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## Efficiency of fluorescence in situ hybridization for bacterial cell identification in temporary river sediments with contrasting water content

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### Abstract

We studied the efficiency of two hybridization techniques for the analysis of benthic bacterial community composition under varying sediment water content. Microcosms were set up with sediments from four European temporary rivers. Wet sediments were dried, and dry sediments were artificially rewetted. The percentage of bacterial cells detected by fluorescence in situ hybridization with fluorescently monolabeled probes (FISH) significantly increased from dry to wet sediments, showing a positive correlation with the community activity measured via incorporation of <sup>3</sup>H leucine. FISH and signal amplification by catalyzed reporter deposition (CARD-FISH) could significantly better detect cells with low activity in dried sediments. Through the application of an optimized cell permeabilization protocol, the percentage of hybridized cells by CARD-FISH showed comparable values in dry and wet conditions. This approach was unrelated to <sup>3</sup>H leucine incorporation rates. Moreover, the optimized protocol allowed a significantly better visualization of Gram-positive *Actinobacteria* in the studied samples. CARD-FISH is, therefore, proposed as an effective technique to compare bacterial communities residing in sediments with contrasting water content, irrespective of differences in the activity state of target cells. Considering the increasing frequencies of flood and drought cycles in European temporary rivers, our approach may help to better understand the dynamics of microbial communities in such systems.

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**Keywords:** Freshwater sediments; Temporary rivers; CARD-FISH; Bacteria; *Actinobacteria*

### Introduction

Drought periods in the Mediterranean area are increasing in both frequency and duration as a consequence of global warming [14]. Often not only the typical temporary streams, but even extended reaches of large rivers are exposed to dry periods. Therefore, interest is increasing to investigate how

ecological processes in river sediments are affected by drastic changes in water content. In this context, methods need to be optimized to compare bacterial communities residing in dry and wet sediments.

The composition of benthic microbial communities can be readily studied by whole-cell fluorescence in situ hybridization [11]. Although FISH with fluorescently monolabeled oligonucleotide probes is an effective tool for studying microbial community structuring in various aquatic environments [2,18], its sensitivity is thought to depend on the growth state (i.e., ribosome content) of

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the target cells [1]. 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 recent years, FISH and signal amplification by catalyzed reporter deposition (CARD-FISH) has developed into a useful approach to study bacterial cells with low ribosome content (e.g., non-growing cells) [8,16,23]. In a previous study [7], we compared the efficiency of FISH and CARD-FISH in freshwater prokaryote communities in different types of submerged benthic substrates. FISH detection rates were significantly lower in communities from substrates with low organic matter content, whereas CARD-FISH did not show any relation with organic matter content. Assuming that the availability of organic matter reflected bacterial activity, we suggested that CARD-FISH results were less dependent on the growth state or activity of cells. Starting from these conclusions, we now hypothesized that CARD-FISH could also be a useful instrument to assess the composition of communities in dry sediments that 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 four temporary rivers of the Mediterranean area 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 radiolabeled tracers 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*.

## Materials and methods

### Microcosm setup

Sediment samples were collected from four rivers that are naturally liable to intermittent drought periods: Mulargia and Tagliamento (Italy), Krathis (Greece), Pardiela (Portugal). For the drying experiment, sediments were sampled on May 2004 from the uppermost oxidized zone (0.5–5 cm depth) and sieved (2 mm mesh). Wet sediment (300 g) was placed in duplicate 11 beakers at room temperature until completely dry. Sub-samples were collected at day 0 (“wet”: water content 100% of

the maximum water holding capacity – WHC), day 32 (“humid”: ~30% of WHC) and day 46 (“dry”: 0% of WHC). The water content was measured as the difference between gross and dry weight (105 °C, overnight). In the rewetting experiment 300 g of artificially dried sediment, collected on November 2004, were placed in duplicate 11 beakers. Filter-sterilized Milli-Q water was added to 100% WHC. Sub-samples for community analysis were collected from dry sediments and 8 days after the rewetting, when bacterial carbon production reached its maximum.

### Bacterial community analysis

At each sampling time-point, three sub-samples from each microcosm were fixed in ethanol (EtOH, 50% vol/vol final concentration) and three in buffered formaldehyde solution (FA, 2% vol/vol final concentration) at room temperature for a maximum of 24 h. Fixed samples were pre-treated in order to chemically and physically detach microbial cells from particles [7]. Briefly sediment (1 g) was placed in a chemical detachment solution (10 ml final volume), consisting of Milli-Q water, NaCl (0.85% wt/vol final concentration),  $\text{Na}_4\text{O}_7\text{P}_2$  (0.1 M final concentration), and Tween20 (0.5% vol/vol final concentration). Samples were then incubated for 30 min under mild agitation at room temperature. Physical detachment was subsequently performed by sonication on ice (20–35 W for 60 s, Microson XL2000 ultrasonic liquid processor with 1.6 mm diameter microtip probe, Misonix, New York, USA). Sub-samples (100  $\mu\text{l}$ ) of sonicated slurry were filtered onto black polycarbonate membrane filters (pore size 0.2  $\mu\text{m}$ , 25 mm diameter, Nuclepore Corporation, Pleasanton, USA) and stained with 4,6-diamidino-2-phenylindole (DAPI) for the regular determination of total bacterial abundances. Next, 1 ml of sonicated slurry was sub-sampled after vortex agitation, and an additional purification step was performed by high-speed centrifugation with the non-ionic density gradient medium Nycodenz (Nycomed, Oslo, Norway); the supernatant was filtered on polycarbonate membrane filters (pore size, 0.2  $\mu\text{m}$ , 47 mm diameter, Nuclepore) by gentle vacuum (<0.2 bar) and the preparations were washed with 20 ml of Milli-Q water. The filters were stored in Petri dishes at –20 °C until further processing.

FISH with fluorescently monolabeled oligonucleotide probes and counterstaining with DAPI was performed according to the protocol of Pernthaler et al. [18]. Probes were commercially synthesized (Biomers, Ulm, Germany) and labeled with the indocarbocyanine dye Cy3. The following oligonucleotide probes were used: probe mix EUB I–III, targeting most *Bacteria* [6] and HGC69a, targeting *Actinobacteria* [19].

CARD-FISH was performed with the probes EUB I-III and HGC69a labeled with horseradish peroxidase. Prior to hybridizations, the filter sections were embedded in low gelling point agarose in order to avoid cell loss [16]. For *Bacteria* and *Actinobacteria* detection, cell permeabilization was performed by lysozyme (62970 Fluka, 20 mg ml<sup>-1</sup>, 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 (A 3547 Sigma-Aldrich, 120 U ml<sup>-1</sup>, dissolved in 0.01 M NaCl, 0.01 M Tris-HCl pH 8.0) for 30 min at 37 °C [7]. In the present study, we also tested a new cell permeabilization protocol maintaining the pre-treatment with lysozyme from our previous protocol, but replacing achromopeptidase by proteinase K (0.1 U ml<sup>-1</sup> in Tris-EDTA buffer) as proposed for marine prokaryotes [17,22]. 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 a Leica DM LB 30 epifluorescence microscope (Leica Microsystems GmbH, Wetzlar, Germany) 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.

## Bacterial carbon production

Bacterial carbon production (BCP) was measured by <sup>3</sup>H leucine incorporation. The protocol by Buesing and Gessner [4] was modified by applying the micro-centrifugation technique proposed for water sample by Smith and Azam [21]. Sediment sub-samples (0.5 g) were dispersed in Milli-Q water (1 ml final volume). Aqueous solution of leucine (50 μM final concentration: unlabeled 49.85 μM, L 8912 Sigma-Aldrich; <sup>3</sup>H labeled 0.15 μM, NEN Life Science Products, Boston, USA) was added to three replicates of each sediment sample. Blank controls were also prepared by adding 100% trichloroacetic acid (TCA, 5% vol/vol final concentration) prior to incubations. Samples were homogenized by vortexing and incubated in the dark at in situ temperature for 60 min. Incubations were stopped by adding 100% TCA.

All tubes were centrifuged at 14,000 RCF for 10 min at room temperature. Next, the supernatant was discarded to separate macromolecules from non-incorporated label. Four additional washing steps by centrifugation were performed thereafter, adding successively 5% TCA, 40 mM unlabeled leucine, 80%

EtOH, Milli-Q water and 1 N NaOH. Samples were then heated for 60 min at 90 °C, cooled and again centrifuged. Liquid scintillation cocktail (1 ml, Ultima Gold, Packard Bioscience, Meriden, USA) was added to 0.1 ml of supernatant and radioactivity was counted in a scintillation counter (TRICARB 4430, Packard). Counts were corrected for quenching and efficiency of the instrument, and the rates of leucine incorporation were transformed into units of C by applying the conversion factor proposed by Buesing and Marxsen [5].

## Data analysis

Statistical analysis of the observed differences was performed using two-way ANOVA on the pooled data set from all rivers followed by Student Newman Keuls (SNK) multiple comparisons. We did not test for differences between rivers, since this was not the scope of the study. We tested if the total cells and the percentages of hybridized fractions of *Bacteria* significantly differed between two levels of fixatives (EtOH, FA) and three levels of hybridization techniques (FISH, CARD-FISH with achromopeptidase pre-treatment, CARD-FISH with proteinase K pre-treatment). Two-way ANOVA was also used to compare the percentage of hybridized *Bacteria* by FISH and by CARD-FISH after permeabilization with proteinase K in dry, humid and wet conditions. In addition, we tested for differences between the percentages of hybridized *Actinobacteria* by FISH and by CARD-FISH with proteinase K pre-treatment. Analysis of covariance (ANCOVA) was performed to compare the relationship between percentages of hybridized cells by the two methods and cell activity. The statistical analysis software SigmaStat 3.1 (Systat Software, Inc., Point Richmond, CA, USA) was used for calculations.

## Results and discussion

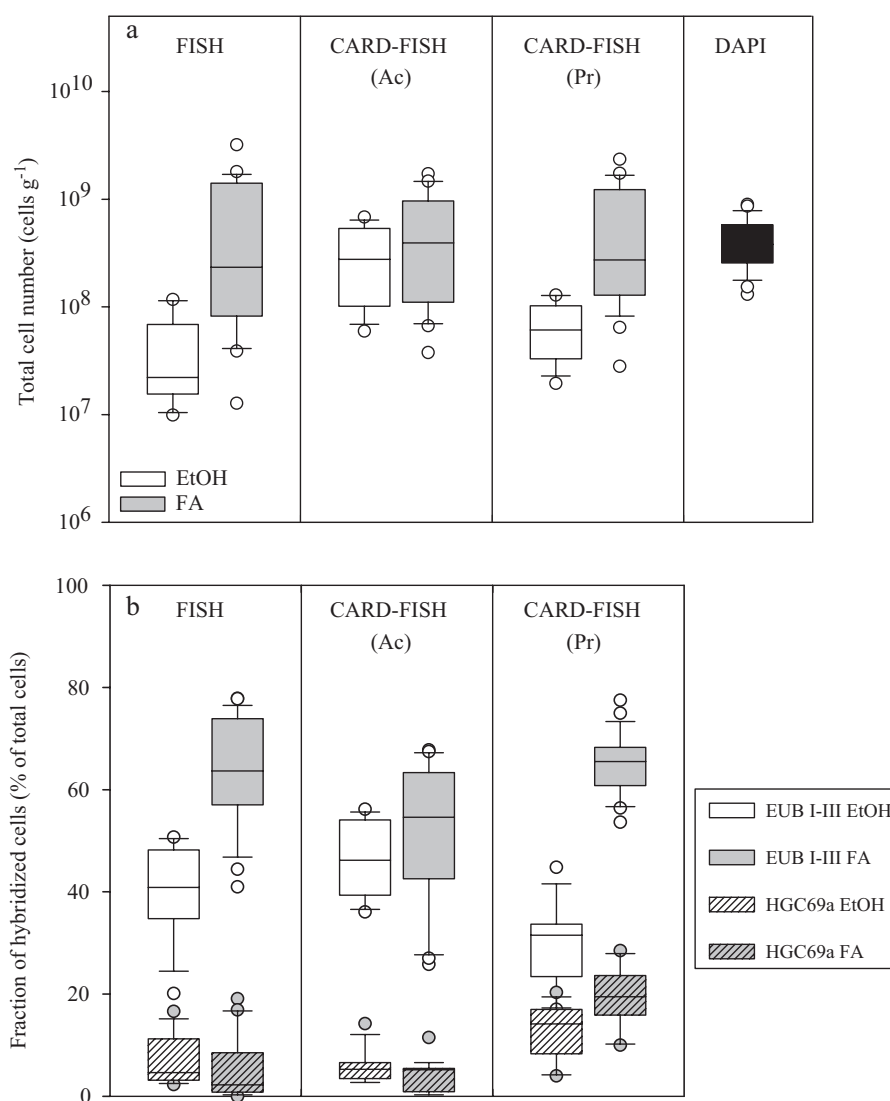
### Potential sources of cell loss during fixation and staining procedure

It has been suggested that total cell counts (i.e., DAPI counts) should not be directly determined from FISH preparations [17] 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 (Fig. 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 FA fixed samples were comparable to those from regular DAPI counts in unhybridized preparations, whereas substantially fewer cells were counted in EtOH fixed samples (Fig. 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 ( $q = 4.99$ ,



**Fig. 1.** (a) Mean values and ranges of total cell number (DAPI stained) after hybridizations on membrane filters ( $\varnothing$  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 unhybridized FA fixed samples on black membrane filters ( $\varnothing$  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.

$p < 0.05$ ). 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 (Fig. 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 [20]. 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 [7]. In FA-fixed samples this pre-treatment resulted in a significantly lower detection of *Bacteria* ( $51.5 \pm 14.6\%$ ; CV = 28.4) than those by FISH with monolabeled probes ( $64.0 \pm 11.0\%$ ; 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 [17,22]. We combined lysozyme from our previous protocol, with proteinase K as a second permeabilizing agent.

This treatment resulted in percentages of hybridized *Bacteria* ( $64.9 \pm 6.1\%$ ; CV = 9.4) comparable (but less variable) to those by FISH with monolabeled probes (Table 1, Fig. 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 1, Fig. 1b) and (ii) substantial cell loss (see above, Fig. 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 [19]. 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 1, Figs. 1b and 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 (Fig. 1a), Gram-positive cells were likely less adversely affected by alcohol fixation than Gram-negative *Bacteria*.

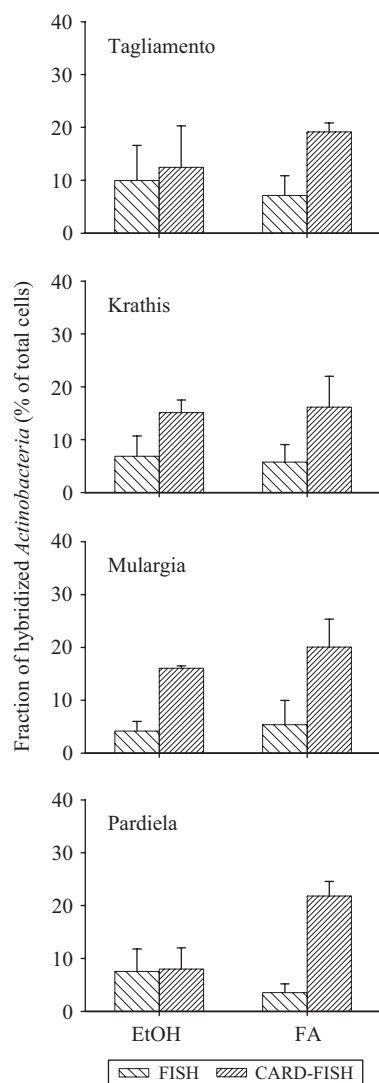
### Effect of microbial activity on FISH and CARD-FISH

During both the drying and rewetting experiments, bacterial communities associated to dry sediments showed the lowest BCP ( $0.05 \pm 0.03 \mu\text{g C h}^{-1} \text{g}^{-1}$ ) in comparison with wet sediments ( $1.37 \pm 0.35 \mu\text{g C h}^{-1} \text{g}^{-1}$ ) (Fig. 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

**Table 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

	FISH (EtOH)		FISH (FA)		CARD-FISH (EtOH, Pr)	
	$q'$	$p$	$q'$	$p$	$q'$	$p$
<i>Probe EUB I–III</i>						
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
<i>Probe HGC 69a</i>						
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

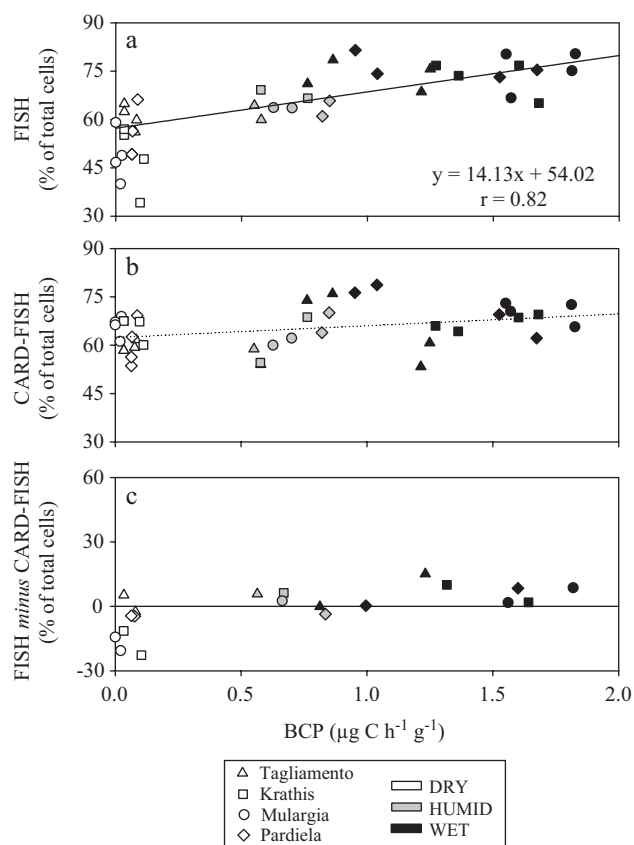
EtOH, ethanol fixation; FA, formaldehyde fixation. Pr, lysozyme and proteinase; K, permeabilization. NS, not significant.



**Fig. 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) and formaldehyde (FA). Error bars indicate standard error.

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 [13], 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 ( $75.5 \pm 2.5\%$ ), to humid ( $64.3 \pm 2.5\%$ ) and dry conditions ( $55.3 \pm 8.0\%$ ), whereas



**Fig. 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 modeled by a linear combination of the two independent variables BCP and percentage of *Actinobacteria* cells (probe HGC69a) ( $y = 9.01 + (5.12 \times \text{BCP}) - (0.58 \times \% \text{ Actinobacteria})$ ,  $r = 0.78$ ,  $p < 0.01$ ).

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 ( $< 48\%$  [11]) and freshwater sediments ( $< 58\%$  [7];  $< 50\%$  [9]).

Moreover, the fraction of cells detected by FISH was linearly related to BCP ( $y = 14.1x + 54.1$ ,  $r = 0.82$ ,  $p < 0.01$ ) 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 (Fig. 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 [10,16,20]. 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 [12], and starving bacterial cell are not quantitatively detected by FISH with monolabeled probes [15]. CARD-FISH overcomes this difficulty by an additional signal amplification step [3]. 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 [7,16]. 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.,  $^3\text{H}$  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 ( $y = 0.2x + 0.8$ ;  $r = 0.70$ ,  $p < 0.05$ ).

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. [20] 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* (Fig. 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 assem-

blages 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.

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