spans with uniform temperature are considered. Then other factors such as substrate availability and grazing become more important (Smith, 1998). The fraction of active bacteria has been shown to increase with nutrient concentrations in both lake, marine and estuarine systems (Del Giorgio and Scarborough, 1995). Accordingly, there is a positive relationship between system productivity and the fraction or abundance of active bacteria, supporting the importance of substrate supply (Yager et al., 2001). In addition, selective grazing on the most active or dividing bacterial cells may have an impact on the active fraction (Gasol et al., 2002; Hahn and Höfle, 2001; Beardsley et al., 2003). Flagellates and other grazers have been found to preferentially feed on metabolically active cells (Del Giorgio et al., 1996). Conversely, other studies show increasing active assemblages in the presence of grazers, due to changes in the bacterial species composition towards more grazing resistant forms (Sherr et al., 1999).

There is a lack of understanding on how the structure and function of benthic bacterial communities respond to environmental perturbation (i.e. water scarcity). In particular, a deeper understanding of microbiological processes is of critical significance in predicting temporary freshwater ecosystem functioning and the C budget in a warmer and drier climate (Darrel Jenerette and Lal, 2005)

Moisture limitation imposes physiological constraints that some bacteria can tolerate by adopting various survival strategies, such as starvation, dormancy and/or sporulation (Bär et al., 2002; Billi and Potts 2002). The uncanny resistance of bacterial spores to adverse environmental conditions (e.g. heat, desiccation and radiation) enable them to persist in changing environments (Nicholson et al., 2000). Furthermore, periods of soil moisture limitation could provoke a strong selective pressure on community structure, modifying resource competition among bacterial taxonomic subgroups and consequently affecting the upper trophic levels (Wardle at al., 1998). Several studies

have recently analyzed microbial responses to drying-rewetting cycles, mainly in terrestrial soil, by measuring biomass or gross microbial processes with chemical determinants or other conventional methods (e.g., fumigation-extraction method, fatty acid analysis) (Steenwerth et al., 2003; Wu and Brookes, 2005). Although useful for general microbiological characterization, analyses at the whole-community level may be not sensitive enough to reveal changes in the structures or activities of small subgroups. In this respect, few authors have analyzed bacterial physiology, viability and diversity at the single-cell level under varying water regimes (Fierer et al., 2003; Griffiths et al., 2003; Pesaro et al., 2004).

1.3 In situ identification of natural bacterial communities

Many bacteria and other microorganisms usually do not have enough morphological detail for easy identification. Traditionally, microbiology has relied on cultivation for identification, which has proven difficult for many environmentally important microbes (Relman 1993; Amann 1995; Amann & Kuhl 1998). Therefore, it has been frequently reported that direct microscopic counts exceed plate counts by several orders of magnitude. Traditional culture-based approaches failed to open the black box of sediment microbial diversity due to selective recovery of only a small sub-population of the microbial communities.

Even though new microorganisms continue to be isolated (Kaeberlein et al., 2002; Connon & Giovannoni, 2002), it is estimated that so far only a small fraction, possibly below 10%, of the extant microorganisms have been grown in pure culture and characterized. Today, about 5,000 species have been described (Bull et al., 1992), with the *Approved List of Bacterial Names* currently containing less than 3,500 entries. Consequently, it is still unlikely to identify many microorganisms and to understand the role of microbes in the regulation of globally important mineralization processes. Therefore, any approach to identify specific microbial populations without cultivation directly in their natural environments has proved to be inappropriate, since it was going to modify the character of microbiology and close the methodological gap that still exists in comparison with botany and zoology (Amann et al., 1995).

In the last years, the application of molecular biological methods (Figure 1.5), mainly derived from medical science and based on

amplification of extracted nucleic acids, enhanced the knowledge of bacterial diversity in different ecosystems (Head et al., 1998; Torvisk and Øvreas, 2002). With the advent of polymerase chain reaction (PCR) (Saiki et al., 1988), a method became available to speed up this quite laborious procedure. By using PCR, 16S rRNA gene fragments can be selectively amplified from mixed DNA. Gene libraries derived from mixed amplification products should contain only defined fragments that can be rapidly sequenced from known priming sites. Moreover, cloning of cDNA transcribed from 16S rRNA with the enzyme reverse transcriptase allows, as in the case of PCR, the selective retrieval of useful rRNA sequence information (Weller and Ward, 1989).

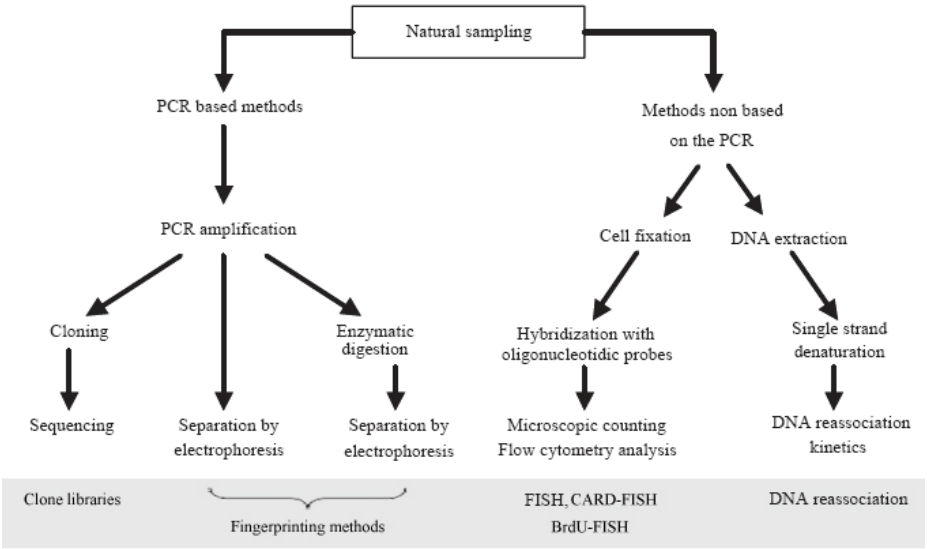


Figure 1.5. Diagram of the different molecular approaches for assessing the genetic diversity of microbial communities. FISH = Fluorescence *In Situ* Hybridization; CARD-FISH = FISH in combination with tyramide signal amplification by catalized reporter deposition; BrdU-FISH = FISH in combination with bromodeoxyuridine incorporation to assess cell specific activity (modified from Dorigo et al., 2005).

The comparative analysis of 16S rRNA sequences is today the most commonly used method for studying the phylogeny of microorganisms, and rRNA sequences can be obtained from environmental samples without cultivation. This direct retrieval is facilitated by PCR exploiting highly conserved primer binding sites on

the 16S rRNA genes. Consequently, the number of publicly accessible 16S rRNA sequences has been increasing rapidly in the last decade. Today, more than 30.000 environmentally retrieved bacterial 16S rRNA gene sequences are available (i.e. Ribosomal Database Project – RDP, Real Environmental Genomics Project - REGX), and many of these sequences represent species that are only distantly related to bacteria available in pure culture. Recent comparative studies of 16S rRNA clone libraries of microbial communities reveal that the natural diversity of prokaryotes by far exceeded the number of Bacteria and Archea that have been cultured. Such microorganisms often prove to be the numerically significant component of microbial communities in natural systems and hence the major challenge in contemporary microbial ecology is to develop and apply molecular methods to better characterize microbial communities within their natural environment (Wagner, 2004). A number of studies based on the analysis of 16S rDNA sequences provided an insight into bacterial diversity and composition of pelagic and benthic communities (e.g. Ravenschlag et al., 2001; Riemann et al., 2000; Urakawa et al., 1999). Novel bacterial lineages were identified as active constituents of sediment communities (Miskin et al., 1999; Stein et al., 2002).

Whereas PCR-based techniques mainly provide information on the qualitative community composition, i.e. on the incidence of a distinct genotype, *in situ* population dynamics can be monitored in a quantitative way only by means of dot- or slot-blot hybridization or fluorescence in situ hybridization (FISH) (Amann et al., 1995). The latter technique has become a powerful tool for ecological investigation of microbes in various aquatic environments (Pernthaler et al., 2001) including sediments (Wobus et al., 2003; Llobet-Brossa et al., 1998). Based on sequences collections, single stranded rRNA-targeted oligonucleotide probes (usually 15–25 nucleotides in length) can be chemically synthesized and designed. These probes may be targeted to signature sites of the rRNA molecules characteristic for defined taxonomic entities such as species, genera, families, orders, or even domains. Sets of probes, therefore, allow for a rapid assignment of cells to major groups.

Identifying the role of bacteria and other microorganisms relies on the simultaneous assessment of their phylogenetic identity and ecological function. Over the last few years various single-cell techniques have been developed for the phylogenetic identification of microbes and for the evaluation of their contribution to the ecosystem

functioning. There were also several interesting technical developments in the area of fluorescence *in situ* hybridization (FISH) and various combinations of these techniques now permit to open the microbial black boxes, by identifying groups of microbes that are functionally important components of ecosystems and defining their main capacity in terms of specific organic substrate uptake. This will lead to advancement from a description of microbial processes to a mechanistic understanding of the functioning of microbial communities, allowing for the resolution of the spatial and temporal scales of *in situ* cell-specific microbial activity, and for the differential response of microbial groups to changing environmental conditions (Amann and Ludwig, 2000; Wagner et al., 2003).

2 METHODS

2.1 Bacterial abundance, biomass and viability

2.1.1 Epifluorescence microscopy and image analysis

Bacterial total abundance (BAB) was determined both for water and sediment samples by epifluorescence microscopy after staining with 4,6-diamidino-2-phenylindole (DAPI), at a final concentration of 1 μg ml^{-1} (Porter and Feig, 1980). Samples were fixed with formaldehyde (2% final concentration), stained for 5 min and filtered through 0.2-μm black polycarbonate filters (pore-size 0.2 μm, 25 mm diameter, Nuclepore Corporation, USA). Filters were mounted on microscope slides with non fluorescent oil (R.P. Cargille Lab) and stored frozen until counted. A Leica DM LB 30 epifluorescence microscope was used at 1000× magnification to manually count a minimum of 300 cells per filter.

For sediments, a pre-treatment was performed in order to detach cells from particles. The optimization of this method is fully described in the chapter 3.1. Briefly, fixed aliquots of sediment (1 g) were placed in a sterile 15 ml falcon tube and diluted (1:10) in a filter-sterilized phosphate-buffered saline (PBS) buffer (130 mM NaCl; 7 mM $Na_2HPO_4 \times 12H_2O$; 3 mM $NaH_2PO_4 \times 2H_2O$, pH 7.4). The chemical detachment solution was amended by adding sodium pyrophosphate (0.1 M, final concentration) as chelating agent and Tween20 (0.5%, final concentration) as detergent. Samples were incubated under shaking on an orbital shaker (720 rpm for 30 min; IKA KS 130 Basic) at room temperature. Efficient dispersion of the sediment slurry was ensured by keeping the tubes in a horizontal position oriented along the movement axis of the shaker. Mild sonication on ice was subsequently performed with a 1.6-mm diameter microtip probe (20 W for 60 sec, Microson XL2000, Misonix, New York). Afterward, the resulting slurry was left for 2 h at 4°C, allowing for sedimentation of coarse particles. Depending on cell abundance, 10–100 μl of the clarified slurry were DAPI stained for BAB estimation. In order to better distinguish cells and detritus particles, a further purification through high-speed centrifugation was performed by the use of Nycodenz (Nycomed, Oslo, Norway; density 1.310 ± 0.002 g ml^{-1}) as density gradient medium. 1 ml of Nycodenz was carefully placed beneath 1 ml of sediment slurry, by using a syringe needle with adequate length to

reach the bottom of the tube. The 2-ml Eppendorf tube was then centrifuged (14,000 g) in a swing-out rotor for 90 min at 4°C. The supernatant, visibly divided in three bands, was entirely pipetted out, filtered and stained. The filters were stored in Petri dishes at -20°C until further processing.

For the automated analysis of fluorescent images, a Leica DC 350 F high-resolution camera (Leica Microsystems GmbH, Wetzlar, Germany) was used to capture 1300 × 1030 TIFF gray-scale images at a color depth of 8 bits and a resolution of 0.1 μm per pixel. Image filtering was performed by the freely available NIH ImageJ software (version 1.37, National Institutes of Health, Bethesda, Maryland, USA). An entropy threshold plug-in filter (Sahoo et al., 1988) was applied to automatically generate binary images for automatic enumeration. The analysis of the binary images allowed rapid estimations of cell morphology and biovolume. Eleven cell morphotypes were automatically identified according to their distinctive shape by the CMEIAS v.1.27 software (Center for Microbial Ecology Image Analysis System; Liu et al., 2001). Depending on the observed prevailing cell morphology, the mean cell biovolume (BV in μm^3) was calculated from the measured mean cell area and perimeter according to Massana el al. (1997). The bacterial total biomass (BB in $\mu g\ C\ g^{-1}$ dry sediment) was calculated by the allometric model ($BB = (104.5 \times BV^{0.59} \times 0.86) \times BAB$) proposed by Fischer and Pusch (2001).

To determine the number of viable bacteria, a two-dye fluorescence bacterial viability kit (Live/Dead® BacLight™, Molecular Probes, Eugene, Oregon, USA) was used to rapidly distinguish live and dead cells (Haglund et al., 2003). Cells with intact membranes stain fluorescent green and are considered to be live while cells with damaged membranes stain fluorescent red. Since the signals of the two dyes can overlap, producing several fading color combinations, a fast semi-automated image analysis was performed to better identify the green-fluorescent live cells, by modifying earlier proposed protocols (Posch et al., 1997; Shopov et al., 2000). A subtraction algorithm operator was applied to combine the two-dye images. The green-fluorescent image (total cells) was used as a template over the red-fluorescent image (dead cells), permitting identification and enumeration of solely green-fluorescent bacteria (live cells). The bacterial total abundance and biomass were multiplied by the percentages of live cells to estimate the live cell abundance and biomass.

2.1.2 Flow citometry

Flow citometry represents a faster and more reliable cell counting method in comparison to microscopy. This technique was utilize to estimate cell abundance after the extraction procedure (see chapter 3.1). Subsamples of purified sediment slurry (1.5 ml) were amended with freshly prepared 1% paraformaldehyde plus 0.05% glutaraldehyde (final concentrations), incubated for 20 min at room temperature in the dark, and then stored at -80°C (Gasol and Del Giorgio, 2000). Before the analyses, samples were thawed, 400 µl was stained with SYTO13 (5 µM final concentration; Molecular Probes), left in the dark for 15 min and run through a Becton Dickinson FACScalibur flow cytometer with an argon laser emitting at 488 nm. Ten microliter of a standard 0.97-µm fluorescent bead suspension (Polyscience) was added to the sample just before counting as an internal reference. The absolute concentration of beads in the suspension was determined by microscopy and the ratio of cells to the beads was used to compute bacterial concentration in the sample. To avoid coincidence, samples were run at various flow rates to keep the number of events below 1000 per second. Data were acquired in log mode until 10000 events had been reached. Bacteria were detected by their signatures in a plot of 90° side light scatter versus green fluorescence (FL1). Different clusters of low and high DNA bacteria were separated in the SSC versus FL1 plot. Cytometric noise was discarded both by setting a threshold on FL1 and by manually separating noise from cells in the FL1 versus FL3 (red) plot (Gasol et al., 1999). Data acquisition was performed with CELL QUEST software and data analysis with PAINT-A-GATE software (Becton Dickinson).

2.2 Bacterial diversity and community composition

2.2.1 FISH with fluorescently monolabeled probes

Fluorescence *In Situ* Hybridization (FISH) with monolabeled oligonucleotide probes was utilized to initially describe the bacterial community composition, in accordance with the protocol of Pernthaler et al. (2001). Despite the lower efficiency in comparison to other *in situ* hybridization techniques, the applied FISH procedure is considerably fast and therefore recommended when many samples need to be promptly analyzed (e.g. environmental monitoring; chapters 3.2 and

3.4). Filter sections were hybridized by the oligonucleotide probes described in Table 2.1. For the *beta-* and *gamma-* subclasses probes unlabeled competitors were used to grant specificity of hybridizations. Stringent hybridization conditions were established by adequate concentrations of formammide in the hybridization buffer (see Table 2 in Glöckner et al., 1999). All probes, 5'-labelled with Cy3 dye, were commercially synthesized (Biomers.net, Ulm, Germany). After hybridization the filter sections were stained with DAPI and mounted on microscope slides in VectaShield medium (Vector Lab., Burlingame, CA, USA). The relative abundance of hybridized cells was estimated as the ratio of hybridized cells counts to counts of DAPI-stained cells.

Target taxa	Probe	Sequence (5'-3')
Bacteria	EUB338	GCTGCCTCCCGTAGGAGT
Bacteria	EUB338-II	GCAGCCACCCGTAGGTGT
Bacteria	EUB338-III	GCTGCCACCCGTAGGTGT
Alpha-Protebacteria	ALF1b	CGTTCGYTCTGAGCCAG
Alpha-Protebacteria	ALF968	GGTAAGGTTCTGCGCGTT
Beta-Proteobacteria	BET42a	GCCTTCCCACTTCGTTT
Gamma-Proteobacteria	GAM42a	GCCTTCCCACATCGTTT
Cytophaga- Flavobacterium	CF319a	TGGTCCGTGTCTCAGTAC
Planctomycetales	PLA46a	GACTTGCATGCCTAATCC
Actinobacteria	HGC69a	TATAGTTACCACCGCCGT
Firmicutes	LGC354a	TGGAAGATTCCCTACTGC
Firmicutes	LGC354b	CGGAAGATTCCCTACTGC
Firmicutes	LGC354c	CCGAAGATTCCCTACTGC

Table 2.1. Oligonucleotide probes used in this study. Further details on the above-mentioned probes are available at *probeBase* (Loy et al., 2003).

2.2.2 CARD-FISH

FISH in combination with signal amplification by catalysed reporter deposition (CARD-FISH) was proposed as a more sensitive technique for microbial community analysis. Specific methodological improvements were tested. Briefly, dried filter sections were embedded in low gelling point agarose, in order to avoid cell loss, and subsequently dried at 35°C for 15 min. Selected sub-samples were initially treated with various permeabilization procedures. Starting from the protocol of Sekar et al. (2003), permeabilization treatments

included: (i) digestion by lysozyme (Sigma-Aldrich) 10 mg ml^{-1} dissolved in 0.05 M EDTA (pH 8) and 0.1 M Tris-HCl (pH 7.4) for 60 min at 37°C followed by incubation with achromopeptidase (Sigma-Aldrich) (60 U ml^{-1}) in a buffer containing 0.01 M NaCl, 0.01 M Tris-HCl (pH 8.0) for 30 min at 37°C; (ii) higher concentration of lysozyme (20 mg ml^{-1}) followed by incubation with different concentrations of achromopeptidase (60, 120, 240 U ml^{-1}). Best results were achieved by a combination of lysozyme digestion for 60 min (20 mg ml^{-1}) followed by incubation with achromopeptidase at a concentration of 120 U ml^{-1} (see chapter 3.2). We also tested a new cell permeabilization protocol maintaining the pre-treatment with lysozyme, but replacing achromopeptidase by proteinase K (0.1 U ml^{-1} in Tris-EDTA buffer), as proposed for marine prokaryotes (Pernthaler et al., 2002a; Sekar et al., 2003). This protocol allowed an improved detection of gram-positive *Actinobacteria* (see chapter 3.3).

After permeabilization, filters were incubated in 0.01 M HCl at room temperature for 10 min in order to inactivate the proteinase K and intracellular peroxidases. The stained filter sections were inspected on by epifluorescence microscopy at 1000× magnification. First, Cy3-stained cells were counted in one microscopic field, and this was followed by determination of total DAPI-stained cells. At least 500 cells were counted in >10 microscopic fields randomly selected across the filter sections. The relative abundance of hybridized cells was estimated as the ratio of Cy3-stained to total cells.

2.2.3 Analysis of 16S rRNA gene sequences

In order to gain specific insights into microbial diversity at a finer taxonomic level, a molecular approach was performed for the 16S rRNA gene sequencing. This analysis allows identifying up to bacterial specie or strain and was therefore essential for the study aim of the chapter 3.6. Briefly, two clone libraries (~100 clones) were generated after 28h and 48h of incubation. Total DNAs of water samples were extracted from polycarbonate filters according to Kirchman et al. (2001b). The specific bacterial primers 8F (GM3, 5'-AGAGTTTGATCMTGGC-3', Muyzer et al. 1996) and 1492R (GM4, 5'-TACCTTGTTACGACTT-3', Kane et al. 1993) were used for PCR amplification (max 30 cycles, Mastercycler personal, Eppendorf, Hamburg) of 16S rDNA in 100µl of DNA-polymerase buffer (Taq-Polymerase by *Thermus aquaticus*, Eppendorf, Hamburg) under standard amplification condition (Suzuki & Giovannoni 1996). The 16S

rDNA fragments were further purified with PCR gel extraction kit (QIAquick, QIAGEN, Hilden). PCR product (1.5 µl) was cloned into the pGEM®-T Easy (Promega, Madison, USA) and transformed by heat shock into chemo-competent E. coli TOP10 strain (Invitrogen, Carlsbad, USA). Partial sequences (~700 bp) were obtained by chain-termination method (Sanger et al. 1977) with the primer 518F (GM1F, 5'-CCAGCAGCCCGGTAAT-3', Saiki et al. 1988). Representative clones with correct size of the insert (~1500 bp) were selected for full sequences analysis. Three overlapping partial sequences were obtained by means of the vector primers 8F, 518F and 1492R. The software Sequencher (GeneCodes, Version 4.5) was used for multiple sequence alignments to reference sequence. About 20 most similar reference sequences were downloaded using BLAST searching on the GeneBank database. Phylogenetic relationships of the isolates and downloaded sequences were constructed by using ARB (version 2.4). Three distance methods (Maximum-Parsimony, Neighbour-Joining, and Maximum-Likelihood) were applied to optimize bootstrap analysis. The 16S rDNA gene sequences reported in the present paper have been submitted to the GeneBank nucleotide sequence database to receive specific accession numbers.

2.3 Bacterial activity

2.3.1 Bacterial carbon production and growth rate

Bacterial carbon production (BCP), measured through the incorporation of ^{3}H-Leucine and ^{3}H-Thymidine, was applied to estimate the total carbon incorporation by bacteria.

For water samples (chapters 3.1 and 3.6), triplicate 1.7-ml samples and one killed control (trichloroacetic acid - TCA; 5% final concentration) were amended with 20 nM radiotracer and incubated for 1 h at 20°C. The extraction, with TCA 5% and subsequent washing with TCA 5% and ethanol 80%, was carried out by the micro-centrifugation method, according to Puddu et al. (2003). To estimate BCP, the rates of leucine incorporation were transformed into units of C by using the conversion factor of 3.1 kg C produced per mole of leucine incorporated (Kirchmann, 2001a). Thymidine incorporation was transformed into cell production by considering the empirical thymidine conversion factor of 2×10^{18} cells mol^{-1} proposed as an average value for marine (Ducklow and Carlson, 1992) and freshwater

samples (Bell, 1993). Rates of cell production were then transformed in C production trough considering a per cell carbon content of 20 fgC $cell^{-1}$ as proposed by Lee and Fuhrman (1987).

For leucine incorporation in sediments (chapters 3.1, 3.3, 3.4, and 3.5) several methodological adaptations were needed, following the method proposed by Buesing and Gessner (2003), in combination with the micro-centrifugation technique applied to soil by Bååth et al. (2001). Briefly, wet sediment (0.5 g) was transferred into 2-ml screw-cap and heat-resistant Sorensen microcentrifuge tubes (Sorenson Bioscience, Salt Lake City, Utah, USA). Ultra-pure water (Millipore Milli-Q) was added to create a final volume of 1 ml in all tubes. A preliminary test showed that leucine saturation was reached in all sediment samples at a final concentration of 50 µM. An aqueous solution of radioactive and non-radioactive leucine (^{3}H-labelled, 0.15 µM final concentration (NEN Life Science Products, USA) and unlabeled, 49.85 µM final concentration (L8912, Sigma-Aldrich) was added to three replicates of each sediment sample. Zero-time controls were run by killing samples with 100% trichloroacetic acid (TCA, 5% final concentration), 15 min before leucine addition. Tubes were homogenized by vortexing and incubated (1 h) at the original temperature in the dark. Incubations were stopped by adding 100% TCA. All tubes were centrifuged at 14000 g for 10 min at room temperature. The supernatant was discarded to separate macromolecules from the non-incorporated label. Four washing steps were then performed adding 1 ml of 5% TCA, unlabelled leucine solution (40 mM), 80% ethanol and Milli-Q water, respectively. To extract protein 1-ml of NaOH (1 N) was finally added to the pellet and the tubes were heated (1 h at 90°C), cooled down on ice and again centrifuged. The supernatant (0.1 ml) was transferred into a 2-ml Eppendorf tube, 1 ml of liquid scintillation cocktail (Ultima Gold, Packard Bioscience, Meriden, Connecticut, USA) was added and radioactivity was detected with the TRICARB 4430 (Packard Bioscience) scintillation counter. The rates of leucine incorporation were converted into units of C per sediment dry weight (µg C h^{-1} g^{-1}) by applying the conversion factor of 1.44 kg C produced per mole of incorporated leucine (Buesing and Marxsen, 2005). Considering the exponential growth model (Koch, 1994) and the live cell biomass (BB_{live}), bacterial growth rates ($\mu = \ln((BB_{live} + BCP)/BB_{live})/h$) and turnover times ($T_2 = (\ln 2)/\mu$) were calculated. The per-cell specific

production (BCPs in fg C h^{-1} $cell^{-1}$) was computed by dividing C production by live cell abundance.

2.3.2 Extracellular enzyme activity

The estimation of the potential extracellular enzyme activities (EEA) were performed fluorometrically (Jasco, FP-6200 spectrofluorimeter) using fluorescent substrates, providing essential information about the mineralization of nutrients and organic matter by benthic bacteria. According to Wobus et al. (2003), 300-mg aliquots of sediments (see chapter 3.4), were diluted with NaCl 0.14M in the ratio 5:1 (g ml^{-1}) and vortexed before and after substrate addition (four replicates per sample). All activities were measured at saturating concentrations (final concentrations: leucine-4-AMC 1mM, 4-MUF-P-phosphate 0.3 mM; 4-MUF-beta-D-glucopiranoside 0.5 mM; 4-MUF-oleate 0.5 mM).Incubations were performed in the dark for 1h (1.5 h for aminopeptidase) at 20°C, under continuous shaking. Substrate blanks were also incubated after boiling the sediments at 100°C for 20 min. After incubation, 1ml of glycine buffer (0.1 M, pH 10) were added to the phosphatase, beta-glucosidase and lipase assay and 1ml Hepes (0.1M, ph7.5) to the aminopeptidase assay. Samples were centrifugated at 7000xg at 6°C for 10min. After the correction of the fluorescence trough the blanks, fluorescence in the supernatant was measured with a standard alkaline solution of AMC (7-amino-4-fluoromethyl-coumarin; at 380-nM excitation, 440nm emission) or MUF (4-methyl-umbelliferone, at 365-nM excitation, 455nm emission). In the water colonization experiment (see chapter 3.6), aminopeptidase and alkaline phosphatase activities were determined at each sampling time-points, as described by Hoppe (1993). Incubations were performed in triplicate (2 ml), in the dark for 2 h at 20°C with enzyme substrates at saturating concentrations (leucine-4-AMC, 250 μM), alkaline phosphatase (4-MUF-P-phosphate,125 μM). Blanks with sterilized artificial freshwater were processed in parallel. The potential hydrolysis rates were calculated following calibration of the fluorescence reading with standards of 7-amino-4-fluoromethyl-coumarin (AMC, at 380-nM excitation, 440nm emission) and 4-methyl-umbelliferone (MUF, at 365-nM excitation, 455nm emission).

2.3.3 DNA-synthesizing cells (BrdU-FISH)

The frequency of DNA-synthesizing cells in different populations was determined by incubation with the halogenated

thymidine analogue bromodeoxyuridine (BrdU) (Pernthaler and Pernthaler, 2005). In combination with CARD-FISH, BrdU-FISH was applied to evaluate the number of replicating cells within a specific bacterial cluster (chapter 3.6). Duplicate water samples (10 ml) were supplemented with BrdU (20 µM) and Thymidine (33 nM) and incubated in the dark at room temperature with mild shaking for two hours and subsequently fixed (FA, 2% final concentration). Afterwards, sub-samples were filtered on polycarbonate membrane filters (pore size, 0.2 µm, 47 mm diameter, Nuclepore) and the preparations were washed with 20 ml of sterilised Milli-Q water. The filters were stored in Petri dishes at -20°C until further processing. Control samples were fixed immediately after sampling and then supplemented with BrdU and thymidine.

2.4 Field study: sampling and sediment characterization

Figure 2.1. Geographic localization of sampling sites. Rivers characterized by temporary (large dots) and permanent (small dots) water regime were selected (see text).

2.4.1 Sampling in rivers characterized by stable water flow conditions

For methodological improvements (chapter 3.1), sediment samples were collected from the upper reach of River Cremera (Formello, Lazio; 42°05'N, 12°23'E), located within the Veio Regional Park (Figure 2.1). This stream is a tributary of River Tevere and has a

length of 37 km, with a catchment extension of approximately 36 km^2 (average elevation of 178m). For the study reported in chapter 3.2, the upper reaches of three streams (River Albegna, River Ente, and River Fiora), located in the same geographical area (Amiata Mountain declivity, Tuscany; 42°53'N, 11°37'E) and with similar hydro-morphological characteristic, were selected based on the different level of anthropogenic influence in the catchments (Figure 2.1). The Albegna catchment, up-stream the sampling area, is covered by natural woods; the upper reach of Ente flows through an area with small villages and small-scale agricultural activities; the selected site at Fiora is situated immediately downstream the Santa Fiora village and receives treated wastewater from a small meat-processing factory. Both Albegna and Fiora upper reaches are part of the Italian Regional network for monthly monitoring; the Integrated Biological Index (APAT and IRSA-CNR, 2003), based on macroinvertebrate community analysis, for the years 2001 and 2002 confirms that the Albegna upper reach belong to the highest water quality class (1st out of five), whereas Fiora is more impacted belonging to the 2nd and 3rd classes (ARPAT, 2002).

Sampling was carried out in mid winter in order to describe the microbial communities that colonize the riverbed in the season when hydrological variability is minimal. Winter was also the period in which all the selected detritus types were most abundant. Three independent replicates of each detritus type were collected in each river; five detritus types (FBOM-L, FBOM-S, Bio-brown, leaves and root debris) were sampled from stream depositional areas (pools) and one (Bio-green) in transport areas (riffles) (Figure 2.2). Sediment samples (about 50 ml) were collected from the top layer (5 cm thickness); biofilms were scraped off from 100 cm^2 of stone surface with a toothbrush; leaves and small roots were gathered by hand. For chemical characterization, additional samples were collected and frozen. Sediments for grain-size characterization were sampled from a standard surface of 150 cm^2 (5 cm thickness) and sieved 2 - 0.5 mm. Each detritus type and each sediment grain size class were dried for 72h at 60°C, weighted, ashed 3 h at 550°C, and re-weighted to determine the ash free dry weight (AFDW). The percentages of total carbon (C_{tot}) and nitrogen (N_{tot}) were determined using a Carlo Erba NA 1500 CHN Analyzer. Organic carbon (C_{org}) was determined after subsamples acidification with 2N HCl. All analyses were performed in triplicates and values were expressed as percentage of dry weight.

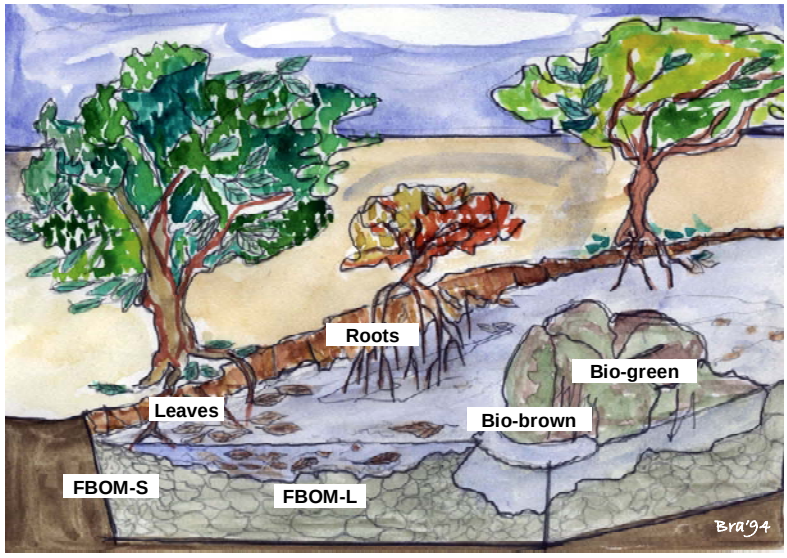


Figure 2.2. Different types of riverine detritus colonized by bacterial community: Fine benthic organic matter from patches dominated by large gravels (FBOM-L) and by small gravels (FBOM-S), biofilm on stones in pools (Bio-brown), biofilm on stones in riffles (Bio-green), Leaves and Roots debris.

2.4.2 Sampling in the temporary river Mulargia

Across the southern European catchments adopted as study sites by the TempQsim project, we selected the River Mulargia (Figures 1.3 and 2.1), a tributary of the River Flumendosa located in the southeastern part of Sardinia (Italy; 39°38'N, 09°11'E). The main reach has a length of 18 km, with a catchment extension of approximately 70 km^2, spanning an elevation range from 250 to 750 m. The river network has an overall length of around 44 km, while the distance from the origin of the stream to the outlet in the reservoir is around 15 km. The morphology of the catchment reflects the very complex geology of the area. Quite commonly carbonaceous layers are interleaved with volcanic formations. Overburden comprises mostly sandy-loam and clay soils whose thickness is variable with a maximum of about 1 m. The temperature regime is typically Mediterranean, with maxima reaching around 40°C and minima seldom below 0°C. The average daily temperature range is around 10°C and it is usually higher in summer. The climate in the area is characterised by an average annual rainfall of around 530 mm mostly concentrated in autumn and winter with usually very dry summers. The strong seasonality of rainfall

events is reflected by the intermittent flow regime of the river, which can become completely dry during summer months (Figure 2.3). However, rainfall amounts vary greatly in space and in time, with much of the annual total pattern concentrated in few. The landscape in the Mulargia catchment is moderately influenced by man's activities. There are three small villages in the catchment, with a total population of around 4,500 inhabitants. Most of the land is used for pasture with a small amount of arable land, whereas industry is largely absent. The River Mulargia delivers to the downstream reservoir low polluting loads on an annual basis. Nevertheless, these loads can become very heavy in correspondence of flood events. The flood events play a great role in the catchment: even if first flushes are accountable only for 10% of total annual flow, they are responsible for the greatest portion of suspended solids and dissolved nutrient transport (Diliberto et al., 2005).

Sediments were sieved (2-mm mesh) immediately fixed after sampling at the river outlet section. The <2-mm fraction was characterized in terms of organic matter by measuring ash free dry weight (AFDW), organic carbon (C_{org}), total nitrogen (N_{tot}) and organic phosphorous (P_{org}), expressed as percentage of dry weight (see chapter 3.4).

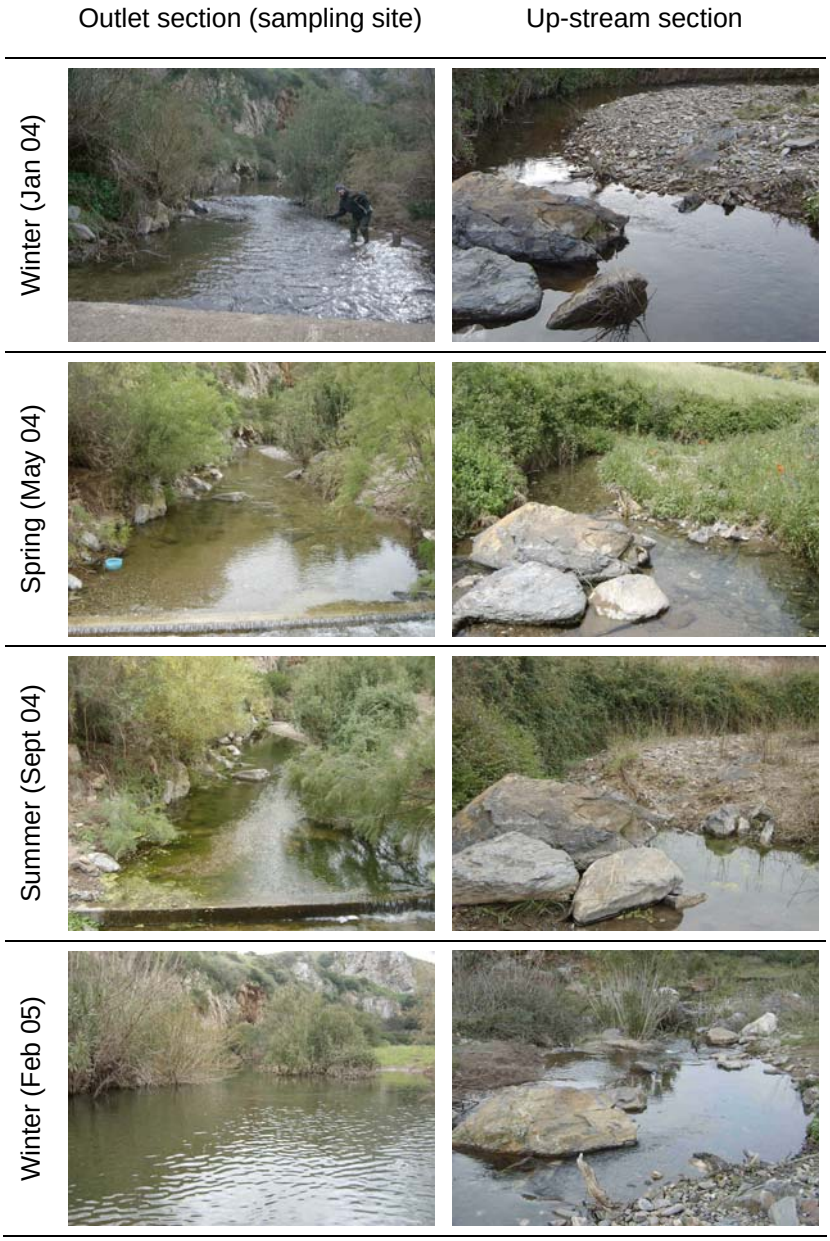


Figure 2.3. Seasonal variability of the Mulargia water regime during sampling survey at the river outlet section. The unexpected lack of drought conditions in the summer 2004 and the low autumnal and winter precipitation patterns produced stable water flow conditions in all the catchment basin.

2.5 Laboratory experiments: microcosm set up

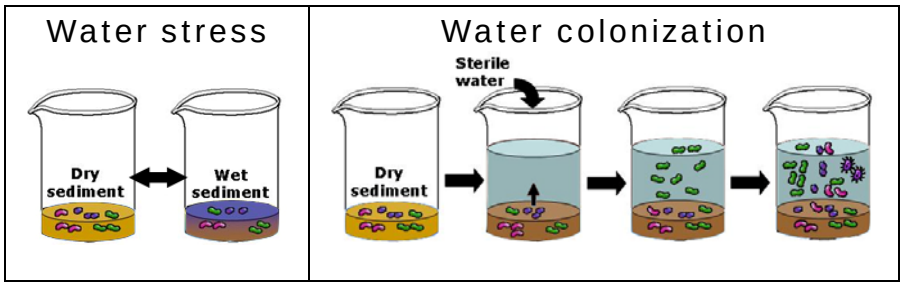


Figure 2.4. Schematic set-up of the microcosm experiments (see text).

2.5.1 Water stress experiment

For the water stress experiment (chapter 3.5), sediments were collected from River Mulargia and from three temporary rivers located in different Mediterranean regions (Figure 2.1), studied by other European partners in the framework of TempQsim project. These selected rivers are typically characterized by the intermittency of the water flow and experience recurrent dry phases of varying duration and spatial extent, usually between June and September (TempQsim-consortium, 2006).

The River Tagliamento (Italy; 46°01'N, 12°55'E) drains an area of approximately 2600 km^2 and flows for 172 km to the northern Adriatic Sea. This braided gravel-bed river is considered the last morphologically intact river in the Alps that has escaped intensive river management. Its hydrology is characterized by a flashy pluvio-nival flow regime with peaks in spring and autumn. First and second order stream segments are intermittent and dry at the surface during low flow periods in winter and summer (Tockner et al., 2003). Sampling took place in an island along a braided reach located in the middle of the river course. The River Krathis (Greece; 38°09'N, 27°17'E) is situated in northern Peloponnesus and flows into the Corinthian gulf. Its length is 30 km and the catchment covers an area of approximately 150 km^2. Urban areas and agricultural plains characterize the lowland region. Sediment samples were collected in the northern part of the river that dries out in late summer-autumn (Tzoraki et al., 2004). The River Pardiela (Portugal; 38°26'N, 07°41'W), a temporary tributary of the long transfrontier River Guadiana, has a catchment area of

approximately 510 km^2 and belongs to the Degebe basin. Like many small streams in the region, during summer months, there is a transition from lotic to lentic conditions (Lillebø et al., *in press*), through fragmentation of the flow channel into a series of isolated pools, where samples were collected.

Sediments were sampled from the uppermost oxic layer (0.5–5 cm depth) of various homogeneous patches and immediately sieved (2-mm mesh). The <2-mm fraction was stored (~3 dm^3) in plastic buckets and kept refrigerated (4°C) until further processing.

The effect of drying on microbial community was analyzed on the four rivers sediments sampled at the end of May 2004. Eight microcosms (two replicates for each river) were set up by placing 300 g of wet sediment (100% of WHC) in 1-liter pre-weighed and acid washed glass beaker (Figure 2.4). All microcosms were incubated at a constant temperature (20°C) in the dark, covered with multilayer sterile gauzes to avoid external contamination yet allowing water evaporation. The experiment ran for 46 days between June and July 2004. The microcosm gross weight (sediment and beaker) was determined regularly to monitor water losses by evaporation, but avoiding excessive sediment manipulation. For microbiological analyses, 20 g of sediment were sampled from each vessel when there was an approximate 30% reduction in moisture. At each sampling, the exact water content was measured as the difference between wet and dry sediment weight. At the beginning of the experiment, owing to practical constraints in collecting homogeneous wet sediment samples, the measured water content was 81.6% ± 8.6% of WHC. Thereafter it decreased to 72.3% ± 2.5% and 32.0% ± 4.8% of WHC at days 17 and 32 respectively, until complete desiccation at day 46 (0% of WHC) (see Figure 4.5.1). Ash free dry weight (AFDW) concentrations and bacterial community composition were assessed at the beginning and at the end of the experiment. For each sediment sample, the electrical conductivity (EC) was determined in a sediment slurry (10 g of dry sediment to 25 ml of filter-sterilized Milli-Q water) after shaking and allowing the suspension to settle for 30 min. The pH was determined in the same slurry amended with $CaCl_2$ (0.01 M). The maximum water holding capacity (WHC) was determined empirically as the ability to retain water against gravity (Hillel, 1971). To estimate the organic matter (OM) content, the ash-free dry weight (AFDW) was determined by subtracting ash weight (500°C, 3 h) from dry weight (105°C,

overnight). Grain size distribution was determined and computed in accordance with the soil textural triangle (Gerakis and Baer, 1999).

The effect of rewetting on microbial community was analyzed on the four rivers sediments sampled in November 2004. All sampled sediments from the four rivers exhibited similar WHC (20% - 25% of wet sediment weight). Wet sediments were initially air-dried to remove the excess of water. The complete desiccation (0% WHC) was reached artificially in the oven at 40°C in order to simulate natural drying conditions, when considering the maximum air temperature (~ 40°C) and average daily temperature drift (>10°C) during Mediterranean summer. Eight microcosms (two replicates for each river) were set up by placing 300 g of dry sediment in 1-liter pre-weighed and acid washed glass beaker (Figure 2.4). Afterwards, Milli-Q water was added up to reach sediment saturation (>100% WHC) as to simulate a natural rainfall event. All microcosms were incubated at a constant temperature (20°C) in the dark and covered with multilayer sterile gauzes to avoid external contamination. Water loss by evaporation was regularly monitored by measuring the gross weight of the microcosms (sediment and beaker). During the experiment, sediment water content never dropped down the 100% WHC. For microbiological analyses three sediment sub-samples were collected from each vessel at the initial time and after 1, 2, 4, 8, 11, 15 and 23 incubation days. Community composition was assessed at the beginning and at the eighth day.

2.5.2 Water colonization experiment

Sediments were collected in February 2005 at the outlet section of the River Mulargia. Dry sediment (200 g) was placed in triplicate sterile beakers and 2 l sterilized artificial freshwater ($NaHCO_3$ 196 mg l^{-1}, $CaSO_4 \times 2H_2O$ 120 mg l^{-1}, $MgSO_4$ 120 mg l^{-1}, KCl 8 mg l^{-1}) was gently added to avoid re-suspension (Figure 2.4). Water samples were collected right the introduction of sterile water (0 h) and at 9 h, 28 h, 48 h. One extra water sampling was carried out at 72 h to have indications on how the microbial community developed after the end of the experiment. An additional microcosm was sterilised by autoclave as control. All microcosms were incubated at a constant temperature (20°C) in the dark, and they were covered to avoid airborne contamination. The water phase of the microcosms was aerated by 0.2 μm filtered air. Dissolved organic matter (DOC) was monitored during the experiment by TOC 5000 Shimadzu analyser (see chapter 3.6).

3 RESULTS AND DISCUSSION

3.1 Optimization of the procedure to extract bacterial cells from freshwater sediments

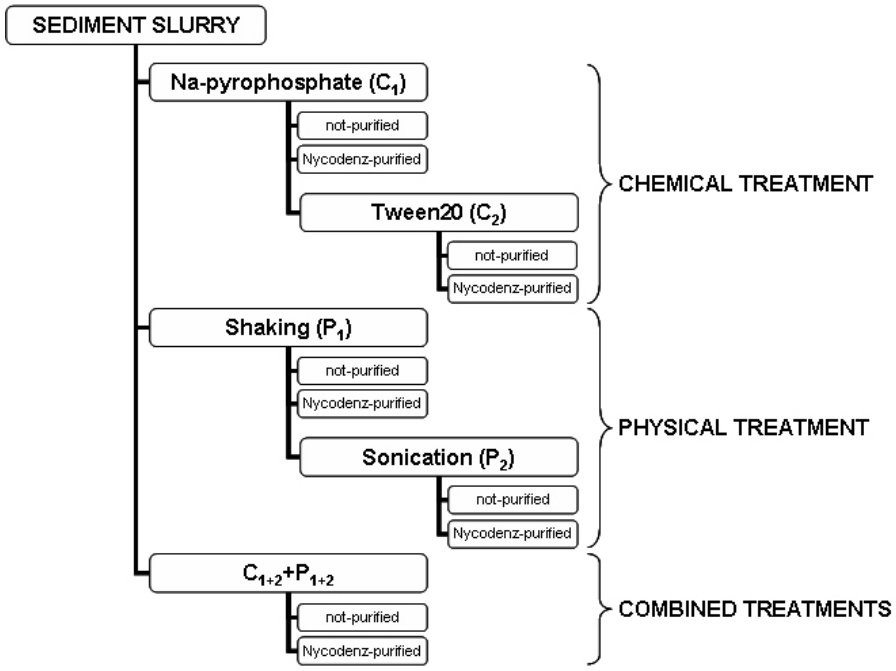
Currently, high purity of the extracted bacteria is required for the advanced application of epifluorescence microscopy (e.g. Griebler et al., 2001), flow citometry (Kalyuzhnaya et al., 2006; Duhamel and Jacquet, 2006), and molecular biological methods (Courtois et al., 2001; Maron et al., 2006). Even following an appropriate dislodgment procedure, the simple and widely used dilution of well-dispersed cell suspension cannot completely exclude detritus masking (Gough and Stahl, 2003). Thus, the reduction of sediment particles prior to microbiological analyses is advantageous to avoid most methodological biases (Boenigk, 2004). The straightforward applicability of fluorescent dyes and probes is limited when non-specific binding to mineral particles may occur. In addition, contaminating impurities such as extracellular DNA and humic substances may interfere with the direct extraction of nucleic acids and gene fragments (i.e. 16S rDNA). Previously described procedures stand on a proper combination of chemical and physical treatments in order to achieve an adequate extraction efficiency with minimal cell loss.

In this work, a common applied detachment procedure was optimized for freshwater sediments in order to assess the relative efficiency of purification and the viability of purified bacteria, as defined by the evaluation of carbon incorporation activity. Natural freshwater samples were used as a particle-free control to estimate the potential damaging effects of the applied procedure in altering bacterial integrity and activity. The successful utilization of flow citometry on sediment purified cell suspension allowed a rapid and multiparametric analysis on the extracted cells.

Effects of detachment and purification procedure

Riverbed sediments from the River Cremera were collected and immediately sieved on 2-mm mesh, in order to the exclude large detritus fraction, wherein microbial dynamics are mainly regulated by biofilm formation (Davey and O'Toole, 2000). Aliquots of sediment (1 g, corresponding to 0.5 cm^3) were placed in a sterile 15 ml falcon tube and diluted (1:10) in PBS buffer solution containing 2% formaldehyde. Data were expressed per cm^3 of sample to allow a direct comparison

among results from sediment and water analyses. All tests were repeated in three replicates. Various combinations of chemical and physical treatments were performed as described in the Scheme 3.1.1.



Scheme 3.1.1. Schematic diagram for the extraction procedure

The chemical treatment facilitates the loosening of the strong hydrogen bindings, van der Waals, electrostatic and chemical forces that tie cells and particles together (Bakken and Lindahl, 1995). The use of Na-pyrophosphate (C_1 - 0.1M final concentration) as chelating agent was tested, also in combination with Tween20 (C_2 - 0.5% final concentration) as detergent. The physical treatment is used to mechanically disrupt the sediment small pores in which bacteria are entrapped (Epstein and Rossel, 1995; Buesing and Gessner, 2002). Orbital shaking (P_1 - 30 min; 720 rpm) was tested, also in combination with sonication (P_2 - 1 min; 20 W).

Cell recovery from sediment varied considerably with regard to the number and type of applied treatments (Figure 3.1.1). Both the first single chemical (C_1) and physical treatments (P_1) did not statistically increase cell numbers with respect to the untreated sample ($2.3\times10^7 \pm 0.3\times10^7$ cell cm^{-3}; $P > 0.05$, Student *t*-test). Conversely, cell counting

following the use of doubled treatments was significantly higher by two folds for chemical, up to three folds for physical combination ($P < 0.05$). The use of Tween20 (C_2) and particularly of sonication (P_2) appeared the most effectives for cell detachment. When combining all treatments (C_{1+2}+P_{1+2}), bacterial abundance reached the highest value ($1.8\times10^8 \pm 0.3\times10^8$ cell cm^{-3}), proving that the extraction efficiency strictly depends on the mutual effects of chemical and physical treatments. In this respect, many authors have been proposed new methods to detach bacterial cells from different surface materials, mostly soil but also sediment and organic aggregates (Buesing and Gessner, 2002; Bockelmann et al., 2003; Boenigk, 2004; Lunau et al., 2005). However, there is no agreement on which procedure gives the best results in relation to different types of substratum. All procedures required a specific optimization with respect to the structural features of the environmental sample. An important detail to consider is that the harshness of extraction conditions may result in a significant cell disruption. This is especially true for sonication treatments (Epstein and Rossel, 1995). The power acting on the sample must be high enough to achieve near-complete detachment of cells from their matrix, but below the level where notable cell loss occurs. The power is influenced by the type and settings of the instrument, the size of the energy-transducing device (e.g. tip diameter of ultrasonic probes) and the proximity of the sample to the power source (Epstein and Rossel, 1995, Mermillod-Blondin et al., 2001).

In view of that, the potential damaging effects of the applied procedures in altering bacterial integrity were assessed on free-living cells in a particle-free water control. A natural freshwater sample (1 liter) was filtered through a 0.8-µm polycarbonate filter (Nuclepore) to remove particulate material as well as larger microorganisms and attached bacteria, which may interfere with the analyses. Aliquots of filtered water (1 cm^3) were placed in a sterile 15 ml falcon tube and fixed with 9 ml of PBS solution containing 2% formaldehyde. Same combinations of chemical and physical treatments were tested (Scheme 3.1.1; Figure 3.1.1). Interestingly, the highest values of bacterial abundance were detected in the untreated sample ($1.8 \times 10^6 \pm 0.3 \quad 10^6$ cell cm^{-3}). Thus, all single (C_1, P_1) or doubled (C_{1+2}, P_{1+2}) treatments appeared to negatively affect cell integrity. Physical treatments and particularly sonication ($1.4 \times 10^6 \pm 0.1 \times 10^6$ cell cm^{-3}) generated the higher cell loss comparing to the chemical combination ($1.6 \times 10^6 \pm 0.2 \times 10^6$ cell cm^{-3}). However, statistical differences were found only when

combined treatments (C_{1+2}+P_{1+2}) were performed ($1.1 \times 10^6 \pm 0.3 \times 10^6$ cell cm^{-3}; $P < 0.05$).

The results of cell recovery analysis both in sediment and water samples showed that the number and type of treatments critically affect the extraction efficiency. In sediment, the detachment procedure allowed for the highest enumeration of benthic bacteria, limiting detritus masking and preserving a better visualization of stained cells.

In addition, the methodological and unavoidable occurrence of cell loss should be considered, when the success of an investigation depends critically on accurate estimates of absolute cell numbers. In this work, the positive effects on cell recovery from sediment largely exceeded the negative effects of cell loss estimated in water samples. This difference is partially explained by considering the different status of attached and free-living bacteria. In sediment, different extracellular polymeric substances (EPS) are mainly responsible for bacterial adhesion mechanisms. Such protected extracellular surroundings, along with the physical texture of the substratum, which from an ecological point of view assure protection against noxious environmental conditions (Neu, 1996), could defend attached cells against lysis due to methodological detachment treatments. Conversely, free-living cells were likely to be more directly affected by the extraction procedure.

In this work, a further purification step by high-speed density gradient centrifugation (14,000g; 90 min; at 4°C) was performed on sediment cell suspensions after all treatments, by using the non-ionic medium Nycodenz to better separate cells and particles. The comparative analysis on water control were only performed on the untreated and full-treated samples (Scheme 3.1.1). Nycodenz purification was already successfully utilized for bacterial community analyses in soils and sediments (Furtado et al., 2000; Courtois et al., 2001; Whiteley et al., 2003). As shown in Figure 3.1.1, Nycodenz purification did not actively promote cell detachment from sediment. Purified samples never exceeded the not-purified ones in terms of bacterial abundance. This was also confirmed when purification was performed on the untreated water sample, since bacterial abundance did not significantly change ($1.6 \times 10^6 \pm 0.1 \times 10^6$ cell cm^{-3}; $P < 0.05$). Conversely, a conspicuous cell loss (74%) was observed when purification was performed on the untreated sediment. In addition, the results of single and coupled treatments confirmed that purification e × clusively works only when performed on well-dispersed cell suspension, since attached cells settled down within the pellet after

centrifugation. In this study, only sonication actively dispersed cells, as proved by the statistical correspondence of cell abundance in sediment sonicated samples before and after Nycodenz purification (P_{1+2} = P_{1+2}(N); C_{1+2}+P_{1+2} = C_{1+2}+P_{1+2}(N); $P < 0.05$). Interestingly, the highest abundance in sediment was detected by applying Nycodenz purification after the use of combined treatments ($2.0 \times 10^8 \pm 0.7 \times 10^8$ cell cm^{-3}), whereas the fully treated water showed the lowest values ($9.6 \times 10^5 \pm 0.4 \times 10^5$ cell cm^{-3}). Probably, two different procedural effects explain such discrepancy. In sediment, cell recovery after the combined treatments overcame by several times the methodological cell loss, and Nycodenz purification allowed a better visualization of stained cells. In water, extraction treatments partially affected the cellular integrity, thus allowing the Nycodenz medium to enter the cells, which would decrease the density and the flotation of cells during Nycodenz density gradient centrifugation (Maron et al., 2006).

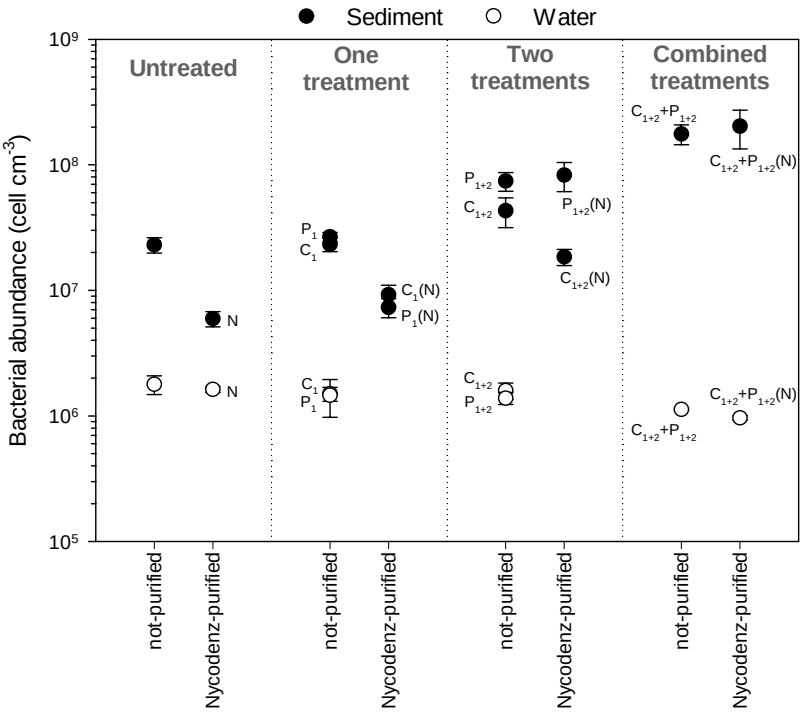


Figure 3.1.1. Bacterial abundance determined following the extraction procedure. C_1 = chemical treatment by Na-pyrophosphate; C_2 = chemical treatment by Tween20; C_{1+2} = coupled chemical treatments; P_1 = physical treatment by shaking; P_2 = physical treatment by sonication; P_{1+2} = coupled physical treatments; C_{1+2}+ P_{1+2} = combined chemical and physical treatments; N = Nycodenz-purification.

Few studies on soil samples have also assessed the physiological state of the Nycodenz-purified bacteria, either indirectly by culture-based methods either directly by detecting the incorporation of radioactive tracers and the heterogeneous staining of active extracted cells (Maraha et al., 2004; Whiteley et al., 2003; Hesselsøe et al., 2001). However, the results of these studies were contrasting, when considering bulk activity measurements. In view of that, the estimation of bacterial activity rates through the incorporation of radioactive tracers (e.g. ^{3}H-Leucine) appears to be the most straightforward approach for gaining reliable information on the bulk activity and growth rates of natural benthic assemblages.

In this work, the microcentrifugation method based on ^{3}H-Leucine incorporation (Smith and Azam, 1992) was performed to estimate bacterial activity in sediment suspension and water samples (not fixed with formaldehyde at sampling time), following the detachment and purification procedure. In line with literature reports (Buesing and Gessner, 2006), the sediment harboured ~10^{3} times the activity of the water sample (on a volume basis). As shown in Table 3.1.1, the highest rates of leucine incorporation was measured in the untreated samples, indicating negative procedural effects on the bacterial bulk activity. Leucine incorporation was almost completely depressed after Nycodenz purification, particularly in purified sediment samples.

	^{3}H-Leucine incorporation (pM h^{-1} cm^{-3})	
	Sediment	Water
Untreated	$1.3x10^{3} \pm 0.1x10^{3}$	1.349 ± 0.208
C_{1+2}+ P_{1+2}	0.014 ± 0.001	0.227 ± 0.041
C_{1+2}+ P_{1+2}(N)	0.005 ± 0.001	0.024 ± 0.003

Table 3.1.1. ^{3}H-Leucine incorporation rates measured following the detachment and purification procedure.

Efficiency of Nycodenz purification

To estimate the efficiency of Nycodenz purification an additional test on sediment was performed on the four distinct phases formed after centrifugation, on the basis of their buoyant density. The separation of bacterial cells by Nycodenz gradient centrifugation and the results obtained from six triplicate sediment samples are shown in Figure 3.1.2.

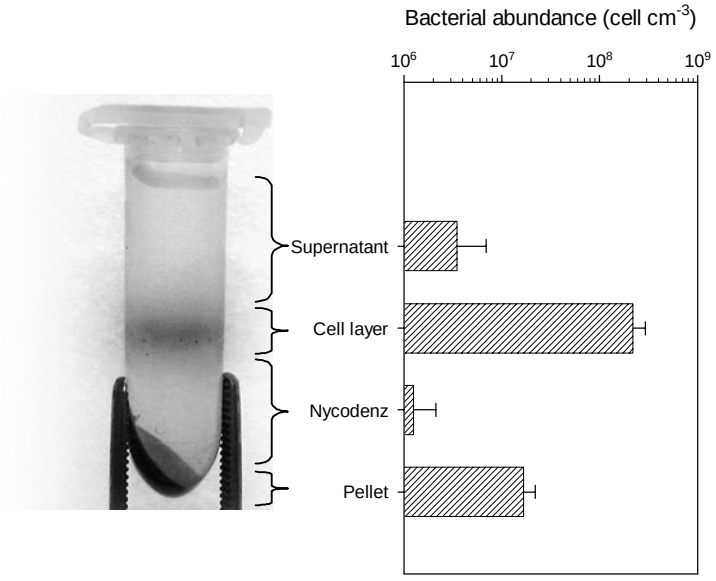


Figure 3.1.2. Phase separation of bacterial cells by Nycodenz gradient centrifugation. The graph shows the bacterial abundance (± standard deviation) estimated within each separated phase.

Fixed amounts of each phase were filtered and cells were quantified by DAPI staining. The remaining pellet was resuspended by subsequent washing with 1 ml of PBS buffer and 10 µl of the resulting slurry were counterstained.

The thin purified cell layer on top of the Nycodenz cushion was clearly visible when the sample was observed against a light source. More than 90% of the detached cells were found in this layer ($2.2 \times 10^8 \pm 0.7 \times 10^8$ cell cm^{-3}). Only 1.5% and 0.5% of detached cells were found in the supernatant and Nycodenz layers respectively. Interestingly, the pellet contained $1.7 \times 10^7 \pm 0.5 \times 10^7$ cell cm^{-3} (6.9%). In order to maximize the recovery of cells from treated samples all the liquid phases (Supernatant, Cell layer and Nycodenz) above the pellet can easily be collected and filtered. Afterward, an additional washing step with ultra-pure water is preferred to better remove impurities (e.g. crystals of salts) from the filtration membrane, which may interfere with the visualization of stained cells.

In our sediment samples, the extraction efficiency of Nycodenz purification, defined as the percentage of purified cells (abundance in the liquid phase) on total detected cells (abundance in liquid phase and pellet) determined by DAPI staining, was higher than that previously

reported for soil samples (<50%) (Mayr et al., 1999; Lindahl, 1996). However, contrasting results have brought to think that the efficiency and biases of the high-speed centrifugation approach were strongly dependent on the examined substratum (Uhlirova and Santruckova, 2003).

In order to better evaluate the Nycodenz efficiency, the tested detachment and purification procedure was applied to sediment sub-samples after granulometric fractionation. The aim was to estimate if the physical sediment texture could influence the extraction efficiency. In laboratory, three granulometric classes (coarse sand, 2-0.5 mm; medium sand, 0.5-0.2 mm; fine sand, <0.2 mm) were further distinguished by sieving on 0.5-mm and 0.2-mm meshes. The coarse sand fraction accounted for the highest percentage of sediment dry weight (87%) and organic matter content (91%), relatively to medium (6% and 4%) and fine (7% and 5%) sands. The mass of 1 cm^3 of the wet sediment was 2.05 g, 2.22 g, 2.92 g, 1.63 g for the original sediment and for coarse, medium and fine sands, respectively.

As shown in Figure 3.1.3, the number of recovered cells after Nycodenz purification was statistically equal to the number of cells observed after the combined detachment treatments ($P < 0.05$). However, higher cell numbers were always detected after high-speed centrifugation, except for the coarse sand fraction. In comparison with the results from the not-sieved original sediment, the medium and fine sand fraction showed higher abundances ($3.1 \times 10^8 \pm 0.2 \times 10^8$ cell cm^{-3}; $3.3 \times 10^8 \pm 0.6 \times 10^8$ cell cm^{-3}, respectively), whereas the coarse fraction the lowest ($1.0 \times 10^8 \pm 0.1 \times 10^8$ cell cm^{-3}). Our findings are supported by other literature studies, where finer texture of the substratum is reported to sustain higher bacterial density, as a consequence of a wider growing surface per unit of volume (Nunan et al., 2003). The efficiency of Nycodenz purification varied among the original sediment and the three sieved sandy fractions. The tested extraction procedure was less efficient (68.9%) when applied on the coarse sand sample, since more than 30% of the total detected cells settled down within the pellet after Nycodenz centrifugation (Figure 3.1.3). In this respect, few studies have highlighted that more powerful chemical and/or physical treatments are preferable in order to remove cells attached and embedded in the porous surface of coarse grains (Lehman et al., 2001; Musat et al., 2005; Wild et al., 2006). Interestingly, the highest extraction efficiency was achieved in the original sediment sample, althought it was largely composed by coarse

sand. This apparent incongruity is probably explained when considering the conspicuous presence of un-attached cells living within the sediment small pores and pore water, which physical fractionation could have removed (Ranjard and Richaume, 2001).

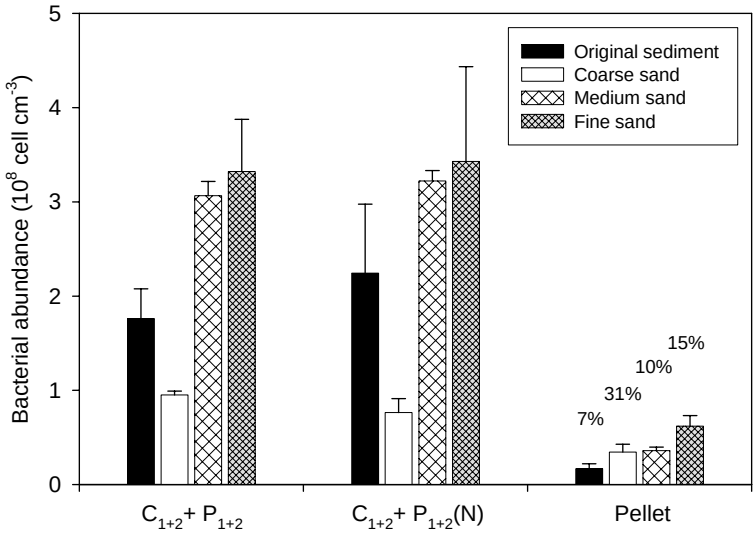


Figure 3.1.3. Bacterial abundance within different sandy fractions, after the extraction with combined treatments (C_{1+2}+P_{1+2}) and the Nycodenz purification (C_{1+2}+P_{1+2}(N)). Values indicate the percentage of total detected cells that settle down within the pellet after Nycodenz purification.

Despite the extraction efficiency variability, a purification step is in most cases necessary to better distinguish cells and particles (Figure 3.1.4a). Cell counting by microscopy was considerably faster for purified samples, as a consequence of precise settling of focus point and accurate determination of cell shape. The high quality of fluorescent images may also allow for the automated cell counting by computerized image analysis (Shopov et al., 2000). Moreover, the high purity of sediment cell suspension suggested us that flow citometry would be suitable to give reliable counts. The comparison of the data obtained by the two counting methods showed that flow citometry gave apparently greater bacterial abundance than microscopy by a factor varying around 3. This was also observed in a very recent study (Duhamel and Jacquet, 2006), accordingly to the high sensitivity of the citometric device to detect even low fluorescent signals.

Although the same number of cells were estimated in the untreated, treated and purified sediment samples by using the standard bead counts, the fraction of counted cells varied greatly (Figure 3.1.4b).

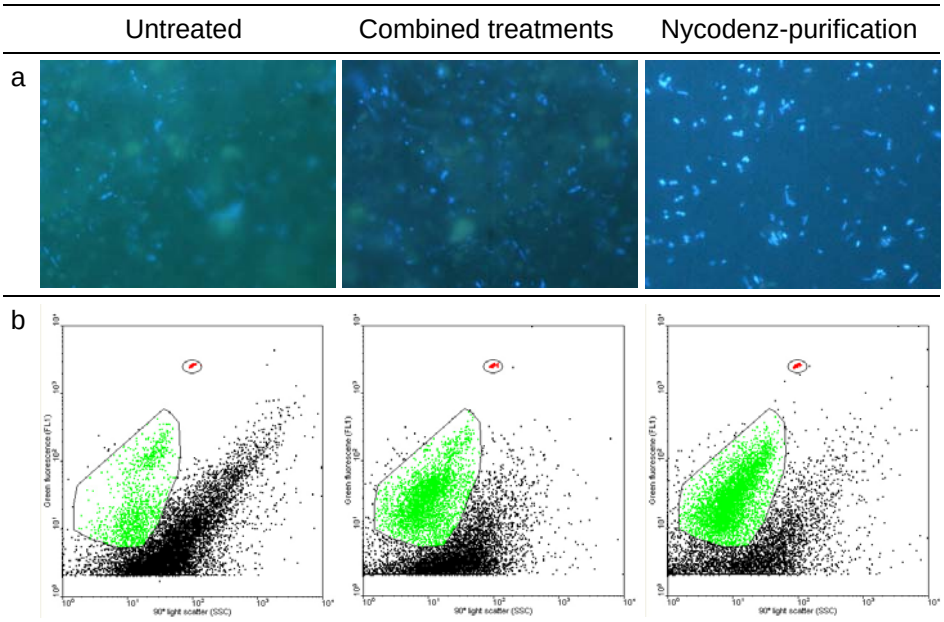


Figure 3.1.4. Sediment samples as visualized by epifluorescent microscopy (a) and flow citometry (b) in the untreated sediment sample and after detachment and purification procedure.

On 10000 total counted events, only 3.7% were gated as bacterial cells when running the untreated sample. This percentage rose by 10 times after the detachment procedure (35.8%), reaching highest values after Nycodenz purification (49.7%). These results are remarkable, when considering that one of the most intriguing potential applications of flow citometry is the possibility of direct extraction of specific subpopulations from environmental samples, omitting the cultivation step, followed by genetic or even genomic characterization (Kalyuzhnaya et al., 2006; Whiteley et al., 2003). Moreover, Button and Robertson (2001) described the evidence of a direct relationship between DNA cellular content and DAPI fluorescence detected by flow citometry. The instrument sensitivity of flow citometry makes it possible to easily discriminate two or more bacterial groups characterized by an increasing apparent DNA content (Button and Robertson, 2001; Li et al., 1995; Casotti et al., 2000). Furthermore, the

intensity of fluorescence emitted by bacterial cells after staining with the SYTO-13 nucleic acid probe can be assumed to be proportional to the cell size (Gasol and Del Giorgio, 2000) and activity (Servais et al., 1999).

In conclusion, our findings highlighted that the extraction procedure needs to be optimized according to the physicochemical structure of the analysed substratum. In view of the high efficiency of Nycodenz purification, enumeration of benthic bacteria can be reliably performed by utilizing the speed and automation of flow citometry, which is more rapid and more sensitive than microscopy. However, cell detachment and purification is to be avoided if the aim is to evaluate bacterial activity.

3.2 Hybridization techniques for community composition analysis in different benthic substrates

According to more efficient extraction procedures, accurate estimates of abundance and community structure, which are critical prerequisites for understanding benthic bacterial dynamics, are actually feasible. In this study, we analyzed benthic microbial communities associated to substrates with different organic matter content. Six types of detritus from three streams in Tuscany (River Albegna, River Ente, and River Fiora), selected for the different anthropogenic impact, were studied: 1) leaves; 2) root debris; 3) fine benthic organic matter (<0.5 mm) from bottom sediment patches dominated by large gravel >2mm (hereafter named FBOM-L); 4) fine benthic organic matter from sediment patches dominated by small gravel <2mm (hereafter named FBOM-S); 5) biofilm on stones in pools (hereafter named Bio-brown); 6) biofilm on stones in riffles, dominated by benthic microalgae and hereafter named Bio-green (except for Albegna River where this type of microhabitat was not found).

For the analysis of microbial community, we compared *in situ* hybridization with fluorescence monolabeled probes (FISH) with catalyzed reporter deposition (CARD-FISH). A suite of four rRNA-targeted probes for large taxonomic units was used for a tentative analysis of community composition. Prior to FISH, a purification step was performed by centrifugation with Nycodenz. Moreover, the permeabilization procedure for CARD-FISH was optimized, balancing permeability with cellular integrity (Pernthaler et al., 2002a).

Differences between sampling sites and detritus types

The grain size distribution of the various detrital samples showed consistent differences between the two bottom sediment patches in all the three rivers, one being dominated by large gravel (average over the three rivers: >2 mm = 81.7 ± 8.7%), the other by fine gravel (<2 mm = 85.5 ± 22.4%). The finest detrital component (<0.5 mm), used for microbial community analysis, showed a relatively low ash-free dry weight (AFDW) content significantly (two-way ANOVA, $P < 0.05$) different either between FBOM-L (average over the three rivers: 4.1 ± 0.8%) and FBOM-S (5.1 ± 0.7%) or among rivers. The organic carbon represented 42.4 ± 9.5% and 48.1 ± 10.5% of the total carbon in FBOM-L and FBOM-S respectively (Table 3.2.1). Biofilms characteristics highly differed among the rivers. In Bio-brown the

AFDW content significantly increased from Albegna (3.2 ± 0.1%) and Ente (5.1 ± 0.2%) to Fiora (7.1 ± 0.2%). The ratio of organic carbon to total carbon (C_{org} : C_{tot}) rose from 17% in Albegna and 50% in Ente to about 100% in Fiora. In Bio-green the AFDW reached even higher values ranging from 16.6 ± 1.8% (Albegna) to 40.3 ± 4.0% (Ente) with a very high C_{org} : C_{tot} ratio (79% Albegna; ~100% Ente). Root debris and leaves showed the highest AFDW content (average over the three rivers: roots 44.7 ± 4.2%, leaves 74.0 ± 3.3%), with an unvarying C_{org} : C_{tot} ratio of about 90–100% in the three rivers (Table 3.2.1).

	AFDW (%)			Total Carbon (%)			Organic Carbon (%)			Total Nitrogen (%)		
	Albegna	*Ente*	*Fiora*	*Albegna*	*Ente*	*Fiora*	*Albegna*	*Ente*	*Fiora*	*Albegna*	*Ente*	*Fiora*
FBOM-L	4.8±0.3	3.2±0.2	4.2±0.0	2.5±0.1	2.9±0.0	2.1±0.1	1.0±0.6	1.5±0.3	0.7±0.1	0.06±0.01	0.04±0.00	0.06±0.00
FBOM-S	4.8±0.2	5.9±0.2	4.5±0.2	2.8±0.2	4.3±0.2	2.7±0.0	1.4±0.3	2.4±0.4	1.0±0.0	0.06±0.00	0.16±0.02	0.09±0.00
Bio-brown	3.2±0.1	5.1±0.2	7.1±0.2	2.9±0.0	4.5±0.1	7.0±1.0	0.5±0.0	2.3±0.4	7.0±0.3	0.05±0.01	0.19±0.01	0.37±0.20
Bio-green	16.6±1.8	40.3±4.0	nd	11.0±0.5	21.9±0.2	nd	8.8±0.9	21.8±2.2	nd	1.38±0.15	3.75±0.36	nd
Roots	49.9±3.6	40.7±7.0	44.6±4.3	44.9±1.9	27.6±3.1	nd	38.6±4.5	24.4±2.7	nd	0.73±0.37	0.85±0.23	nd
Leaves	77.3±4.1	70.7±7.0	74.0±5.3	41.0±2.2	39.8±1.4	40.4±1.3	46.2±3.5	42.3±2.6	43.1±2.5	1.31±0.87	1.63±0.09	1.42±0.11

Table 3.2.1. Ash Free Dry Weight (AFDW), Total Carbon (C_{tot}), Total Organic Carbon (C_{tot}), Total Nitrogen (N_{tot}) of six types of organic matter across the 3 streams. FBOM-L= FBOM from patches dominated by large gravel; FBOM-F= FBOM from patches dominated by fine gravel; Bio-brown= biofilm on stones in pools; Bio-green= biofilm on stones in riffles dominated by benthic algae. Data are expressed as average of three replicated measurements ± 1 standard deviation. nd= not detected.

Bacterial abundance on different types of detritus

Detritus samples were fixed in buffered formaldehyde solution (3.7% final concentration) and analysed for bacterial abundance and community composition. A two-way ANOVA for three levels of rivers and five levels of detritus type showed a statistically significant difference both among rivers ($F = 8.73$; $P < 0.001$) and detritus types ($F = 41.13$; $P < 0.001$), as well as significant interaction between these two sources of variance ($F = 10.13$; $P < 0.001$). Pairwise multiple comparisons (Student-Newman-Keuls method) of bacterial cell numbers showed no significant differences between Albegna and Ente, while both of them significantly differed from Fiora. Two-way ANOVA also showed that in Albegna and Ente bacterial abundance was significantly higher in Bio-green when compared with Bio-brown. Cell counts in the detritus types with low OM content (FBOM-L,

FBOM-S and Bio-brown) ranging from 3×10^{10} to 2×10^{11} cells per gram of AFDW (in line with findings by Hudson *et al*., 1992; Findlay *et al*., 2002), were 10-fold higher in Fiora than in Albegna and Ente. Considering that AFDW slightly differed among rivers (Table 3.2.1), bacterial abundance in Fiora was even higher if expressed in number of cells per unit of dry weight.

Moreover in Fiora, FBOM and biofilms supported significantly higher bacterial abundance than debris from leaves and roots (Figure 3.2.1). This implies that the microbial densities in riverine benthic surface layers with low OM content may be related to water nutrient concentration, as suggested by Claret and Fontvielle (1997). In Fiora sampling site, downstream of a meat factory, water showed substantially higher concentrations of total phosphorous and nitrogen in comparison with the more pristine sites at Albegna and Ente (total phosphorus: Albegna 0.008 mg l^{-1}, Ente 0.038 mg l^{-1},Fiora 0.237 mg l^{-1}; total nitrogen: Albegna 0.8 mg l^{-1}, Ente 1.2 mg l^{-1}, Fiora 1.5 mg l^{-1} – Erba et al., 2004). bacterial abundance in OM-rich detritus types (roots and leaves), varying between 2×10^{10} and 6×10^{10} cells per gram of AFDW, was also in line with literature values (Findlay *et al*., 2002; Rier *et al*., 2002).

For root debris we could not find literature data for comparison. As in OM-rich detritus types no differences in bacterial abundance were observed between rivers, microbial community might be less dependent on water chemistry when growing on substrates with high organic carbon and nitrogen content (Figure 3.2.1).

These results might contribute to the definition of the types of benthic detritus to be mainly considered in the assessment of river ecosystems. To date, the standardized river monitoring systems take into consideration macroorganisms and cultivable bacteria; wider analyses on the natural microbial assemblages associated to low OM substrates, and therefore highly responsive to water quality, might open new perspectives for river assessment and for a better understanding of human impact on stream ecosystem functioning.

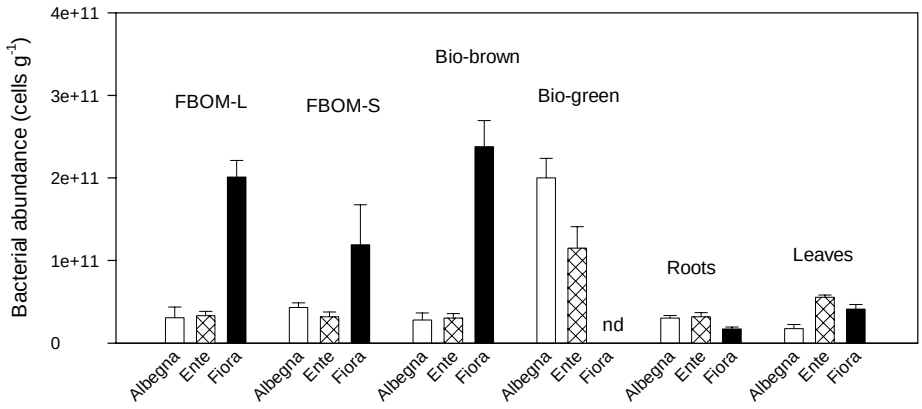


Figure 3.2.1. Bacterial abundance, expressed as number of cells per AFDW (g), for the six types of organic matter collected from the three streams. Data are means of three independent samples each analyzed in two replicates (n=6) ± standard error. nd= not detected.

Cell purification for FISH by density gradient centrifugation

A high background fluorescence and non-specific binding of probes to non-bacterial particles can hamper the enumeration of cells in sediments after FISH (Amann and Ludwig, 2000). Therefore, we performed additional cell purification by Nycodenz high-speed centrifugation, following the procedure described in chapter 3.1. Griffiths et al. (2003) argued that Nycodenz purification would permit comparison between equally treated samples even if the total cell numbers were slightly underestimated. Moreover, Nycodenz-purified cell suspensions appear to be representative of the original community (Whiteley *et al*., 2003).

The efficiency of Nycodenz extraction was tested by comparing the total cell numbers (cell ml^{-1}) in the supernatant above the Nycodenz cushion, inside the Nycodenz cushion and in the pellets beneath the cushion (re-suspended in 2 ml of ultra-pure water). In five samples from various detritus types, 90.5 ± 7.4% of the total cells were present within the supernatant above the Nycodenz cushion, about 9.0% within the cushion and merely 0.3% in the pellets (Figure 3.2.2). Thus, the cell loss during the preparations was comparatively low. Our results show that Nycodenz density gradient centrifugation highly improved the purity of bacterial cell suspensions that originate from freshwater

detritus communities, thus making the counting both faster and more reliable.

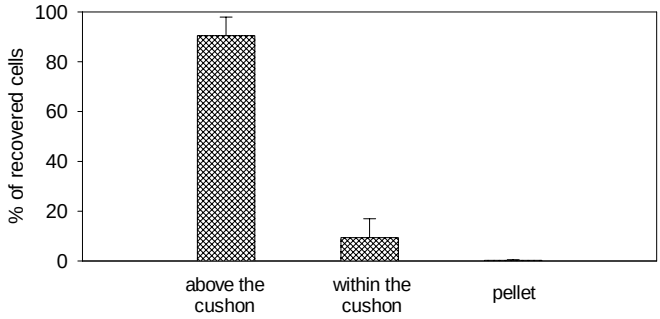


Figure 3.2.2. a) Efficiency of the Nycodenz purification step for the recovery of sediment bacteria. Data are expressed as percentage of total cells in the supernatant above the Nycodenz cushion, within the cushion and in the pellets.

Cell detection after FISH staining

Fluorescence *in situ* hybridization with fluorescently monolabelled (5'-Cy3) oligonucleotide probes, counterstaining with DAPI, and microscopic evaluation was performed according to the protocol by Pernthaler et al. (2001). The percentages of cells in Nycodenz purified cell suspensions visualized by FISH with the fluorescently monolabelled probes EUB338, EUB338-II and EUB338-III (EUB I-III), targeted to most bacteria (Daims *et al*., 1999), ranged between 19% and 60%. This is similar to values reported for freshwater sediments by other authors (Araya *et al*., 2003; Bouvier and Del Giorgio, 2003). Bacteria hybridized by probe EUB338 represented on average 34.8% of the DAPI-stained objects, while cells hybridized by probes EUB338-II and EUB338-III, which are targeted to the *Planctomycetes* and *Verrucomicrobiales* lineages, represented only 2.4%. The highest values were found in OM-rich detritus – leaves and Bio-green – (Figure 3.2.3b). The fraction of bacteria visualized by FISH in FBOM and Bio-brown was consistently higher in Fiora (range 38–41%) than in Ente and Albegna (range 19–36%); this trend was not observed for bacteria from leaf detritus. The percentage of cells detected by FISH with fluorescently monolabelled EUB I-III was significantly correlated to the detritus AFDW ($P < 0.05$; $r = 0.67$), C_{tot} ($P < 0.05$; $r = 0.66$) and C_{org} ($P < 0.05$; $r = 0.67$). These correlations were not observed for the cells detected by CARD-FISH (see below). This suggests that the sensitivity of FISH with monolabelled probes

depends, in part, on the activity state of the target cells (Figure 3.2.3b). The detection efficiency of bacterial cells by this FISH approach is related to the cell-specific rRNA content (Oda *et al*., 2000), which itself has been suggested as an index of cell activity (Williams *et al*., 1998). Rapidly growing bacteria contain higher ribosome densities (Kerkhof and Kemp, 1999; Luna *et al*., 2004) binding proportionally more probe molecules per cell (Oda *et al*., 2000). This might explain why the highest percentage of hybridized bacteria on OM-poor detritus was found in samples from the Fiora River, which also featured the highest water nutrient concentrations. Fluorescence *in situ* hybridization with signal amplification by catalysed reporter deposition, with probe EUB I-III and subsequent signal amplification with fluorescein labeled tyramides was performed basically following the protocols of Sekar et al. (2003) and Pernthaler et al. (2004). However, we optimized the permeabilization treatment for our samples from freshwater detritus (Figure 3.2.3a). It is likely that the higher enzyme concentrations (as compared with Sekar *et al*., 2003) were required because the concentration of fixative in our samples was relatively high (3.7%). The percentage of bacteria hybridized by EUB I-III after CARD-FISH ranged between 23% and 58%. There were no significant differences in the percentage of cells visualized by FISH with the fluorescently monolabelled probe or by CARD-FISH with respect to the whole data set (Mann–Whitney Rank Sum Test: $n = 14$, $P = 0.39$). However, the percentage of cells hybridized by CARD-FISH was significantly higher in samples from detritus types with low OM content – FBOM-S, FBOM-L and Bio-brown – (Mann–Whitney Rank Sum Test: $n = 9$, $P < 0.05$) (Figure 3.2.3b). In addition, cells stained by CARD-FISH showed a more intense fluorescence signal. We furthermore observed a lower overall variability of the fractions of cells that could be hybridized in the different rivers and detritus types (coefficients of variation: CARD-FISH, 23%; FISH, 31%). The percentage of cells visualized by CARD-FISH did not show any significant correlation with detritus AFDW, C_{tot}, or C_{org}. Thus, CARD-FISH results were not dependent on substrate quality. This suggests that bacterial cells could be visualized even if their activity (ribosome content) was low. Therefore, CARD-FISH with our optimized protocol likely represents a superior alternative to FISH with monolabelled probes for an accurate analysis of microbial community structure in riverine detritus. This is in agreement with reports from marine sediments (Pernthaler *et al*., 2002a; Ishii *et al*., 2004).

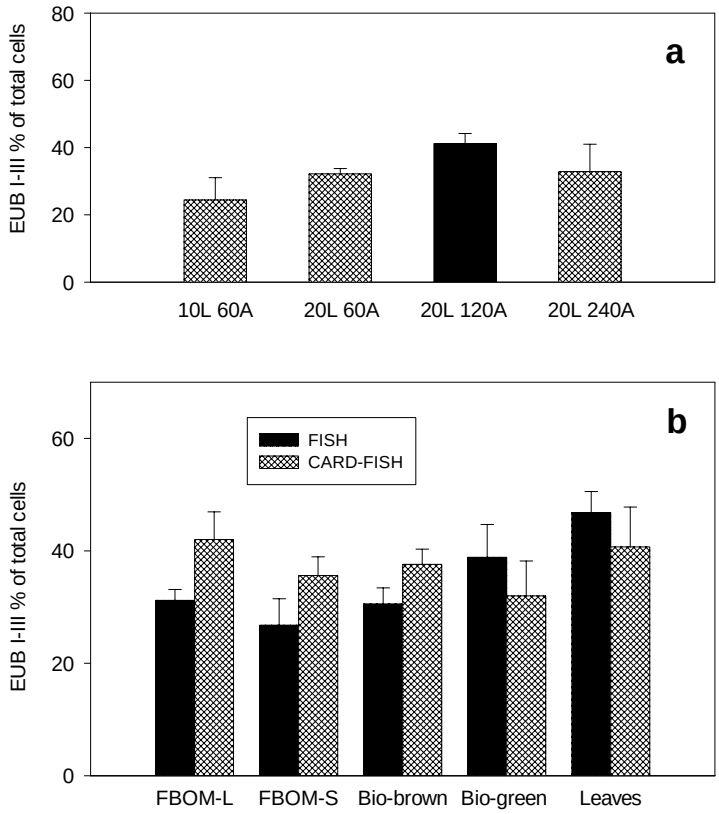


Figure 3.2.3. a) CARD-FISH detection rate of bacteria with the general bacterial probe EUB338 after incubation at different dilution of lysozyme (L - 10 and 20 mg ml^{-1}) and achromopeptidase (A - 60, 120, 240 Unit ml^{-1}). Best results were achieved by lysozyme digestion for 60 min (20 mg ml^{-1}, in 0.05 M EDTA [pH 8)] and 0.1 M Tris-HCl [pH 7.4], 37°C) followed by incubation with achromopeptidase for 30 min (120 U ml^{-1}, in 0.01 M NaCl and 0.01 M Tris-HCl [pH 8.0)], 37°C). Even higher achromopeptidase concentration (240 U ml^{-1} for 15 min) did not further increase the fraction of hybridized cells, but rather caused a disintegration of cells. The darker bar represents the enzymes concentration selected for the whole set of samples evaluation. Data are expressed as percentage of DAPI stained cells and are means of duplicates over the three rivers (n=6) ± standard error.

b) Detection of bacteria by EUB338 probe with fluorescently monolabeled probes (black bars) or by CARD-FISH (shaded bars) in the different detritus types. The order of the detritus types follows the increasing percentage of AFDW. Data are expressed as percentage of DAPI stained cells and are means of duplicates over the three rivers (n=6) ± standard error.

Bacterial community composition

A tentative community analysis was performed using the fluorescently monolabeled probes ALF1b, BET42a, GAM42a for the *alpha-*, *beta-*, *gamma-*subclasses of *Proteobacteria*, respectively, and CF319a, targeted to many groups from the *Cytophaga-Flavobacterium* (*CF*) cluster of the *Bacteroidetes* (Amann *et al*., 1995). For the probes BET42a, GAM42a unlabelled competitors were added. The description of microbial assemblages by probes for large phylogenetic lineages can only provide limited information about the complexity of the microbial community structure, as there might be different physiological types of bacteria within these broad categories. However, it can be helpful to gain first insight about the affiliation of ecologically relevant bacteria and to follow their spatial-temporal dynamics and activity in natural environments (Bouvier and Del Giorgio, 2002; Kirchman *et al*., 2004).

In our study, between 9% and 40% of the total DAPI cell counts could be identified by these four probes (Figure 3.2.4). On average 40% of the cells targeted by EUB I-III remained unaffiliated, which is in line with data from marine sediments (Llobet-Brossa *et al*., 1998). Thus, the majority of the FISH detectable bacteria could be assigned to known groups. *Proteobacteria* belonging to the *alpha-* and *beta-*subclasses formed the largest fraction of hybridized cells in the microbial communities on FBOM and Bio-brown (5.6% and 9.0% of total DAPI-stained cells respectively). Bio-green showed a higher relative abundance of *alpha-Proteobacteria* (15.4 ± 0.5 in Albegna and 21.4 ± 3.3 in Ente) and of members of *CF* (up to 6% in Albegna). In leaf detritus, *beta-Proteobacteria* and bacteria related to *CF* reached the highest densities, ranging around 16.0% and 10.4%, respectively. Members of the *CF* cluster were not found in leaves from the Ente River. The *gamma-Proteobacteria* did not constitute a numerically important phylogenetic group, forming less than 2% of DAPI-stained cells in samples from all detritus types. Bacterial community composition seemed to be related to detritus quality (Figure 3.2.4).

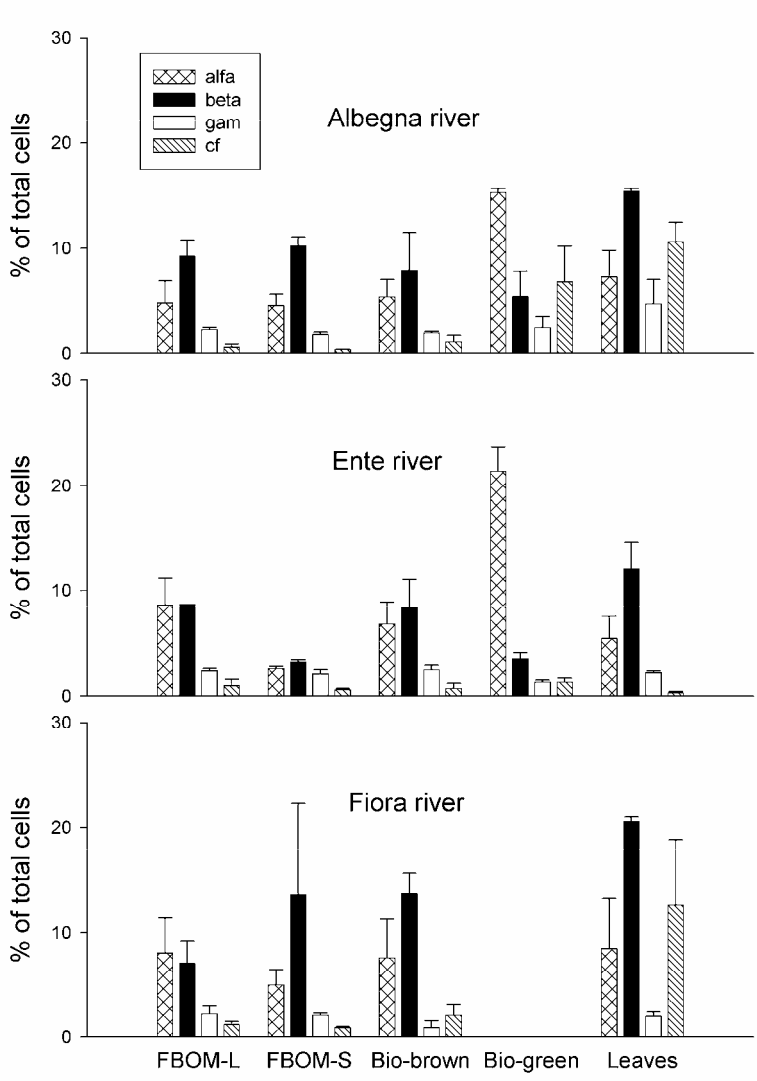


Figure 3.2.4. Taxonomic composition of the bacterial communities as analyzed by FISH. Data are values of three independent samples purified by high-speed centrifugation with Nycodenz. Values are expressed as percentages of hybridized cell counts of total counts of DAPI-stained cells. Error bars indicate the range of duplicates.

There was a significant positive correlation between the percentages of cells detected by probes for the *beta-Proteobacteria* and for *CF* cluster and AFDW (*beta-Proteobacteria*, $r = 0.55$; *CF*, $r = 0.67$; $P < 0.05$), C_{tot} (*beta-Proteobacteria*, $r = 0.56$; *CF*, $r = 0.67$; $P < 0.05$) and C_{org} (*beta-Proteobacteria*, $r = 0.58$; *CF*, $r = 0.67$; $P < 0.05$). In contrast, *alpha-Proteobacteria* were significantly correlated with detrital nitrogen content ($r = 0.82$, $P < 0.05$).

Our results largely agree with the known distribution patterns of the four phylogenetic groups in oxic freshwater sediments and on suspended particles (Spring *et al*., 2000). The prevalence of bacteria from the *beta*-subclass of *Proteobacteria* and the *CF* cluster in substrates highly loaded with organic carbon might be a general feature of microbial communities in such habitats. *Beta-Proteobacteria* are important in the plankton of lakes, rivers and reservoirs (Glöckner *et al*., 1999; Kirchman *et al*., 2004), on lake snow (Simon *et al*., 2002) and in biofilms of drinking water systems, streams and rivers (Brümmer *et al*., 2003). Such bacteria are believed to be of particular importance during early biofilms succession (Davey and O'Toole, 2000; Jackson *et al*., 2001). The presence of a large fraction of *beta-Proteobacteria* could be also attributed to their ability to oxidize ammonia or degrade organic pollutants (Araya *et al*., 2003). *Cytophaga-Flavobacteria* phylotypes have been found in freshwaters, and in pelagic and benthic marine habitats (Llobet-Brossa *et al*., 1998; Glöckner *et al*., 1999; Kirchman, 2002). They constitute a significant proportion of the microbial communities on suspended organic particles (Simon *et al*., 2002) and in stream and river biofilms (O'Sullivan *et al*., 2004). Currently, their role in OM processing is poorly understood. Some members of the *CF* cluster are specialized for the degradation of complex macromolecules (Kirchman, 2002).

Our findings highlight that the composition of microbial assemblages appears to be related to the quality of the detritus. Future studies might thus be aimed at determining which physiological properties favor the occurrence of specific bacterial taxa in benthic detritus, e.g. by combining *in situ* cell identification with activity measurements.

3.3 Hybridization techniques for community composition analysis in sediments with different moisture content

So far, investigations by FISH in freshwater sediments have been performed at conditions of water saturation only. However, it has not been considered how the low metabolism of microbes under conditions of water scarcity may affect the efficiency of this approach. *In situ* hybridization methods need to be optimized to compare bacterial communities residing in sediments, which likely feature a high percentage of cells with low activity. In experimental microcosms we investigated the efficiency of FISH and CARD-FISH as tools for the analysis of microbial communities associated to river sediments with contrasting water content. Sediments from the four Mediterranean temporary rivers were exposed to artificial changes of moisture conditions: wet sediment was dried and dry sediment was rewetted. Changes of bulk community activity were determined via the incorporation of ^{3}H-Leucine and related to the efficiency of cell detection by FISH or CARD-FISH. In addition, the cell fixation and permeabilization protocol for CARD-FISH was optimized for the visualization of *Bacteria* from dry sediments, with particular focus on the detection of gram-positive *Actinobacteria*.

Potential sources of cell loss during fixation and staining procedure

It has been suggested that total cell counts (i.e., DAPI counts) should not be directly determined from FISH preparations (Pernthaler et al., 2004) because the cell distribution on membrane filters with a diameter of 47 mm may significantly deviate from randomness. However, one can at least partially compensate for this statistical shortcoming by producing average values from counts of several pieces of such a larger filter, which are then based on both a substantially higher number of counted cells and on a larger fraction of the total filter area. In the context of our study we applied this approach only for the estimation of potential cell loss due to the combined effects of the different fixation and hybridization treatments.

While the overall variability of the DAPI counts determined from FISH-preparations was visibly higher than from standard preparations, it was nevertheless still possible to establish a significant difference between the two fixation protocols (Figure 3.3.1a). The observed differences between these treatments clearly exceeded the variability of

cell abundance from the experimental conditions (moisture content, sediment origin and sampling time-point). The mean DAPI cell counts after hybridization of formaldehyde (FA) fixed samples were comparable to those from regular DAPI counts in unhybridized preparations, whereas substantially fewer cells were counted in ethanol (EtOH) fixed samples (Figure 3.3.1a). Two-way ANOVA suggested significant differences of total (DAPI) cell numbers between fixatives ($F = 12.5$, $P < 0.05$), whereas there was no difference between hybridization techniques ($F = 0.1$, $P = 0.92$) and no interaction between these two sources of variance ($F = 0.9$, $P = 0.40$). Subsequent pair-wise SNK multiple comparisons confirmed significant differences only between FA and EtOH fixed samples ($q = 4.99$, $P < 0.05$). We assume that cell loss on filters hybridized with monolabeled probes was due to the lack of agarose embedding. By contrast, the reduced cell numbers in EtOH fixed samples after CARD-FISH appears to be related to the permeabilization strategy: the highest difference between EtOH and FA fixed samples was observed after permeabilization by proteinase K (Figure 3.3.1a). This could explain why no comparable cell loss was observed after EtOH fixation and CARD-FISH of *Bacteria*, pre-treated by achromopeptidase, from the water column of lakes (Sekar et al., 2003). EtOH fixed cells seemed to be more affected by the lack of agarose embedding and more sensitive to a stronger pre-treatment (i.e., by proteinase K).

Fixation and permeabilization strategy for CARD-FISH

We recently reported a procedure for CARD-FISH staining of *Bacteria* from freshwater sediments based on increased concentrations of lysozyme and achromopeptidase during cell permeabilization (Fazi et al., 2005). In FA-fixed samples this pre-treatment resulted in a significantly lower detection of *Bacteria* (51.5% ± 14.6%; CV = 28.4) than those by FISH with monolabeled probes (64.0% ± 11.0%; CV = 17.2) ($q = 5.28$, $P < 0.05$). Protocols for CARD-FISH staining of marine prokaryotes suggest the use of either lysozyme or proteinase K (Pernthaler et al., 2004; Teira et al., 2004). We combined lysozyme from our previous protocol, with proteinase K as a second permeabilizing agent.

This treatment resulted in percentages of hybridized *Bacteria* (64.9% ± 6.1%; CV = 9.4) comparable (but less variable) to those by FISH with monolabeled probes (Table 3.3.1, Figure 3.3.1b) and significantly higher than CARD-FISH after permeabilization by

lysozyme and achromopeptidase ($q = 5.66$, $P < 0.05$). By contrast, the pre-treatment with proteinase K clearly showed adverse effects on CARD-FISH cell detection after EtOH fixation. Altogether, EtOH fixation appears to be less efficient when combined with CARD-FISH for the staining of *Bacteria* in freshwater sediments after pre-treatment with proteinase K because it resulted in (i) reduced hybridization efficiency (Table 3.3.1, Figure 3.3.1b) and (ii) substantial cell loss (see above, Figure 3.3.1a).

EtOH has been suggested as a better fixative for the hybridization of gram-positive *Bacteria* than aldehydes, enhancing both cell wall and membrane permeability (Roller et al., 1994). In our samples, the detection of *Actinobacteria* by FISH and by CARD-FISH after a pre-treatment with lysozyme and achromopeptidase was unaffected by the fixative. By contrast, significantly more *Actinobacteria* cells could be visualized by CARD-FISH after a pre-treatment with lysozyme and proteinase K, but only in FA-fixed samples (Table 3.3.1, Figures 3.3.1b and 3.3.2). This combination thus appears to be the optimal treatment for the detection of *Actinobacteria* in freshwater sediments. We also noted that *Actinobacteria* typically represented a higher fraction of all hybridized bacterial cells in EtOH-fixed samples than in FA-fixed samples (data not shown). Considering the substantial loss of cells during this treatment (Figure 3.3.1a), gram-positive cells were likely less adversely affected by alcohol fixation than gram-negative *Bacteria*.

	FISH (EtOH)		FISH (FA)		CARD-FISH (EtOH, Pr)	
Probe EUB I-III	q'	p	q'	p	q'	p
FISH (FA)	7.70	<0.05				
CARD-FISH (EtOH, Pr)	2.64	NS	10.85	<0.05		
CARD-FISH (FA, Pr)	7.99	<0.05	0.39	NS	11.15	<0.05
Probe HGC 69a						
FISH (FA)	0.92	NS				
CARD-FISH (EtOH, Pr)	2.74	NS	4.08	<0.05		
CARD-FISH (FA, Pr)	6.66	<0.05	9.07	<0.05	3.61	<0.05

Table 3.3.1. SNK multiple comparisons of the percentage of hybridized cells by FISH and CARD-FISH with probes EUB I-III (*Bacteria*) and HGC69a (*Actinobacteria*) using different fixation. EtOH, ethanol fixation; FA, formaldehyde fixation. Pr, lysozyme and proteinase K permeabilization. NS, not significant.

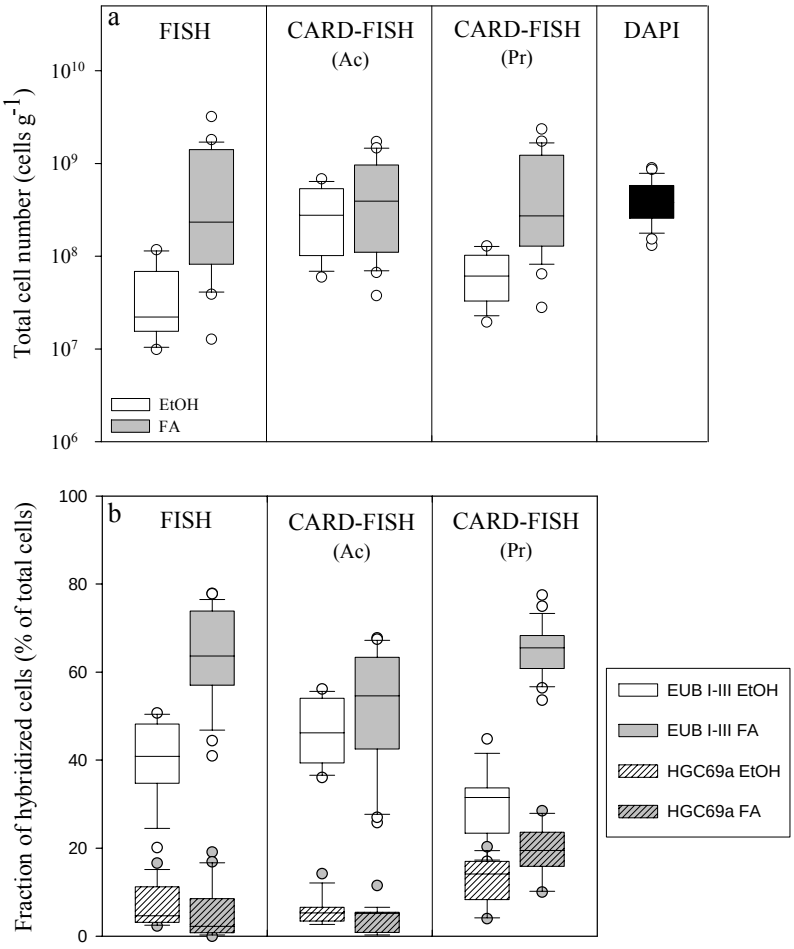


Figure 3.3.1. a) Mean values and ranges of total cell number (DAPI stained) after hybridizations on membrane filters (Ø 47 mm) with different protocols. EtOH, ethanol fixation; FA, formaldehyde fixation; Ac, lysozyme and achromopeptidase permeabilization; Pr, lysozyme and proteinase K permeabilization. The black box plot gives the abundance obtained by regular DAPI counts of un-hybridized FA fixed samples on black membrane filters (Ø 25 mm). Data are expressed in number of cells per gram of dry sediment. b) Fractions of *Bacteria* (cells hybridized by probe EUB I-III) and *Actinobacteria* (probe HGC69a) detected by FISH and CARD-FISH using different fixatives or permeabilization pre-treatments. Lines within the boxes, boundaries, error bars and dots mark respectively median, 25th - 75th percentiles, 10th - 90th percentiles, and outliers. Cleveland linear interpolation method was used to compute the percentile values.

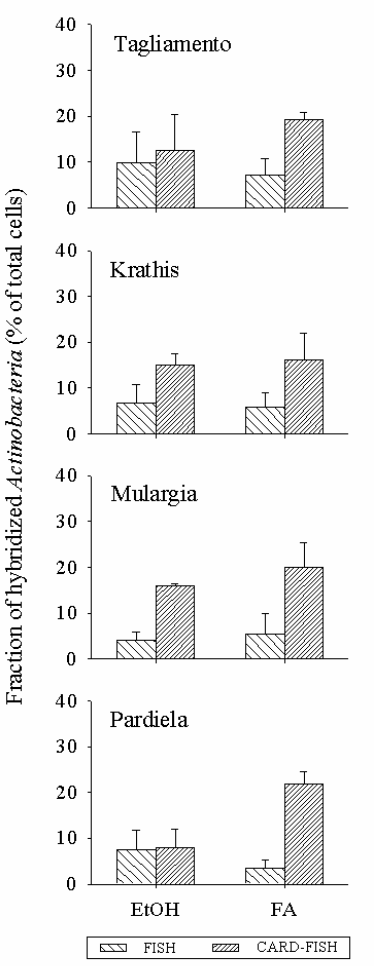


Figure 3.3.2. Fractions of *Actinobacteria* (HGC69a) detected either by FISH or by CARD-FISH after permeabilization by lysozyme and proteinase K in sediments from different rivers when using two different fixatives: ethanol (EtOH), formaldehyde (FA). Error bars indicate standard error.

Effect of microbial activity on FISH and CARD-FISH

During both the drying and rewetting experiments, bacterial communities associated to dry sediments showed the lowest BCP (0.05 ± 0.03 μg C h^{-1} g^{-1}) in comparison with wet sediments (1.37 ± 0.35 μg C h^{-1} g^{-1}) (Figure 3.3.3). Bacterial assemblages in wet sediments at the beginning of the drying and at the end of the rewetting experiment exhibited similar values of BCP ($F = 0.96$, $P = 0.36$). The same was true for dry sediments in the two experiments ($F = 3.56$, $P = 0.11$), and there were also no significant differences in BCP between sediments from the different rivers. The relatively low values and the narrow range of BCP across all microcosms is in line with reported data from other freshwater sediments (Kirschner and Velimirov, 1999), probably reflecting the low organic matter content of our samples.

A two-way ANOVA showed no overall differences between bacterial cells detected by FISH and CARD-FISH with proteinase K pre-treatment ($F = 0.03$, $P = 0.87$), but significant difference between water content conditions ($F = 25.41$, $P < 0.01$) and a significant interaction between these two sources of variance ($F = 8.24$, $P < 0.01$). Pair-wise SNK multiple comparisons showed that the cell detection by FISH significantly decreased from wet (75.5% ± 2.5%), to humid (64.3% ± 2.5%) and dry conditions (55.3% ± 8.0%), whereas there were no such differences for CARD-FISH.

In our study the percentage of Bacteria cells visualized by EUB I-III probes both by FISH and CARD-FISH with proteinase K was higher than data reported for marine (< 48%, Llobet-Brossa et al., 1998) and freshwater sediments (< 58%, Fazi et al., 2005; <50%, Gao et al., 2005). Moreover, the fraction of cells detected by FISH was linearly related to BCP ($y = 14.1 x + 54.1$, $r = 0.82$, $P < 0.01$) and the slope of this regression was significantly different from zero (ANCOVA, $F = 37.9$, $P < 0.01$). By contrast, the percentage of bacterial cells detected by CARD-FISH did not show any significant relation with BCP (Figure 3.3.3).

CARD-FISH represents a superior alternative to FISH with monolabeled probes for the analysis of microbial community structure in various environments, such as the water column of lakes and oceans, and freshwater and marine sediments (Ishii et al., 2004; Pernthaler et al., 2002a; Sekar et al., 2003). Small environmental *Bacteria* with low numbers of ribosomes offer few targets for the binding of fluorescently labeled FISH probes. The ribosome content of slowly growing microbial cells may be related to growth rate (Kemp et al., 1993), and starving bacterial cell are not quantitatively detected by FISH with monolabeled probes (Oda et al., 2000). CARD-FISH overcomes this difficulty by an additional signal amplification step (Bobrow et al., 1989). It has been speculated that CARD-FISH allows a more precise quantification than FISH because it is independent on the activity of the studied assemblage (Pernthaler et al., 2002a).

So far, there was no direct evidence to support this hypothesis. Thus, the novelty of this work lies in the comparison of FISH and CARD-FISH results with bacterial production (i.e., ^{3}H-Leucine incorporation). Our results showed that the percentages of hybridized *Bacteria* by FISH with monolabeled probes were significantly lower than those detected by CARD-FISH in dry sediments, where cells exhibited low activity. On the other hand, the efficiency of these two techniques was similar when analyzing more active assemblages residing in wet sediments. In contrast to FISH, CARD-FISH was unaffected by different levels of cell activity, thus expanding the possibilities to perform in situ studies of bacterial community composition in dry habitats.

Overall, the ratio of the fraction of bacterial cells detected by FISH and CARD-FISH was significantly related with C production, ranging from 0.8 (range 0.6-1.1) in dry sediments to 1.1 (range 1.0-1.3) in wet sediments ($y = 0.2 x + 0.8$; $r = 0.70$, $P < 0.05$).

The differences between the numbers of cells that can be detected by FISH or by CARD-FISH appear to be also related with taxonomic composition of the studied microbial assemblage. For example, Sekar et al. (2003) reported substantially higher detection of *Bacteria* by CARD-FISH in samples from various lakes, as compared to FISH with monolabeled probes. This difference was mainly due to the high numbers of freshwater *Actinobacteria*. In our study the optimized CARD-FISH protocol also significantly enhanced the detection of *Actinobacteria*.

The fraction of *Actinobacteria* cells detected by FISH (range 0-14% dry; 0-19% wet) accounted for about 20% of those hybridized by CARD-FISH (range 17-29% dry; 10-28% wet) and this ratio was not related to cell activity state. In fact, the difference between the fractions of *Bacteria* in our samples that were detected by FISH and by CARD-FISH could be readily modelled by a linear combination of the two independent variables BCP and percentage of *Actinobacteria* (Figure 3.3.3c). This illustrates that the value of FISH with monolabeled probes is doubtful when comparing communities associated to wet and dry sediments in these temporary rivers. However, in active bacterial assemblages FISH still remains a valuable technique and there may be instances in which FISH with the general probe for *Bacteria* might serve as an easy parameter of the activity state of the microbial assemblage. The technically more demanding CARD-FISH in combination with appropriate cell permeabilization treatment is insensitive to either environmental characteristics that affect microbial metabolism (e.g., water content) or taxonomic composition of the microbial assemblages (e.g., fractions of gram positive *Bacteria*). Thus, it could be applied for the long-term (i.e., seasonal) assessments of microbial communities in the benthic zone of temporary rivers that are increasingly exposed to cycles of flood and drought in Southern European Regions.

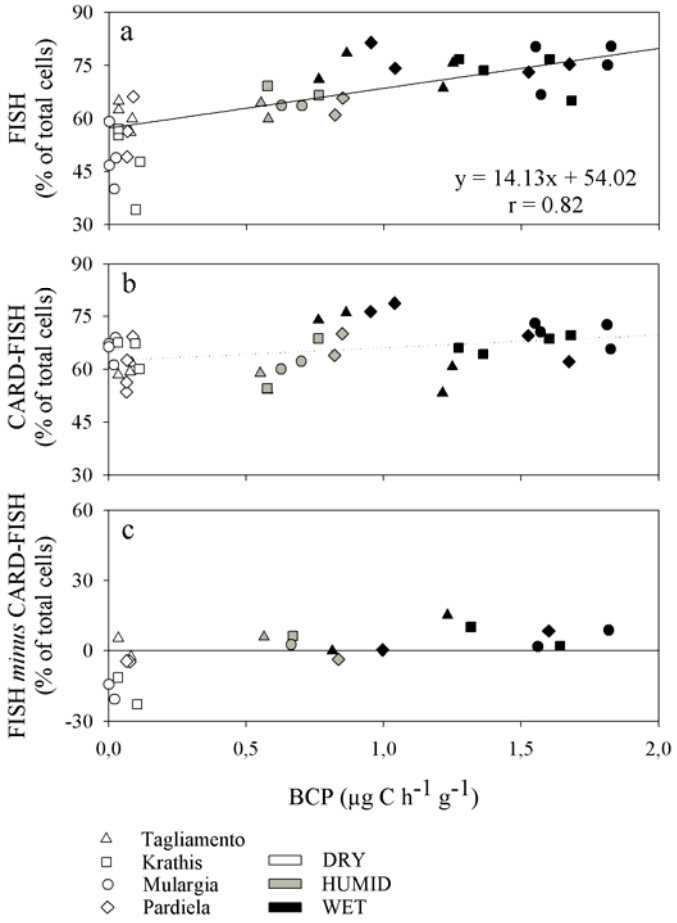


Figure 3.3.3. Relationship between bacterial carbon production (BCP) and the fraction of *Bacteria* (cells stained by probe EUB I-III) detected either by a) FISH or b) CARD-FISH, fixed in formaldehyde, after pre-treatment with lysozyme and proteinase K. c) Cell detection efficiency expressed as difference between the fractions of *Bacteria* detected by FISH and by CARD-FISH. This difference could be readily modelled by a linear combination of the two independent variables BCP and percentage of *Actinobacteria* ($y = 9.01 + (5.12 \times BCP) - (0.58 \times \%$ *Actinobacteria*), $r = 0.78$, $P < 0.01$).

3.4 Seasonal characterization of benthic bacterial community during the environmental monitoring of River Mulargia

This study was part of a wider environmental monitoring activity integrating investigations on hydrological dynamics, water quality, ecosystem processes and surface-subsurface exchange patterns along the main longitudinal axis of the River Mulargia (Sardinia, Italy). Environmental data were collected at four sampling sites, including sediment physicochemical characterization and major microbiological analyses, in the framework of the EU project TempQsim (www.tempqsim.net).

In this work, I integrated the monitoring activity by carrying out a seasonal in-depth study on benthic bacterial composition and activity in the river outlet section (Figure 2.3). Sediments were collected bimonthly from January to September 2004, with an additional sampling in February 2005.

River sediment characterization

Chemical and biological determinations were carried out only on the fine fraction (< 2 mm) where the majority of microbiological activities are resident (Fischer and Pusch, 2001). About 95% of the fine fraction was sand (80% coarse and 15% fine sands), while silt and clay represented only 3% and 2%, respectively of the total fine fraction. Ash free dry weight (AFDW) varied between 1.80% (January 2004) and 0.41% (September 2004). The highest percentages of C_{org} were found in March 2004 (0.42%) and February 2005 (0.28%). Values of N_{tot} decreased from January 2004 (0.06%), up to reach the lowest percentage in July 2004 (0.01%). Concentrations of P_{org} showed similar values in all samples (0.03-0.04%) (Barra Caracciolo et al., *unpublished data*). Based on these results, the analyzed sediments showed low nutrient and organic matter content with relative low variability of chemical and physical properties, as reported for other freshwater sandy sediments (Fischer et al., 2002a-b).

Flood events are extremely rapid and intense runoff of organic matter typically occur at the end of the dry period, following the heavy summer storms. In a parallel survey, Diliberto et al. (2005) monitored the Mulargia runoff events for two years by collecting hydrometric, meteorological and water quality data at daily intervals from the river headwater to the outlet. The results of this study highlighted that the flushing phenomenon was particularly evident in October 2003, in

consequence of a long and dry summer (Figure 3.4.3). In winter 2004, stable water flow conditions prevented the accumulation of particulate materials on riverbed sediment, therefore heavy spring storms (i.e. in April and May 2004) did not produce a conspicuous OM flushing. The unexpected absence of extreme conditions of drought in the summer 2004 and the lack of alluvial phenomena of strong intensities in the following autumnal and winter months produced a regular water flow in all the catchment basin and in particular at the river outlet section, where my in-depth study was carried out.

Although the lack of the water surface flow, a hyporheic flow persisted even in summer periods. Consequently, sandy sediments maintained stable moisture content.

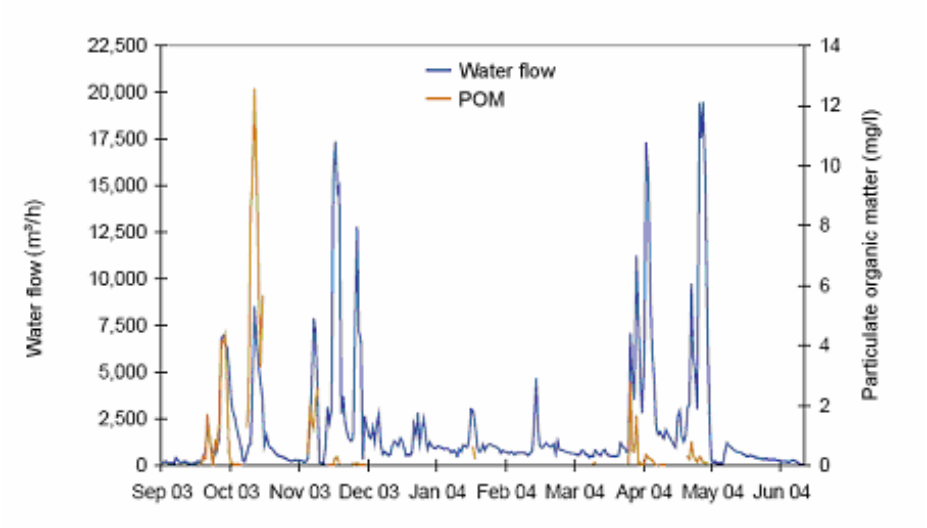


Figure 3.4.3. Water flow and particulate organic matter (POM) concentration monitored at the Mulargia outlet section (modified from Diliberto et al., 2005).

Bacterial community structure

In sampled sediments, the bacterial abundance (BAB) ranged between $1.8 \times 10^9 \pm 0.3 \times 10^9$ cell g^{-1} and $5.7 \times 10^8 \pm 1.4 \times 10^8$ cell g^{-1} (March 2004 and February 2005, respectively). Overall, BAB was about one order of magnitude lower in comparison with other freshwater aerobic sediments (Findlay et al., 2002; Fischer et al., 2002b), but comparable with data from sediments with similar low OM content (Fischer et al., 2002a; Fazi et al., 2005).

For a prompt monitoring of the microbial phylogenetic structure, benthic community composition was analyzed by FISH. A broad suite of six oligonucleotide probes specific for a high taxonomic level was used as in other studies on river sediments (Spring et al., 2000; Gao et al., 2005). However, comparative data for temporary rivers are not available.

Bacteria marked by the generic probe EUB I-III reached high detection percentages in March 2004 (79.3% ± 1.0%), while in February 2005 the lowest values were detected (61.3% ± 6.3%). These percentages are relatively high in comparison to reported data for sediments (Bouvier and Del Giorgio, 2003). Comparable results were reported for eutrophic water systems (Castle and Kirchman, 2004) and well-balance nutrient substrata (Fazi et al., 2005).

Since the percentage of hybridized cells is, to some extent, dependent on cell activity (i.e. ribosome content; Oda et al., 2000), the low variability of percentages of hybridized Bacteria during the whole sampling survey remarks the stable and active metabolic state of benthic bacterial communities in sampled sediments.

At division level (Figure 3.4.1), *alpha*-Proteobacteria dominate over the other taxa during the whole year of sampling, with percentages varying from 30.2% ± 2.1% (January 2004) to a minimum of 21.3% ± 3.0% (February 2005). Relatively high percentages of *beta*- (18.3% ± 1.4%; February 2005) and *gamma*-Proteobacteria (15.7% ± 0.2%; March 2004) were also detected. *Cytophaga-Flavobacterium* (range 12.0% - 2.3%), *Planctomycetes* (range 7.7% - 3.0%) and *Firmicutes* (range 0.45% - 0.35%) were respectively found into a smaller amount.

Considering the relative percentages of affiliated-Bacteria cells, it is interesting to note that in January 2004 and February 2005 bacterial communities were almost completely characterized by the use of six generic probes. In the other sediment samples, up to 30% (i.e. July 2004) of Bacteria detected by EUB I-III remained unaffiliated. The occurrence of unknown groups could indicate an increase of the microbial diversity during spring and summer periods.

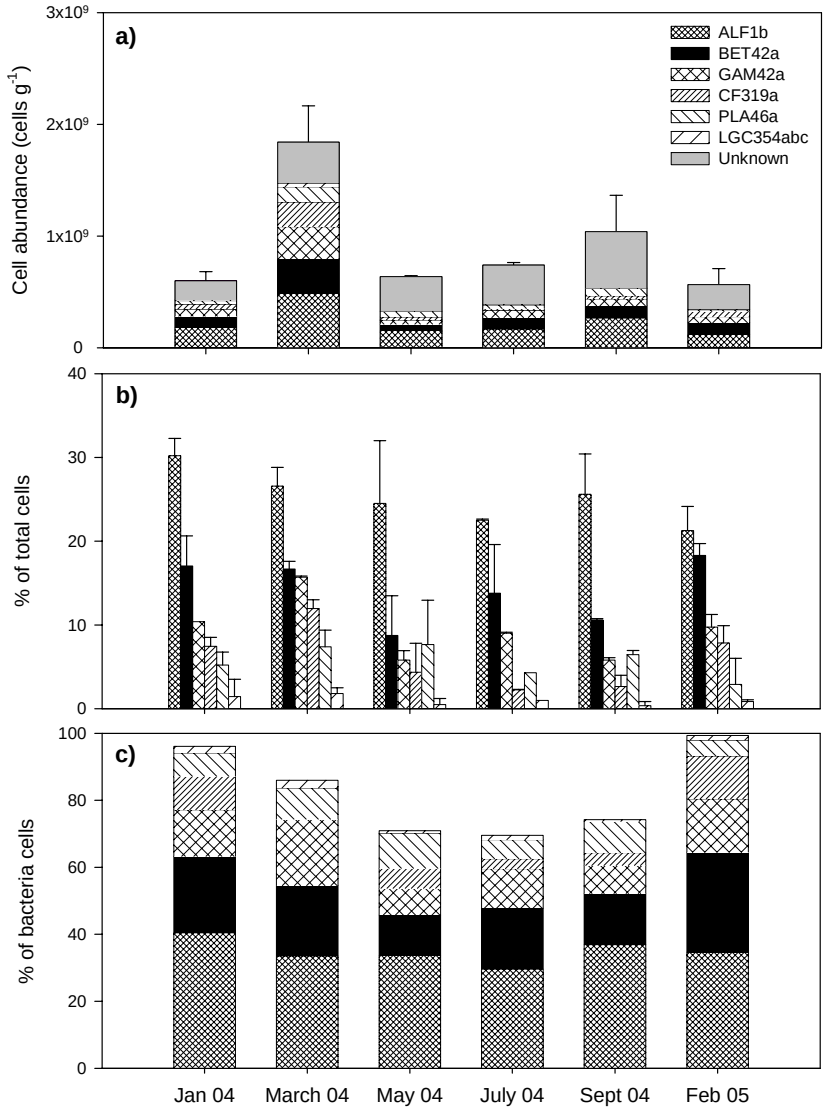


Figure 3.4.1. Structure of bacterial communities associated to Mulargia sediments as analyzed by FISH. ALF1b = *alpha*-Proteobacteria; BET42a = *beta*-Proteobacteria; GAM42a = *gamma*-Proteobacteria; CF319a = *Cytophaga-Flavobacterium*; PLA46a = *Planctomycetales*; LGC354abc = *Firmicutes*.
a) Contribution of the six analyzed taxa to the total cell abundance. Error bars indicate standard deviation of total DAPI-stained cells (n=12). b) Taxonomic composition expressed as percentages of total DAPI-stained cells. Error bars indicate the range of duplicates. c) Taxonomic composition expressed as percentages of bacterial cells hybridized by the probe EUB I-III. Values are means of duplicate analyses.

Bacterial activity in natural sediments

Bacterial carbon production (BCP), estimated through the incorporation of ^{3}H-Leucine and expressed in terms of carbon incorporation rates per gram of dry sediment, represents an estimation of new bacterial biomass production, and therefore it is assumed as an index of metabolic activity (Del Giorgio and Cole, 1998). As presented in Figure 3.4.2a, bacterial metabolic activities ranged between 170 ± 9 nmol C g^{-1} h^{-1} and 69 ± 4 nmol C g^{-1} h^{-1} (March 2004 and July 2004, respectively), falling into the lowest range reported in literature (25 - 2500 nmol C h^{-1} g^{-1}, Kirschner and Velimirov, 1999; ~250 nmol C h^{-1} g^{-1}, Fischer et al., 2002b; ~420 nmol C h^{-1} g^{-1}, Buesing and Gessner, 2006). Overall, the variability of BCP was low comparing to other studies, where C production rates highly varied in relation to the various physicochemical and biological characteristics of the sediments (Fischer & Pusch 2001; Buesing & Gessner 2006).

However, the ratio between bacterial production and abundance was in line with data reported for river sediment layers that provide "hot spots" of bacterial activity (0.03 - 0.36 fmol C h^{-1} $cell^{-1}$; Fischer et al. 2002a; Buesing and Marxen, 2005). In our samples, the relatively low values of cell abundance and metabolic activity reflected the low organic load, since these parameters are assumed to be, at least partially, a function of the sediment OM content and composition (Findlay et al., 2003; Fischer et al., 2002a).

The low molecular weight substances can be directly transformed by bacteria into biomass and ultimately mineralized to CO_2, whereas complex organic substrates need to be firstly degraded by extracellular enzymes. Therefore, the microbial degradation and transformation of particulate and dissolved organic matter in the sediment are key processes with regard to the carbon cycling in aquatic systems. According to the fundamental role of extracellular enzymes (EEA) in the process of OM decomposition, the patterns of their activities may provide specific insights on the available OM sources in natural systems, as well as functional profile of microbial communities in substrate uptake processes and nutrient cycling (Wobus et al., 2003). During the annual sampling survey, EEA was assessed to measure changes in the organic matter utilization, particularly related to the main constituents of sinking organic matter (organic phosphorous, proteins, lipids, derived cellulose organics).

High contribution of alkaline phosphatase (APA) and amino peptidase (AMA) activities either implied a fast phosphorous and

nitrogen re-cycling, either reflected a nutrient-impoverished environment, since nutrient limitation may induce bacterial cells to synthesize specific enzymes (Taylor et al., 2003).

The potential hydrolysis of phosphorilated organic compounds by APA contributed on average for 35.8% ± 11.2% of the total EEA activities measured in this study. Similarly, the hydrolysis of proteinaceous material AMA contributed for 34.7% ± 7.6%. Interestingly, APA/AMA ratio was on average 1.1 ± 0.5, indicating an equal nutrient microbial demand. These trends could also confirm the oligotrophic nature of sampled sediments. On the annual scale, APA was significantly related to bacterial abundance ($r = 0.80$, $P < 0.05$), revealing the essential role of APA in fueling bacterial cells with phosphorous, which is essential for the synthesis of new biomass. The highest contribution of APA was measured in March 2004 (140.5 ± 4.2 nmol MUF h^{-1} g^{-1}), when the early spring conditions stimulate microbial growths (i.e. high BCP rates) and higher nutrient demand. Moreover, photosynthetic organisms could compete with bacteria for phosphorous uptake, thus contributing to APA production. On average, lipase and beta-glucosidase activities gave a more limited contribution to the total activity (16.0% ± 3.8% and 13.5% ± 10.2%, respectively), indicating that the hydrolysis of lipids and polysaccharides assumed a minor importance for the benthic microbial community. The only exception was observed in February 2005, when beta-glucosidase activity reached the highest hydrolytic rate (178.7 ± 14.7 nmol MUF h^{-1} g^{-1}), probably as consequence of changed conditions in food sources caused by the overflow event registered at that sampling time-point.

Overall, stable seasonal EEA dynamics (Figure 3.4.2b) were observed, with the relatively low activities in winter (January 2004) in comparison to warmer months. In this respect, water temperature (e.g.; Wilczek et al. 2005) could have regulated EEA, thus having produced the minimal rates observed in this study.

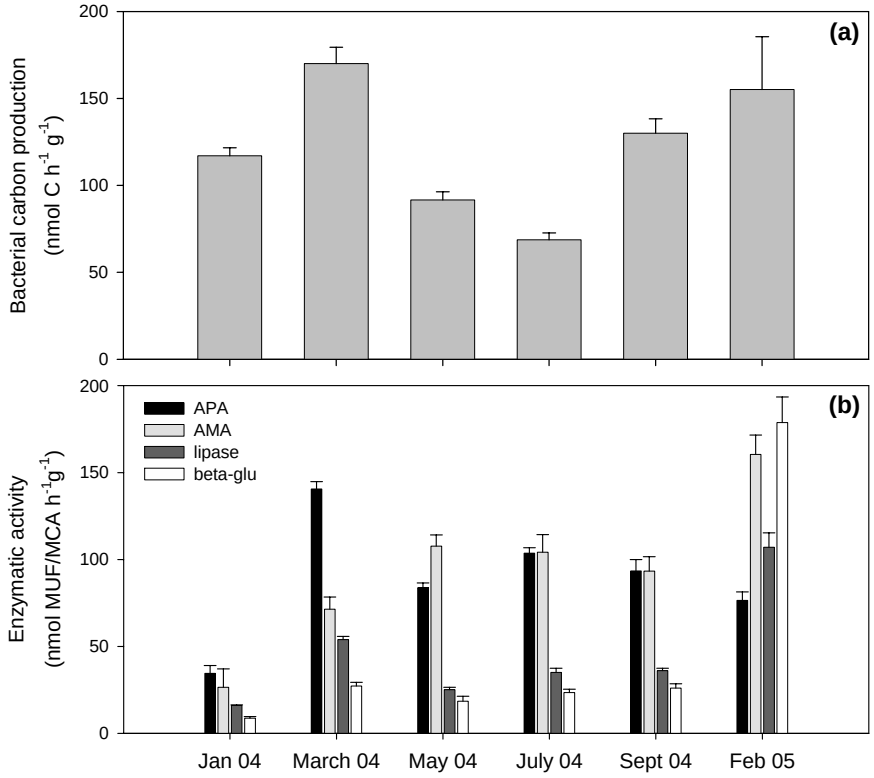


Figure 3.4.2. a) Annual trend of bacterial carbon production estimated through the incorporation rates of ^{3}H-Leucine b) Annual trend of extracellular enzymatic activities determined fluorometrically using MUF/MCA fluorescent substrates. APA = alkaline phosphatase; AMA = aminopeptidase; beta-glu = beta-glucosidase. All data are expressed per gram of dry sediment. Error bars indicate standard deviations of three independent measurements.

In summary, the low variability of all measured microbiological parameters could reflect the unchanged chemical and physical properties of sampled sediments. From an ecological point of view, despite the microbial heterotrophic activity were slightly affected from the minimal variations of the OM content observed on sediments samples (Findlay et al., 2003; Fazi et al., 2005), the effects of the "nearly surface flow" on bed sediment processes did not allow to clearly assess the effects of water stress on natural microbial community. This specific issue was aimed by the experimental approach described in the next chapter.

3.5 Bacterial physiological responses to water stress

Owing to the stable moisture content of natural sediments in River Mulargia during the field survey in 2004, it was difficult to provide clear evidences on the effects of water stress on microbial dynamics. To experimentally describe benthic bacterial responses to the drying and rewetting processes, we simulated desiccation and re-inundation of sediments collected from four European temporary rivers (River Mulargia and Tagliamento - Italy; River Krathis - Greece; River Pardiela - Portugal) in artificial microcosms.

Sediment characterization

The <2-mm sediment fractions were mainly composed of sand (>80%). Clay was below 2% in all sediments. Silt was 12.2% and 7.6% in Tagliamento and Krathis respectively, whereas in Mulargia and Pardiela it was only 3.0% and 1.2%. Sediments from Tagliamento and Krathis were classified as loamy sand, Mulargia and Pardiela as coarse sand. The physicochemical and microbiological parameters measured in sediments at the beginning of the experiment are summarized in Table 3.5.1 Sediment from Pardiela differed from the others by having the lowest pH and conductivity, while sediment from Mulargia was characterized by the highest conductivity and ash-free dry weight content. At the end of the experiment, the AFDW concentrations were not significantly different from the initial values ($P > 0.05$). The maximum water holding capacity showed similar values for all sediments. Microbiological characterization showed no major differences among the sediments from the four rivers in term of bacterial total abundance, biomass and C production (Student-Newman-Keuls test, $P > 0.05$). *In situ* bacterial abundance, determined in Mulargia sediments at the same sampling site and date when samples were collected for microcosm set-up, showed values ($6.4 \pm 0.1 \times 10^8$ cells g^{-1}) not significantly different from those found at the beginning of the experiment (Student *t*-test, $P < 0.05$). Moreover, *in situ* C production (1.04 ± 0.03 µg C h^{-1} g^{-1}) was statistically different (Student *t*-test, $P > 0.05$) but still comparable to the microcosm initial rate (1.47 ± 0.01 µg C h^{-1} g^{-1}), when taking into account the wider range of BCP registered in Mulargia sediments during the annual monitoring activity (0.6 - 3.5 µg C h^{-1} g^{-1}).

	Tagliamento	Krathis	Mulargia	Pardiela
pH	8.3	8.2	8.1	7.3
EC (μS cm^{-1})	88.5	89.1	104.3	47.7
WHC (g g^{-1} %)	19.5	26.0	21.4	21.1
AFDW (mg g^{-1})	5.9 (0.6)	4.4 (0.7)	9.2 (1.2)	4.5 (0.2)
BAB (10^8 cells g^{-1})	6.9 (0.30)	8.9 (0.68)	7.0 (0.15)	8.1 (0.42)
BB (μg C g^{-1})	38.8 (0.9)	40.3 (3.6)	35.2 (2.8)	46.0 (3.8)
BCP (μg C h^{-1} g^{-1})	1.00 (0.01)	1.07 (0.04)	1.47 (0.01)	1.30 (0.06)

Table 3.5.1. Physicochemical and microbiological parameters of the sediments from the four rivers at the beginning of the experiment. The maximum water holding capacity (WHC) is expressed as percentage of the weight of retained water versus dry sediment weight. Ash-free dry weight (AFDW), bacterial total abundance (BAB), biomass (BB) and C production (BCP) are expressed per gram of dry sediment as the average of three replicates with standard deviation in parentheses.

During desiccation, water content in all the microcosms decreased linearly (white symbols in Figure 3.5.1) without any significant difference among the four rivers (ANCOVA, $P > 0.05$). After 35 days of incubation, the amount of water lost by evaporation was no longer detectable. The direct measurements of the water content by sediment dry weight determination (black symbols in Figure 3.5.1) revealed a slower decrease until day 46, when 0% of WHC was reached.

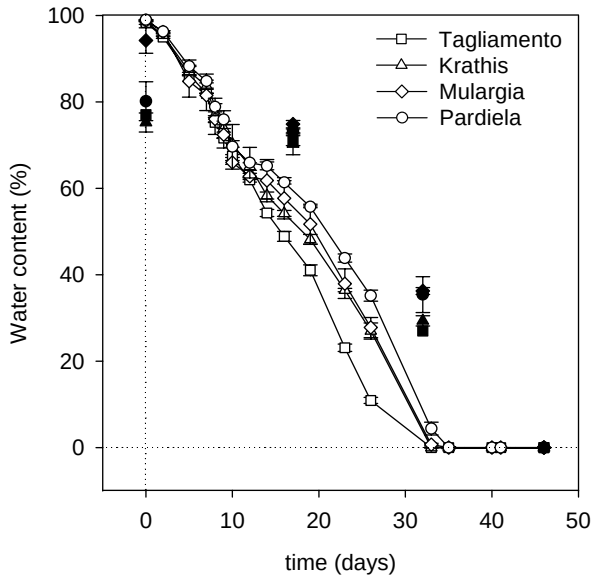


Figure 3.5.1. Downward trend in sediment moisture. White symbols indicate the frequent estimations of microcosm water content by gross weight determination, expressed as a percentage of the initial value (± 1 SD). Black symbols indicate the sediment water content, determined at each sampling time as the difference between sediment gross and dry weight and expressed as a percentage of WHC (± 1 SD)

Effect of drying on bacterial abundance and activity

Bacterial total abundance was significantly related to the sediment water content, following an exponential pattern (Tagliamento $r = 0.89$, Krathis $r = 0.84$, Pardiela $r = 0.89$, $P < 0.05$), with the exception of Mulargia where the best interpolation was linear ($r = 0.92$, $P < 0.05$). Taking into account the whole set of data a significant exponential relation was found ($y = 2.0 \times 10^8 \times e^{0.01x}$, $r = 0.83$, $P < 0.05$) (Figure 3.5.2a). On average, total abundance decreased by 74%, passing from $7.7 \pm 1.0 \times 10^8$ cells g^{-1} in the wet sediments down to $2.0 \pm 0.7 \times 10^8$ cells g^{-1} in dry sediments. The percentage of live cells also decreased from 48.3 ± 3.7% to 31.1 ± 6.6% of the total abundance on passing from wet to dry conditions. Altogether, the live cell abundance was exponentially related to sediment water content ($y = 7.0 \times 10^7 \times e^{0.02x}$, $r = 0.87$, $P < 0.05$) with an overall reduction of 84% of the initial value. During the whole experiment, image analysis revealed that coccus-like cells represented the prevailing cell morphological type (average 82.5 ± 7.9%) followed by regular rods (average 11.8 ± 5.5%). Cell spherical shape was therefore considered to estimate the mean cell biovolume. No significant correlation was found between biovolume and sediment water content ($r = 0.26$, $P > 0.05$). Nevertheless, Two-Way ANOVA showed a significant difference in biovolume between wet (0.41 ± 0.10 μm^3) and dry conditions (0.27 ± 0.09 μm^3), with no difference among rivers and no interaction between these two variables (i.e., moisture content and rivers; $P < 0.05$). Bacterial total biomass exponentially decreased with increasing dryness ($y = 9.4 \times e^{0.02x}$, $r = 0.81$, $P < 0.05$), with an overall reduction of 78%. When only live cells were considered, biomass was also correlated to sediment water content ($y = 3.0 \times e^{0.02x}$, $r = 0.84$, $P < 0.05$) (Figure 3.5.2b). Live cell biomass drastically decreased by 86% from the beginning (19.5 ± 2.6 μg C g^{-1}) to the end of the experiment (2.7 ± 1.3 μg C g^{-1}).

The average bacterial carbon production was 1.21 ± 0.21 μg C h^{-1} g^{-1} in wet sediments and progressively decreased, until reaching a minimum value of 0.06 ± 0.03 μg C h^{-1} g^{-1} in dry sediments. Only in the two microcosms with sediments from Mulargia, BCP completely ceased showing no differences with the zero-time control (Student-Newman-Keuls test, $P > 0.05$). BCP was exponentially related to sediment water content (Tagliamento $r = 0.77$; Krathis $r = 0.92$; Mulargia $r = 0.90$; Pardiela $r = 0.91$, $P < 0.05$) with no differences among sediments from the four rivers (ANCOVA, $P > 0.05$). Taking into account the whole set of data, the significant exponential relation

($y = 0.07 \times e^{0.03x}$, $r = 0.89$, $P < 0.05$) showed the highest slope value in comparison to those found for total abundance and biomass (Figure 3.5.2c). Mean per-cell specific production ranged from 3.3 ± 0.7 to 1.1 ± 0.8 fg C h^{-1} $cell^{-1}$ when passing from wet to dry conditions. As long as sediment moisture persisted, bacterial growth rate was on average 0.06 ± 0.02 h^{-1} but decreased in completely dry conditions (μ= 0.03 ± 0.02 h^{-1}) when the turnover time reached its maximum (Figure 3.5.3).

Bacterial abundance and activity detected in Mulargia sediments were comparable to those found at the beginning of the experiment. Due to logistic constraints, such direct comparison was not possible for the other three rivers. However, the similarity among sediments in terms of physicochemical and microbiological characteristics, allowed us to consider the stored samples from the other rivers suitable to achieve the aim of this study. In addition, Fletcher et al (2001) did not observe major changes in benthic community in sediments stored over several weeks.

Overall, bacterial total abundance was about one order of magnitude lower in comparison with other freshwater aerobic sediments (Findlay eta l., 2002; Fischer et al., 2002b), and bacterial C production fell into the lowest reported range, recalculated on a dry-weight basis (0.3–30 µg C h^{-1} g^{-1} (Kirschner and Velimirov, 1999), ~3 µg C h^{-1} g^{-1} (Fischer et al., 2002b), ~5 µg C h^{-1} g^{-1} (Buesing and Gessner, 2006). Bacterial abundance and activity are assumed to be, at least partially, a function of the sediment OM content and composition (Findlay et al., 2003; Fischer et al., 2002a). Hence, the relatively low values of these parameters could be mainly due to the low organic matter in our samples. In this context, the small differences in the sediment OM content among the four rivers were reflected by similar initial bacterial biomass and activity (Table 3.5.1). Moreover, the ratio between bacterial C production and total abundance was in line with those reported for river sediment layers that provide “hot spots” of bacterial activity (0.4–4.4 fg C h^{-1} $cell^{-1}$ (Fischer et al., 2002a). By considering the relatively high values of cell biovolume (Buesing and Marxsen, 2005), bacterial total biomass was comparable to previous reports (~50 µg C g^{-1} dry sediment (Fischer and Pusch, 2001). Hence the ratios between C production and total biomass (specific growth rate) also fell within the reported range (0.004–0.3 h^{-1} (Fischer et al., 2002a), 0.1 h^{-1} (Buesing and Gessner, 2006), indicating that the organic matter was efficiently utilized in water saturation conditions. Considering a bacterial growth efficiency of 10–40% (Kirschner and

Velimirov, 1999), we estimated that at the beginning of the experiment bacteria were able to process 70–290 µg C per gram of dry sediment per day, representing 1-3% of the organic matter of our samples.

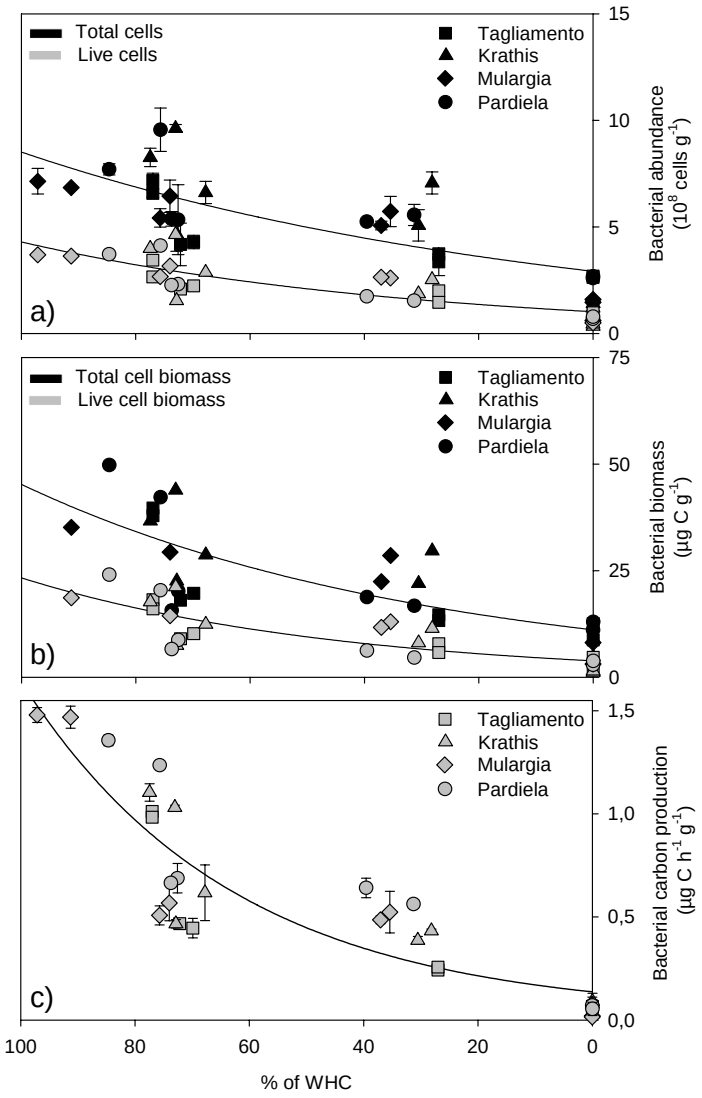


Figure 3.5.2. Total and live cell abundance (a), biomass (b) and bacterial carbon production (c) *versus* sediment water content, expressed as a percentage of WHC. Note the *x*-axis reverse scale: 100% identifies wet sediment at the beginning of the experiment, 0% corresponds to the ending dry sediment. All data are normalized to gram of dry sediment. Error bars indicate standard deviations of three independent measurements. Regression curves are shown (see text).

The results clearly showed that bacterial growth and metabolic rates were dependent on sediment moisture. During desiccation, a concomitant decreasing trend in the availability of organic matter and nutrients may occur (Baldwin and Mitchell, 2000). However, the organic substrate should not represent a limiting factor in our experimental microcosms, since it largely exceeded the estimated bacterial carbon demand. This is also supported by the similar values of AFDW percentages registered at the beginning and at the end of the experiment.

When water was below the sediment saturation level, the first 30% decrease in water content induced a substantial reduction of bacterial C production (54%), abundance (31%) and consequently biomass (46%). Thereafter, C production exponentially decreased, nearly ceasing in dry conditions, followed by a slower decrease in bacterial abundance and biomass. It cannot be excluded that longer incubation times of the leucine incorporation assay could lead to a higher estimate of BCP, even in dry sediments. However, the linear relation between leucine incorporation and incubation time is not always guaranteed after 90 minutes (Buesing and Marxsen, 2005), and prolonged incubations could provoke a shift of bacterial metabolism (Buesing and Gessner, 2003).

Bacterial communities responded to dryness through cell death and a break in carbon incorporation in the remaining live cell biomass. Indeed, the removal of water through air drying is know to be lethal to bacterial cells by damaging membranes, proteins and nucleic acids (Billi and Potts, 2002). Since the per-cell production and turnover time did not show major variations while sediment moisture persisted, the reduction in community C production was mainly attributable to the decrease in cell numbers. Conversely, in dry sediments at the end of the experiment, a conspicuous number of live cells were still abundant but mostly depressed in their main metabolic activity. Therefore, the significant decrease in per-cell production resulted in a drastic increase in the community turnover time (Figure 3.5.3). Our results indicated that complete desiccation of sediments led to a delay in mineralization processes and synthesis of new biomass.

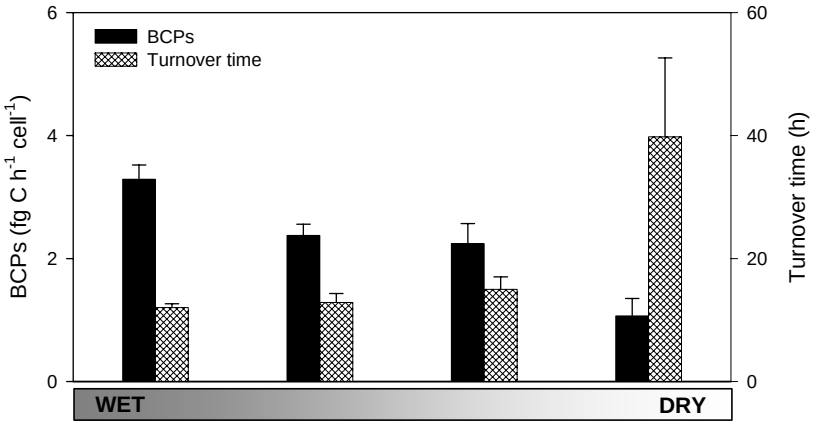


Figure 3.5.3. Per-cell specific carbon production (BCPs) and turnover time at the four sampling times during the drying process (shading bar). Data are means of values from the four river sediments (± 1 SE).

The temporarily limited microbial degradation could affect the accumulation of organic matter. New inundation might release organic matter to downstream reaches without prior decomposition thus allowing loss of higher quality organic matter than would occur under constant flow conditions. Nevertheless, bacterial communities continue to be metabolically active even in sediment with reduced water content. During no-flow periods, when hyporheic waters sustain sediment moisture, microbial degradation may still occur and influence the sediment organic matter content and composition, though with lower rates in comparison with those found in water saturation conditions. The considerable loss of microbial organic carbon and the reduction of size of resistant cells during desiccation could also have a direct repercussion on the upper trophic levels. As soon as the sediment is re-hydrated, only a minor part of the initial live cell biomass (14%) is available to immediately start the re-activation of the aquatic microbial food web.

It should be noted that the experiment was carried out under controlled laboratory conditions and thus not taking into consideration the combined effect of the dry-heat condition, as well as the duration and the rate of drying. The combination of these factors could significantly influence bacterial physiology (Keryn, 2001). Moreover protistian predation, a major factor in limiting bacterial abundance and

biomass (Gücker and Fischer, 2003), could differentially affect bacterial communities in wet and dry sediments, since the increasing sediment aggregate structure during desiccation could restrict the movement of protists and their grazing efficiency (Ekelund et al., 2002).

Effect of drying on bacterial community composition

In the initial wet sediments, Bacteria visualized by FISH accounted for 74.8 ± 2.3% of the DAPI stained cells, whereas in dry conditions they decreased to 55.7 ± 8.9%. Archea were detected only in dry conditions representing 2.0 ± 0.9% of the microbial community. On average 58.3 ± 16.7% of Bacteria in wet sediments and 68.1 ± 13.2% in dry sediments were affiliated to the seven analyzed phylogenetic groups. The relative distribution of these clusters changed during desiccation. In wet sediments, Proteobacteria accounted for 79.8 ± 7.5% of the affiliated cells (*alpha-* = 12.8 ± 3.1%, *beta-* = 48.3 ± 11.3% and *gamma-* = 20.5 ± 9.2%). Dry sediments from Mulargia, Krathis and Tagliamento rivers were mostly dominated by *beta*-Proteobacteria (73.1 ± 9.7%). Only in dry sediments from the River Pardiela, Proteobacteria remained more widely distributed, with *alpha*-Proteobacteria passing from 13% to 32% and *beta*-Proteobacteria decreasing from 56% to 39% of the affiliated cells (Figure 3.5.4).

To the best of our knowledge, the reported FISH results are the first attempt to describe bacterial community composition in sediment from temporary rivers, suggesting an inherent resistance to drying of a part of the benthic bacterial communities. Water availability seemed to influence community composition at least at division-level, even if the use of generic probes may hide additional variability at finer taxonomic levels.

Both in wet and dry sediments, the percentage of Bacteria cells visualized by EUB338 probes was higher than data reported for other freshwater sediments (Fazi et al., 2005; Gao et al., 2005). However, the reduced percentages of hybridized cells in dry sediments were probably due to the observed decrease in cell activity. In this respect, recent evidence highlights that the low ribosome content of slowly growing cells may affect the detection efficiency of rRNA-targeted probes (Zwirglmaier, 2005).

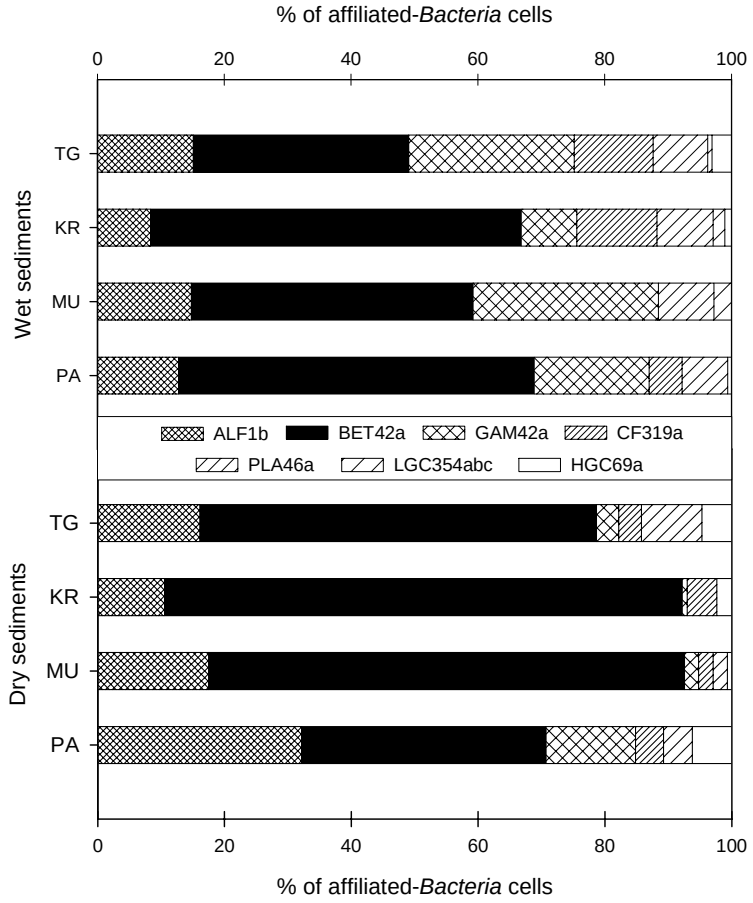


Figure 3.5.4. Taxonomic composition of the bacterial communities as analyzed by FISH at the initial wet and final dry conditions. Values are means of duplicate analyses, expressed as relative percentages of affiliated-Bacteria cells. The range of duplicates never exceeded 9% of mean values. TG, Tagliamento; KR, Krathis; MU, Mulargia; PA, Pardiela. ALF1b = *alpha*-Proteobacteria; BET42a = *beta*-Proteobacteria; GAM42a = *gamma*-Proteobacteria; CF319a = *Cytophaga-Flavobacterium*; PLA46a = *Planctomycetales*; LGC354abc = *Firmicutes*; HGC69a = *Actinobacteria*.

Passing from wet to dry conditions there was an increase of about 10% in the total *Bacteria* affiliated to the seven analyzed groups, probably indicating a lower occurrence of other groups in dry sediments. Although all bacterial taxonomic groups decreased in term of abundance by more than 70%, an increase of the relative percentage of *Bacteria* affiliated to *beta-Proteobacteria* division was observed,

except for Pardiela sediments where *alpha-Proteobacteria* increased. The *alpha-* and *beta-Proteobacteria* divisions contain several cosmopolitan freshwater clusters and often represent the highest proportion of the domain *Bacteria* in freshwater systems (Crump et al., 2003; Gao et al., 2005; Zwart et al., 2002; Zwisler et al., 2003). During the annual monitoring activity of Mulargia sediments, these two groups together represented about 50% of the whole community at the end of winter period (March 2004), constantly increasing up to 70% at the end of summer (September 2004). In accordance to our findings, these clusters appear to dominate the microbial community of temporary river sediments and they could be considered as primary targets for finer phylogenetic analyses.

Considering the lag between sediment sampling and the setting-up of the microcosms, our experimental design could have limitations for community composition analysis, such as the possible growth of microbes quite different from the native assemblage. However, the distribution of taxa within the source community and the prevailing environmental conditions are considered the most relevant factors in regulating the community composition dynamic (Fenchel and Findlay, 2004; Kirchman et al., 2004; Lagenheder et al., 2006). Therefore, the comparison between wet and dry conditions still allowed us to estimate how drying affects the bacterial community structure.

Similar bacterial metabolism rates among different river sediments could indicate that drying stress conceivably led to comparable physiological responses not completely related to the initial community structure. Drying imposes a consistent bottleneck on function and relative composition despite potential differences in the original community. Differently composed communities are able to cope with a wide range of environmental conditions, owing to the presence of generalist species, maintaining similar aggregated functions, such as biomass, production and respiration (Lagenheder et al., 2006). Alternatively, drying stress severely affected bacterial metabolism to such an extent as to overcome differences in community composition. In conclusion, our findings clearly showed a substantial influence of drying stress on the bacterial community, affecting organic matter mineralization by temporarily limiting metabolic activity and reducing microbial biomass.

Effects of sediment rewetting

A parallel sediment rewetting test was performed in order to specifically assess the potential reactivation of viable cells, residing in a state of low-activity in dry sediments.

Total and live bacterial abundance did not remarkably change after sediment rewetting, showing a narrow range of variation among rivers (range: 2.8-3.4 × 10^8 cells g^{-1}). Nevertheless, the percentages of intact-membrane cells increased on average from 20% to 30% of total abundance in the first 24h, reaching 40% at day 8.

As shown in Figure 3.5.5, bacterial carbon production reached the highest rates (1.0±0.3 μg C h^{-1} g^{-1}) within 8 days from the rewetting event. These results were comparable with values measured in the initial wet sediments (Table 3.5.1). Cell-specific C production, exclusively referred to live cells, reached considerably high values at day 8 (8.9 ± 0.5 fg C h^{-1} $cell^{-1}$).

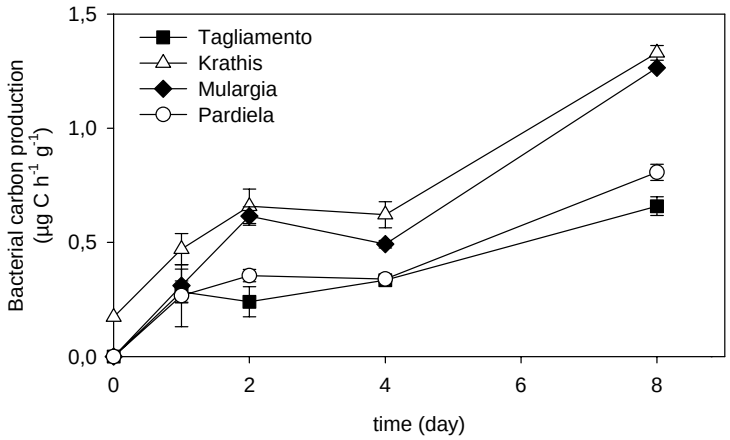


Figure 3.5.5. Bacterial carbon production after rewetting dry sediments. All data are normalized to gram of dry sediment. Error bars indicate standard deviations of three independent measurements.

Despite differences in the origin of the sediments, results showed that the recovery of BCP occurred with similar patterns in the four sediments (10% ± 0.5% increase per day). Interestingly, the full recovery of C production could be mainly attributable to the increase of cell-specific production and live cell percentage, instead of total bacterial abundance.

3.6 Dry sediments as a source of microbial populations

In this study, we simulated the re-inundation of dry sediment in microcosms analyzing composition and activity of the bacterial community that primary colonized the water phase. In particular, the study could contribute to better understand the ecological role of those benthic microbes that reside in a state of low-activity in dry sediments and promptly colonize the new incoming water at the end of the dry period.

DOC, bacteria and grazers dynamics

DOC concentration in the initial artificial water was about 21 μM and it reached a concentration of 340 ± 14 μM (overall average) right after water entered in contact with dry sediment. In the sterile control DOC concentration was 553 ± 50 μM, indicating that sterilization in itself increased the release of dissolved matter into the water phase.

In the water phase of the sterilized microcosm it was possible to visualize $1.4 \times 10^4 \pm 0.24 \times 10^4$ cell ml^{-1} (overall average) since the starting of the experiment without any change within 48 h. This value was not significant different from the number of cells found in the experimental microcosms within the first nine incubation hours ($1.7 \times 10^4 \pm 0.26 \times 10^4$ cell ml^{-1}). After a lag phase of at least 9 hours from the rewetting, bacteria increased in number reaching $3.4 \times 10^6 \pm 0.16 \times 10^6$ cell ml^{-1} at 28 h and stabilizing thereafter. Ciliates and flagellates were not detectable within the first nine hours; at 28 h they reached an abundance of 110 ± 34 cell ml^{-1} and 220 ± 88 cell ml^{-1} respectively. Thereafter ciliates did not significantly change in abundance (170 ± 40 cell ml^{-1} at 48h) while flagellates increased to 1100 ± 140 cell ml^{-1} at 48 h (reaching 5000 ± 380 cell ml^{-1} at 72h) (Figure 3.6.1).

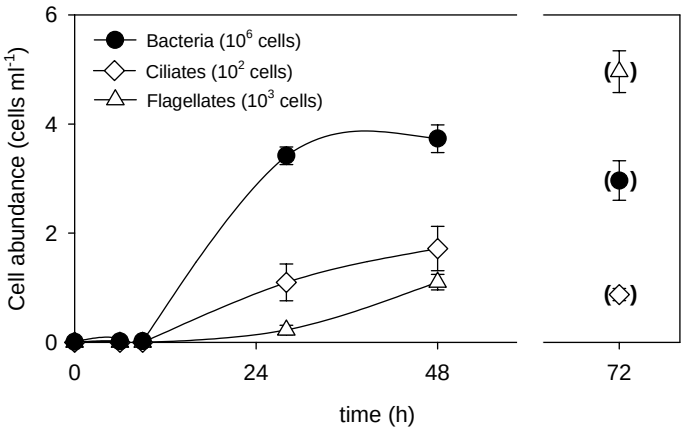


Figure 3.6.1. Bacteria, ciliates and flagellates abundance expressed as number of DAPI stained cells. Data are means of duplicate samples in three independent microcosms. Error bars indicate standard deviation.

Within river sediments large quantities of dissolved and particulate organic matter, both autochthonous and allochtonous, are available to the heterotrophic bacteria and they can support the productivity of the water phase (Cole, 1999). In temporary water bodies, this benthic and pelagic coupling is not constant in time, organic matter can accumulate during drought in the sediments to be promptly resuspended in the column water after the first water flush.

Our results showed a high release of bacterial cells from dry river sediments. The not significant difference between the abundance of floating cells in the test microcosms and in the sterilized control could indicate that water colonization was initially induced by passive dispersion. After an initial lag-phase, during which bacteria residing in dry sediments probably re-activated their metabolic activities, bacterial community colonized the water phase reaching the highest abundance between 9 h and 28 h. In parallel, grazer abundance (ciliates and flagellates; Figure 3.6.2) started to increase becoming an important control of bacterial abundance.

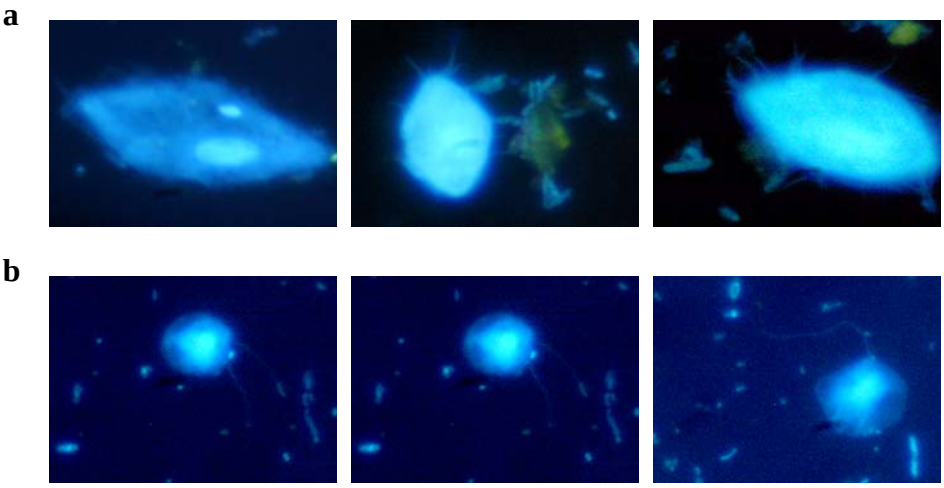


Figure 3.6.2. Ciliates (a) and flagellates (b) stained with DAPI at 28h, 48h and 72h water samples.

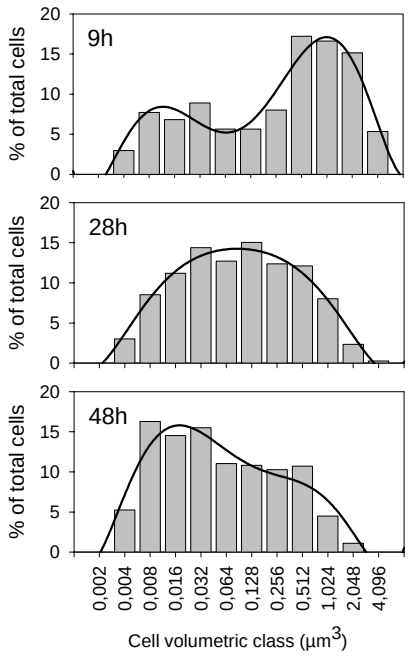


Figure 3.6.3. Distribution of bacterial cells in volumetric classes at time 9h (n= 348) 28h (n= 1196) 48h (n= 915).

Bacterial biovolume decreased in time from 0.58 ± 0.08 μm^3 at 9 h to 0.13 ± 0.01 μm^3 at 48 h; the overall bacterial distributions in dimensional classes are reported in Figure 3.6.3. Biovolume analysis clearly showed that, as bacterial community developed in water, cells became significantly smaller, confirming a possible top-down control of bacterial populations. Size selective grazing of protists tends to select small bacteria in most natural waters (Hole and Boraas, 1991). Boenigk et al. (2004) recently reported that for ultramicrobacteria affiliated with *Polynucleobacter* cluster grazing-related mortality was lower than for larger *Bacteria*. Moreover, cell miniaturization seems to have specifically evolved in response to grazing mortality (Pernthaler, 2005).

Bacterial carbon production and extracellular enzyme activity

Bacterial carbon production estimated by ^{3}H-Leucine (^{3}H-Leu) incorporation (i.e. protein synthesis) showed higher rates with respect to ^{3}H-Thymidine (^{3}H-TdR) incorporation (i.e. new cell production) (Figure 3.6.4). ^{3}H-Leu incorporation showed a rapid increase in time reaching its maximum 28 h after sediment rewetting almost stabilising thereafter. Per cell carbon production did not change between 28 h and 48 h (overall average $1.37 \times 10^{-4} \pm 0.10 \times 10^{-4}$ nM C h^{-1} $cell^{-1}$). ^{3}H-TdR incorporation reached its maximum rate at 48 h and it was possible to estimate that cell production increased in time passing from $3.7 \times 10^{7} \pm 0.44 \times 10^{7}$ cell l^{-1} h^{-1} (28 h) to $9.4 \times 10^{7} \pm 1.40 \times 10^{7}$ cell l^{-1} h^{-1} (48h). Moreover, the ratio between pico-mole of ^{3}H-Leu and ^{3}H-TdR incorporated decreased from 98 (28h) to 42 (48 h). Soon after the water addition, extracellular enzyme activities (EEAs) were not significantly different from blanks. AMA steady increased in time (Figure 3.6.4), reaching at 28 h about 70% of the highest values registered at 48 h (430.7 ± 22.6 nM MCA h^{-1}). APA showed a similar trend with a prompt reactivation at the 28 h (83% of the maximum activity) and a peak at 48 h (98.6 ± 4.3 nM MUF h^{-1}). Interestingly, during the experiment AMA:APA ratios passed from 3.5 at 28 h up to 4.4 at 48 h (reaching 5.0 at 72 h) showing a increasing higher contribution of AMA.

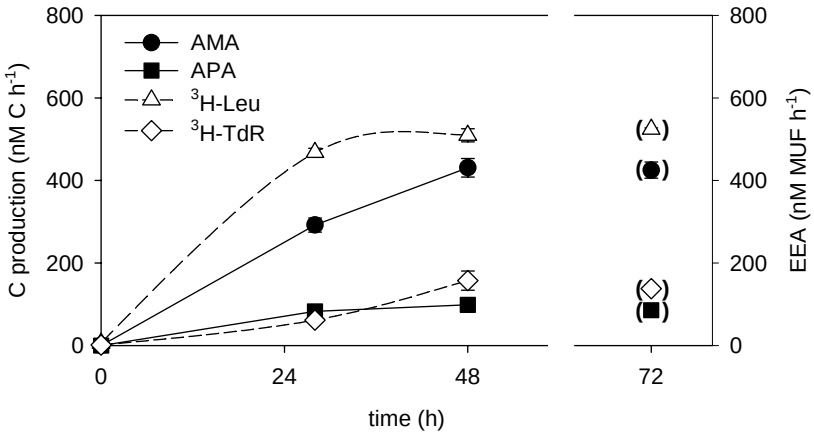


Figure 3.6.4. Bacterial carbon production and extracellular enzymes activity rates. Data are means of duplicate measurements in three independent microcosms. Error bars indicate standard deviation. APA= hydrolysis rate of organic phosphorous; AMA= hydrolysis rate of proteins.

The two methods utilised for the estimation of bacterial carbon production address two different physiological processes: radiolabelled leucine incorporates into proteins while thymidine incorporates into bacterial DNA. In our study Bacteria community expressed a C production rates, as protein incorporation, similar to those measured in highly productive eutrophic systems (i.e. estuaries or marshes, Ducklow and Carlson 1992). The different temporal trends of ^{3}H-Leu and ^{3}H-TdR incorporation could be the consequence of a possible uncoupling between protein and DNA synthesis. These differences could suggest an unbalanced growth at the beginning of the water colonization with bacterial populations devoting cellular resources to biomass synthesis rather than to cell division. Afterwards the increasing cell production not followed by a proportional increase in C production could indicate a decrease in size of the new cells. Taking into account that the per cell ^{3}H-Leu incorporation rates did not vary between 28 h and 48 h, the decreasing ratio of leucine to thymidine incorporation rates could confirm that cell size decrease in time. The ^{3}H-Leu : ^{3}H-TdR ratio values observed in this study were in line with literature values (Ducklow, 2000). Most of the dissolved organic matter re-suspended in the water column is of high molecular weight and it has to be cleaved by hydrolytic enzymes before it can be utilised by bacteria (Chrost, 1990). In this experiment we focused on the dynamics of aminopeptidase and alkaline phosphatase because their crucial roles in the nutrient cycling (C, N and P). The hydrolysis of proteinaceous substances by AMA implies the release of labile substrates, mainly constituted by aminoacids, and the fast recycle of nitrogen. The increase in APA may improve the flux of phosphorous in limited conditions by enhancing hydrolysis of organic phosphorous compounds.

During this experiment, APA and AMA were tightly coupled ($P < 0.01$ $n = 24$), although AMA gave a relatively higher contribution. The narrow link between these two enzymatic activities is regulated by bacterial nutrient demands (Taylor et al. 2003; Hoppe, 2003; Zoppini et al., 2005). AMA and APA were also significantly correlated to bacterial carbon and cell production ($P < 0.01$; $n = 12$) showing the key role played by EEAs in linking the synthesis of both the intracellular proteins and the production of biomass to the high molecular weight organic matter originated from the sediment.

In aquatic systems it has been widely recognised that most EEAs are of bacterial origin (e.g Chrost, 1992; Sala and Gude 1996) and in

our study this is confirmed by the significant relation with bacterial abundance ($P < 0.01$; $n = 12$). However, the protozoan community and algae (i.e. APA) might be responsible for particle degradation through enzyme – catalysed hydrolysis (Hoppe, 2003). Our experimental conditions allow us to exclude algae contribution, but not with flagellates and ciliates. Only ciliates resulted correlated to both the measured EEAs ($P < 0.01$) but it should be noted that ciliates followed a temporal trend similar to bacteria ($P < 0.01$) and their cell abundance was four orders lower.

The lower hydrolysis rates of organic phosphorus with respect to proteins, and the increasing AMA:APA ratio from 3.5 at 28 h to 4.4 at 48 h, can reflect a slower phosphorous cycling with respect to nitrogen, during the microbial community colonization of the water phase. Alternatively, taking into account the extra sampling at 72 h, when AMA:APA ratio reached a value of 5.0 and flagellates an abundance of 5000 cell ml^{-1}, it could be postulated that *Bacteria* express a relatively lower APA because phosphorous was available, released by the high grazing pressure. Protistian grazing on *Bacteria* is an important mechanism of nutrient regeneration, in particular, of nitrogen and phosphorous (Pernthaler, 2005).

Community composition and DNA-synthesizing cells

In dry sediment cells hybridized by EUB I-III probe targeting most bacteria represented about 60% of the total bacteria. Cells affiliated to the six analyzed clusters represented about 77% of Bacteria with a prevalence of *alpha*- and *gamma*-Proteobacteria (21% and 25% respectively) followed by gram-positive bacteria with high (16%) and low (9%) GC content of DNA and *beta*-Proteobacteria (7%).

In water samples, Bacteria cells represented ranged between 94% - 96% of total cells. After 9 h from the rewetting event the water phase was mainly colonized by *Firmicutes* (9 h = 74% of bacterial cells), whereas *alpha*- *beta*- and *gamma*-Proteobacteria emerged at 28 h representing 19%, 3% and 6% respectively. At 48 h the water phase was colonized by a more heterogenic community, where *alpha*- *beta*- and *gamma*-Proteobacteria (19%, 7% and 18% respectively) and *Firmicutes* (14%) were followed by the appearance of *Actinobacteria* (3%) and *Cytophaga-Flavobacteria* (2%) (Figure 3.6.5).

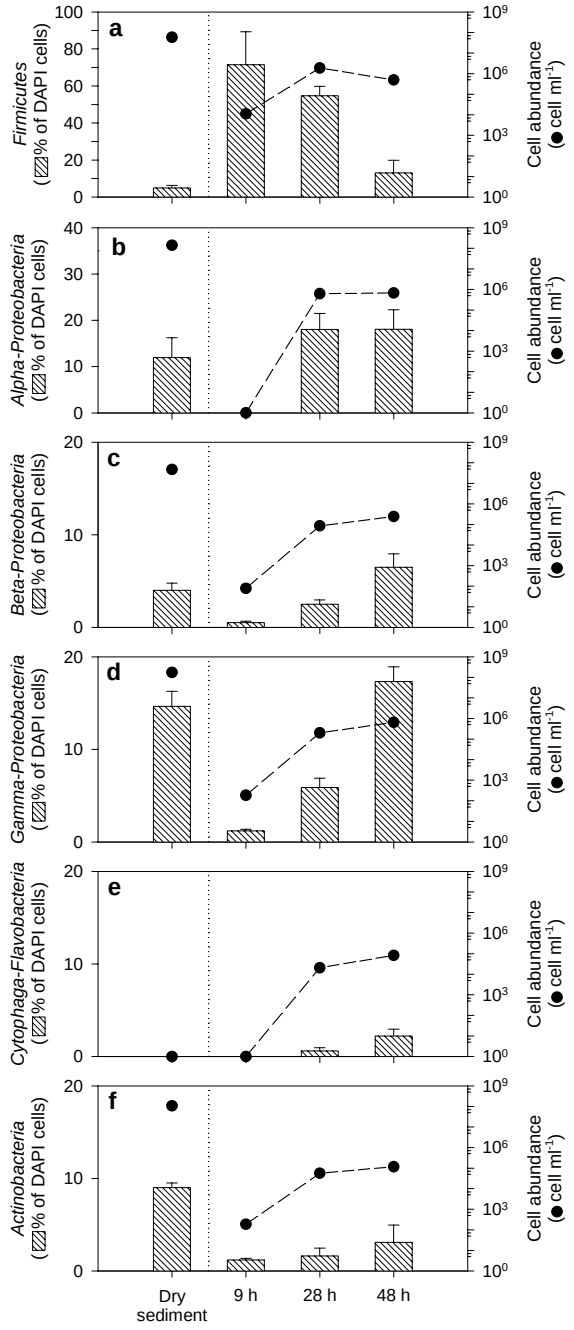


Figure 3.6.5. Taxonomic composition of bacterial community as analysed by CARD-FISH. Values are expressed as: a) Percentage of hybridised cells counts to total counts of DAPI stained cells (left axis); b) Cell abundance expressed as cells per ml (right axis, log scale). Error bars indicate standard deviation.

Moreover image analysis of *Firmicutes* and *alpha*-Proteobacteria hybridized cells by CARD-FISH showed a difference in cells dimension among these two clusters (Figure 3.6.6).

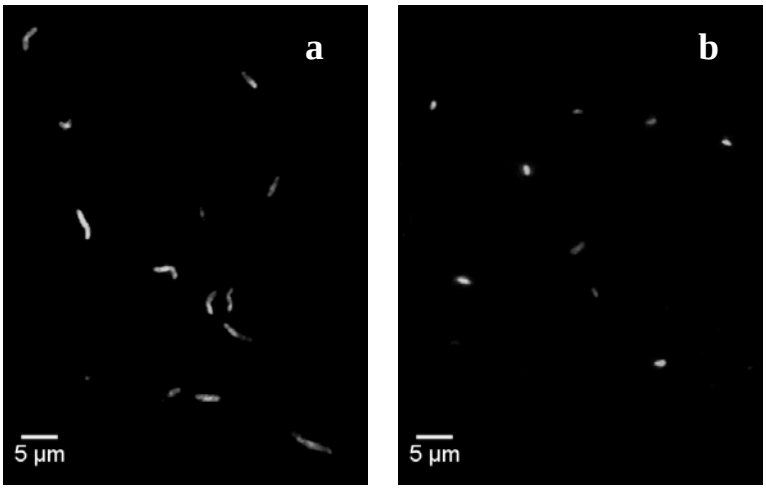


Figure 3.6.6. Examples of bacterial cells hybridised by HRP- labelled oligonucleotide probes by CARD-FISH: a) *Firmicutes* at time 9 h; b) *alpha*-Proteobacteria at time 48h.

The 16S rRNA-based phylogenetic relationships showed that our isolates belonged to at least 5 major phylogenetic groups: *Firmicutes*, *alpha- and gamma*-Proteobacteria, *Cytophaga-Flavobacterium* and *Actinobacterium*. In both 28h and 48h water samples, isolates related to *Firmicutes* formed the largest cluster in term of diversity and relative clone abundance (53 in total; 81.5% of all isolates) with at least 6 phylogenetically distinct organisms. The most frequently recovered clusters of isolates were members of the genus *Bacillus* (39 in total; 60.0% of all isolates) and *Planococcus* (8 in total; 12.3% of all isolates), which were the only groups represented in both the clone libraries. In 48 h water sample, three *Firmicutes* isolates belonged to the genus *Exiguobacterium* (4.6% of all isolates), whereas only one isolate grouped with each genera of *Paenibacillus*, *Sporosarcina* and *Clostridium*. Phylogenetic relationships of the seven most recovered and full sequenced *Firmicutes* isolates are shown in Figure 3.6.7.

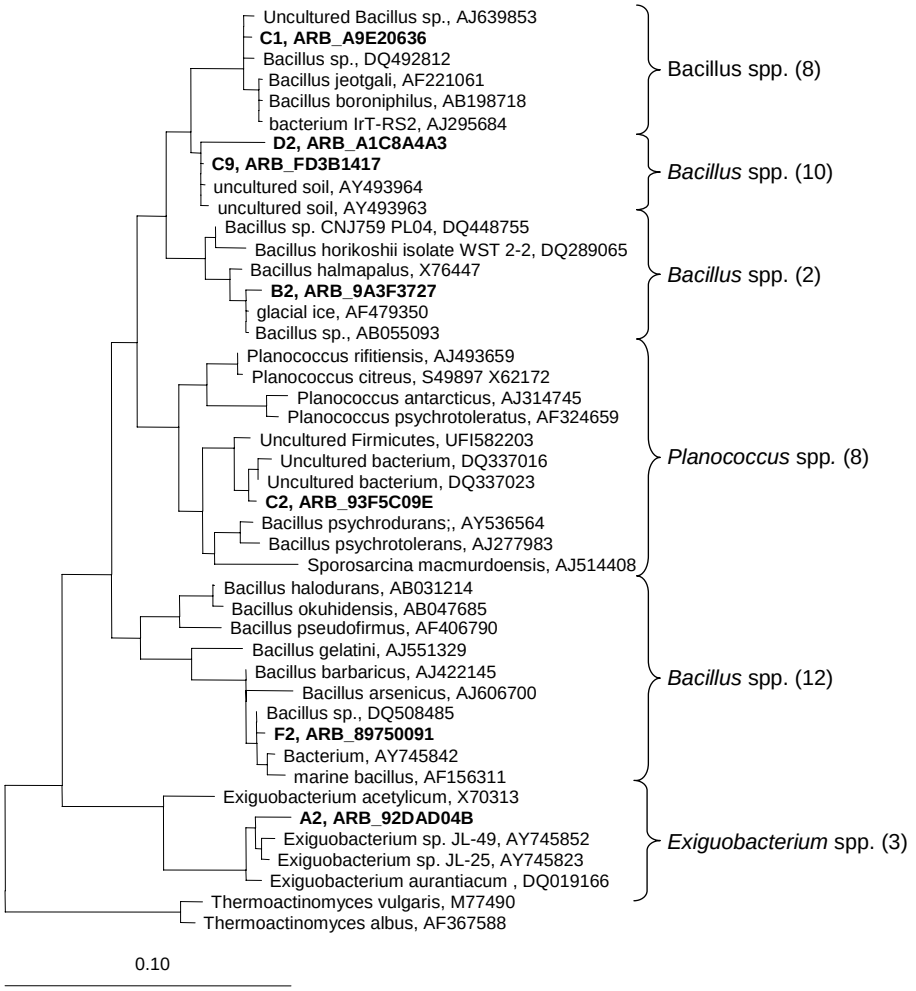


Figure 3.6.7. Maximum-Likelihood tree indicating phylogenetic relationships of *Firmicutes* isolates (in bold) from 24h and 48h water samples and their closest relatives based on GeneBank 16S rRNA sequences. Scale bars indicate p distances.

Members of different phylogenetic groups were exclusively recovered from 48 h water sample. *Alpha*-Proteobacteria (4 in total; 6.2 % of all isolates) were represented by the genera *Brevundimonas* and *Caulobacter*, whereas *gamma*-Proteobacteria (5 in total; 7.7 % of all isolates) by *Xanthomonas* and *Pseudomonas*. Two isolates grouped with the genus *Pontibacter* of *Cytophaga-Flavobacterium* division, whereas only one belonged to the genus *Tetrasphaera* of *Actinobacteria* division.

The Fraction of *Bacteria*, *Firmicutes* and *alpha*-Proteobacteria, related-cells with visible DNA *de novo* synthesis expressed as percentage of total DAPI-stained cells are reported in Figure 3.6.8a. After 9 h of incubation about 20% of the hybridized Bacteria cells were BrdU-positive. This fraction remained fairly constant during the first 28 h and decreased at 48 h (10.5 ± 2.1%). At time 9 h and 28 h, Bacteria incorporating BrdU were mainly members of the *Firmicutes,* whereas *alpha*-Proteobacteria active cells appeared at 28 h (2.2 ± 1.1%). In Figure 3.6.8b-c, *Firmicutes* and *alpha*-Proteobacteria BrdU-positive cells are reported as percentage of cells hybridized by LGC354a and ALF1b probes and as actual active cells number. The BrdU-positive cells fraction of *Firmicutes* constantly increased in percentage from 27.9 ± 11.4% at 9 h to 63.2 ± 18.6% at 48 h, resulting in an increase in the number of active *Firmicutes* cells passing from 9 h to 28 h to 48 h. The active fraction of *alpha*-Proteobacteria slightly decreased from 11.7 ± 4.2% at 28h to 6.0 ± 4.0% at 48 h and the abundance of active cells affiliated to this cluster did not significantly changed from 28 h to 48 h.

The primary microbial colonizers of the water phase during the first 48 h - as determined by 16S rDNA gene sequence analysis and CARD-FISH (probe LGC354a) - were related to at least seven different genera of *Firmicutes* that dominated (70%) the initial bacterial community released in the water phase within the first 9 h. At the beginning of the experiment *Firmicutes* represented 4.9 ± 1.4% of the DAPI stained cells associated to dry sediments, in line with the percentages found in the Mulargia River sediments, monitored during a one-year period. In the same sampling site where sediment was collected for microcosms set-up, we registered an average percentage of *Firmicutes* of 1.3 ± 0.9% (range 0.2% - 2.8%). Moreover, parallel batch experiments with sediments from Mulargia River showed that *Firmicutes* ranged between 0.7% and 6.2% (personal observation). Various clades of *Firmicutes* are reported to be adapted to environmental changes by forming endospores (i.e. Bacillus spp.). This survival strategy could allow to better tolerate desiccation and to promptly spread into the new available water phase (Figure 3.6.9).

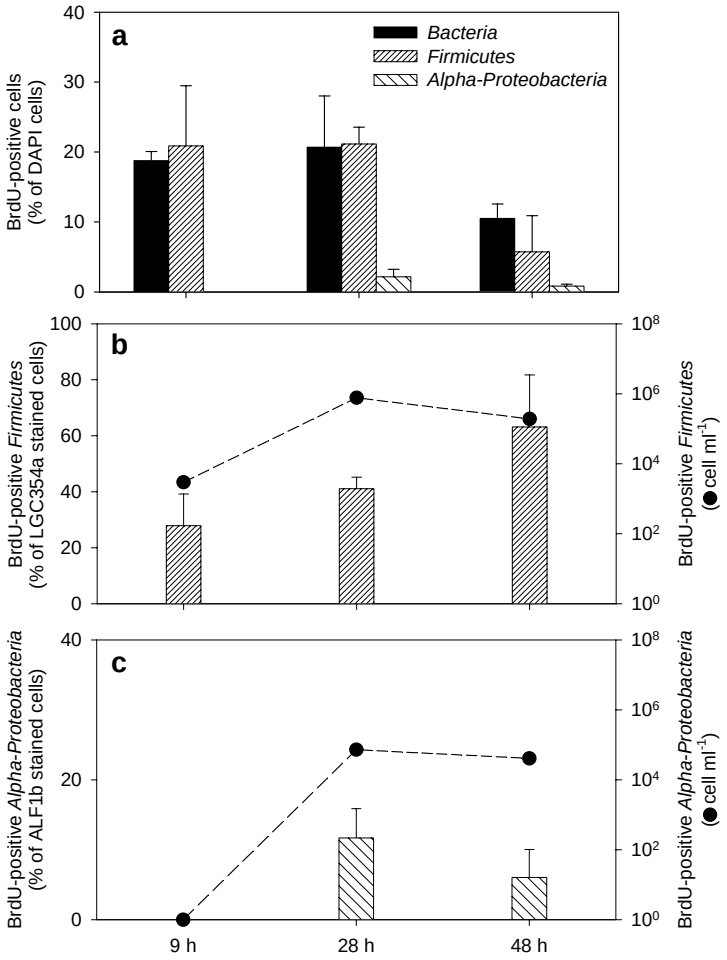


Figure 3.6.8. a) Fraction of Bacteria, *Firmicutes* and *alpha*-Proteobacteria, related-cells with visible DNA *de novo* synthesis (BrdU-positive) expressed as percentage of total DAPI-stained cells; b) *Firmicutes* BrdU-positive cells expressed as percentage of cells hybridized by LGC354a probes (left axis) and as actual active cells number (right axis). c) *alpha*-Proteobacteria BrdU-positive cells expressed as percentage of cells hybridized by ALF1b probes (left axis) and as actual active cells number (right axis). Error bars indicate standard deviation.

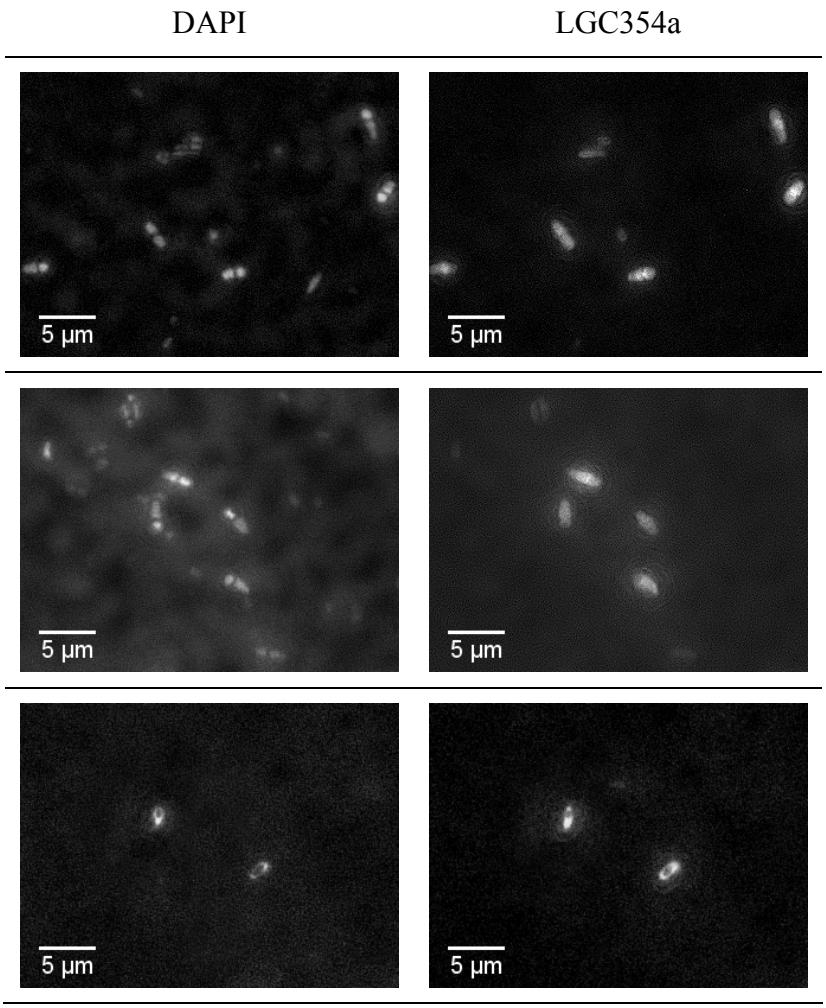


Figure 3.6.9. Microscopic images showing the possible presence of endospores within *Firmicutes* cells, stained both with DAPI and Cy3-labeled probe LGC354a.

From 28 h, the relative importance of *Firmicutes* decreased as community started to be more widely distributed among other clusters (i.e. *alpha*- and *gamma*-Proteobacteria). *Beta*-Proteobacteria and *Cytophaga-Flavobacterium*, typically associated to substrate rich in organic matter, more gradually colonized the water phase. It should be noted that water colonization, especially in the case of these two latter clades, could be enhanced by sediment resuspension that was avoided in our experiment.

Our results showed that after 9 h from the rewetting event, DNA-synthesizing bacterial cells (BrdU-positive) represented about 20% of total bacterial cells decreasing to about 12% at 48 h. Immunocapture of BrdU labeled DNA was already applied to freshwater and soil samples (Urbach et al., 1999; Yin et al., 2000). However, this method in combination with fluorescence *in situ* hybridization was firstly optimized for marine bacterioplankton (Pernthaler et al., 2002b), so far it was not applied to freshwater samples. Pernthaler et al. (2002b) found that within 20 h incubation the detectable DNA-synthesizing fraction increased to 14%. More recently Pernthaler and Pernthaler (2005) reported that in natural samples from coastal North Sea waters about 40% of all hybridized *Bacteria* were also BrdU positive. In seawater incubation experiments Hamasaki et al. (2004) found that free-living BrdU-positive cells were already detectable after 1 h of incubation reaching the maximum values of 10% after 8 h and 25% after 24 h in seawater without and with enrichment respectively. Particle-attached bacteria the percentage of BrdU-positive cells reached the maximum value of about 10% after 50 h incubation.

At 9 and 28 h, DNA-synthesizing cells were mainly members of the *Firmicutes* clades; confirming that in our microcosms these clades were the most reactive colonizers of the water phase. This observation could allow us to estimate *Firmicutes* cell loss rate during community development. At 28 h, by ^{3}H-TdR incorporation, it was estimates a cell production rate of 3.7×10^{4} cell ml^{-1} h^{-1}. Being *Firmicutes* the main DNA-synthesizing bacterial cells, after 20 hours we could expect a theoretical increase of *Firmicutes* of about 7.4×10^{5} cell ml^{-1}. Starting from an abundance of 1.9×10^{6} cell ml^{-1} observed at 28 h, we should theoretically expect that at 48 h *Firmicutes* abundance reached 2.6×10^{6} cell ml^{-1}. This theoretical value is about 9 fold higher that the observed *Firmicutes* abundance at 48h (3.0×10^{5} cell ml^{-1}) and it was possible to estimate a very high cell loss rate of 1.2×10^{5} cell ml^{-1} h^{-1}.

At 28 h, DNA-synthesizing *alpha*-Proteobacteria cells (BrdU-positive) represented about 2.2% of total bacteria, about 10% of DNA-synthesizing bacterial cells. Therefore, it is possible to estimate that in 20 hours they contribute to cell production for about 7.4×10^{4} cell ml^{-1}, reaching at 48 h a theoretical abundance of 6.9×10^{5} cell ml^{-1}, only 0.3 fold higher that the observed *alpha*-Proteobacteria abundance at 48 h (6.2×10^{5} cell ml^{-1}) with a cell loss rate of 8.1×10^{2} cell ml^{-1} h^{-1}.

This different loss rate between *Firmicutes* and *alpha*-Proteobacteria could be due to a different effect of grazing on these two

clusters with a lower grazing pressure on *alpha*-Proteobacteria. It should be noted that we registered an overall decrease in cells size in time and a decrease in the ratio of ^{3}H-Leu and ^{3}H-TdR incorporation, confirming the new production of smaller cells. Moreover the microscopy observation revealed the smaller size of *alpha*-Proteobacteria in respect to *Firmicutes.* The smaller *alpha*-Proteobacteria could be more protected from grazing. Salcher et al. (2005) recently reported that, under the effect of grazing by mixotrophic flagellate *Ochromonas sp*., *alpha*-Proteobacteria formed very small single cells until day 35 of incubation. Grazing induced changes in bacterioplankton community composition have been extensively reported both in laboratory and field study (reviewed by Salcher et al., 2005). Strain-specific variations in grazing sensitivity even between closely related bacteria are high and predator-prey interaction could be an important factor in the evolution and maintenance of microbial microdiversity (Boenigk et al., 2004).

In synthesis we observed a succession of bacterial clusters, although analyzed at large taxonomic scale, with the initial bloom of *Firmicutes*. The selective top-down control appears not only to control the overall bacteria abundance but also to reduce the out-breaking of opportunistic microbes favoring the establishment of a more widely distributed community. This water colonization pattern by bacteria from dry sediments opens new questions on the quality of down-stream water-body if pathogenic bacteria would be the first active colonisers. In this respect, some pathogens are reported as endospore forming and thus could promptly occupy the "bloom niche" in the water flush rich in organic matter realised by the same sediments. Thus, further studies might be aimed at determining the dynamic of bacteria at finer taxonomic scales in heavily contaminated sites and could be of interest to follow the structuring of community development for longer periods after rewetting.

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ANNEX I

Publications related to the Doctorate Thesis

Fazi S., **Amalfitano S**., Pernthaler J., Puddu A. 2005. Bacterial communities associated with benthic organic matter in headwater stream microhabitats. Environmental Microbiology 7 (10) 1633-1640.

Fazi S., **Amalfitano S**., Pizzetti I., Pernthaler J. 2007. Efficiency of fluorescence in situ hybridization for the detection of bacterial cells in river sediments with contrasting water content. Systematic and Applied Microbiology (*in press*).

Amalfitano S., Fazi S, Zoppini A., Barra Caracciolo A., Grenni P., Puddu A. Responses of benthic bacteria to experimental drying in sediments from mediterranean temporary rivers (*passed first revision for Microbial Ecology*).

Amalfitano S. and Fazi S. Methods for the analysis of bacteria in river sediments. (*submitted to Journal of Microbiological Methods*).

Fazi S., **Amalfitano S**., Piccini C., Zoppini A., Puddu A., Pernthaler J. Colonization of the water phase by bacteria from dry river sediments. (*Manuscript*).

Zoppini A., **Amalfitano S**., Fazi S., Puddu A. Extracellular enzymatic activity in temporary river sediments under drying and rewetting conditions (*Manuscript*).

Amalfitano S., Fazi S., Corno G. Bacterial community composition in river sediments and debris. (*Manuscript*).

Tockner K., Uehlinger U., Doering M., Langhans S., Tzoraki O., Nikolaidis N.P., Amaxidis Y., Skoulikidis N.T., Puddu A., **Amalfitano S**., Barra Caracciolo A., Fazi S., Zoppini A., Morais M., Froebrich J. Heterogeneity of Ecosystem Processes in Temporary Streams: The Role of Drying and Rewetting. (*Draft*).

Lo Porto A., De Girolamo A., Diliberto L., Botti P., Canè G., Puddu A., Fazi S., **Amalfitano S**., Zoppini A., Barra Caracciolo A. Hydrology and nutrient processes in a semi arid catchment in Sardinia (Italy) (*Draft*).

Technical reports

Puddu A., **Amalfitano S**., Barra Caracciolo A., Fazi S., Grenni P., Zoppini A., Bacciu F., Falconi F. 2005. River bed sediments. In: Mulargia (Sardinia, Italy) Conceptual model, TempQsim project report - ed. TempQsim (EVK1/2001/005100).

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Amalfitano S., Barra Caracciolo A., Fazi S., Grenni P., Pizzetti I., Arnone R., Zoppini A., Puddu A. 2004. Microbial community response to water stress in a temporary river ecosystem. XIV congresso nazionale della Società Italiana di Ecologia: conservazione e gestione degli ecosistemi, Società Italiana di Ecologia, p125, 44. Siena, 04-06/10/2004.

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Fazi S., **Amalfitano S**., Piccini C., Zoppini A., Puddu A., Allers E., Pernthaler J. 2006. Colonization of the water phase by bacteria from dry river sediments. International Symposium on Microbial Ecology - ISME 11th, International Society for Microbial Ecology – ISME. Vienna, 20-25/08/2006.

Amalfitano S., Zoppini A., Della Chiesa E., Puddu A., Fazi S. 2006 Recovery of bacterial communities residing in dry river sediment

after a rewetting event. XVI Congresso SITE: cambiamenti globali, diversità ecologica e sostenibilità, Viterbo, 19-22/09/2006.

Fazi S., **Amalfitano S**. 2006 New molecular approaches for the assessment of bacterial diversity and single-cell activity in benthic freshwater systems. XVI Congresso SItE: cambiamenti globali, diversità ecologica e sostenibilità, Viterbo, 19-22/09/2006.

Amalfitano S., Fazi S., Zoppini A., Puddu A. 2006. Responses of benthic bacterial communities to river sediment drying: changes in metabolism and community composition. XVI Congresso SITE: cambiamenti globali, diversità ecologica e sostenibilità, Viterbo, 19-22/09/2006.

ANNEX II

January 2004	EAWAG, Department of Limnology (dr. N. Buesing e dr. M. Gessner), Lucerne, Switzerland. Technical improvements for the analysis of bacterial activity in sediment samples.
May 2005	Molecular Ecology Departement, Max Planck Institute for Marine Microbiology (dr. J. Pernthaler), Bremen, Germania. Learning and application of bromodeoxyurdine incorporation in combination with fluorescent oligonucleotidic probes (BrdU-FISH), to estimate the replicative activity of specific benthic bacterial cluster.
July- August 2005	Molecular Ecology Departement, Max Planck Institute for Marine Microbiology (dr. J. Pernthaler e dr. E. Allers), Bremen, Germania. Molecular analyses for sequencing of nucleic acids extracted from sediment samples.
October 2006	Molecular Ecology Departement, Max Planck Institute for Marine Microbiology (dr. B. Fuchs), Bremen, Germania. Elaboration of Flow Citometry data.
November 2006	Limnological Station, University of Zurich, Svizzera. Elaboration of 16S ribosomal RNA sequences and construction of phylogenetic trees by using the software ARB.