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Effects of submarine groundwater discharge on coastal bacterioplankton communities from the NW Mediterranean

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CERTIFIES:

That the research work entitled "Effects of submarine groundwater discharge on coastal bacterioplankton communities from the NW Mediterranean" has been carried out by Olena Maister in deparment of marine biology and oceanography under the supervision of Dr. Clara Ruiz-González from the of the Instituto de Ciencias del Mar (ICM-CSIC) in order to achieve 30 ECTS as a part of the MER Master program.

In Barcelona, 31 July 2018

Signed:

Manu Soto

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Abbreviations

BONCAT	Bioorthogonal non-canonical amino acid tagging
CARD-FISH	Catalyzed Reported Deposition Fluorescence in situ hybridization
DAPI	4`6-diamidino-2-phenylindole stain
DOC	Dissolved organic carbon
HMW	High molecular weigh compounds
LMW	Low molecular weigh compounds
MGV	Mean grey value
PCA	Principal component analysis
SGD	Sea groundwater discharge
UVR	Ultraviolet radiation

SUMMARY

Bacteria play major roles in biogeochemical cycles across all kinds of terrestrial and aquatic ecosystems. Although there has been plenty of research on the ecology and diversity of bacterial communities from surface terrestrial and aquatic ecosystems, we know very little about bacteria inhabiting subsurface environments like groundwater aquifers. In particular, coastal groundwater aquifers are gaining increasing attention because submarine groundwater discharge (SGD) has recently been shown to be extremely important for coastal ecosystems, delivering large amounts of nutrients. Due to our limited knowledge of groundwater microbial diversity, however, whether these coastal aquifers also represent reservoirs of bacterial diversity for coastal bacterial communities remains completely unknown. Here, we first aimed at characterizing the taxonomic composition and activity of bacterial communities in various coastal groundwater aquifers and their adjacent marine coastal sites along the NW Mediterranean coast, where SGD has been shown to be much more important than previously believed. Second, we experimentally explored the responses of marine bacterial communities to groundwater additions, as well as the capacity of groundwater taxa to grow under marine conditions. Using catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) coupled with bioorthogonal non-canonical aminoacid tagging (BONCAT), we could quantify the abundances of major bacterial groups and their single-cell activity across aquifers, or in response to the experimental mixing of seawater and groundwater. Our results show that Mediterranean groundwater aquifers show much lower bacterial abundances and activity than surface sea- or river water, and that are much more heterogeneous in terms of taxonomic composition at the spatial scale, probably due to higher isolation and more variable physico-chemical conditions between aquifers. Interestingly, our mixing experiment showed fast but transient increases in activity of marine opportunistic bacterial groups after groundwater addition. Moreover, despite the low *in situ* abundance and activity of groundwater bacteria, we observed a large potential of some groundwater bacterial groups such as Gammaproteobacteria, *Roseobacter* and Bacteroidetes to grow when exposed to marine conditions. This work represents the first attempt to describe spatial variation in bacterioplankton communities in the Mediterranean aquifers. Moreover, our results provide evidence that groundwater discharge to the ocean could also transport some viable bacterial taxa able to grow in the ocean. All this will enlarge our knowledge on the influence of SGD on marine bacterioplankton communities, as well as on the bacterial potential to persist under unfavourable conditions and grow in different environments.

SECOND LANGUAGE SUMMARY (RESUMEN)

Las bacterias desempeñan un papel muy importante en los ciclos biogeoquímicos en todo tipo de ecosistemas terrestres y acuáticos. Aunque ha habido muchos estudios sobre la ecología y la diversidad de las comunidades bacterianas de ecosistemas terrestres y acuáticos superficiales, sabemos muy poco sobre las identidad y función de las bacterias que habitan en entornos subsuperficiales como los acuíferos subterráneos. En particular, los acuíferos subterráneos costeros están atrayendo interés debido a que se ha demostrado recientemente que la descarga de aguas subterráneas submarinas (SGD) es extremadamente importante en los ecosistemas costeros, ya que aporta grandes cantidades de nutrientes. Sin embargo, debido a nuestro limitado conocimiento de la diversidad microbiana del agua subterránea, se desconoce si estos acuíferos costeros representan también reservorios de diversidad microbiana para las comunidades bacterianas costeras. En este estudio, quisimos en primer lugar caracterizar la composición taxonómica y la actividad de las comunidades bacterianas presentes en varios acuíferos costeros y en la costa adyacente a lo largo de la costa mediterránea noroccidental, donde se ha observado que la SGD es mucho más importante de lo que se creía anteriormente. En segundo lugar, exploramos experimentalmente las respuestas de las comunidades bacterianas marinas a las adiciones de agua subterránea, así como la capacidad de las bacterias de aguas subterráneas para crecer en condiciones marinas. Mediante el uso combinado de las técnicas de microscopia CARD-FISH *(catalyzed reporter deposition-fluorescence in situ hybridization)* y BONCAT (*bioorthogonal non-canonical aminoacid tagging*) pudimos cuantificar la abundancia de los principales grupos bacterianos y su actividad en los distintos acuíferos, así como en respuesta al experimento de mezcla de agua de mar y agua subterránea. Nuestros resultados muestran que los acuíferos mediterráneos esconden una abundancia y actividad bacteriana mucho menor que las aguas superficiales de mar o de río, y que son mucho más heterogéneos en términos de composición taxonómica a escala espacial, probablemente debido a un mayor aislamiento y condiciones fisicoquímicas más variables entre distintos acuíferos. Por otra parte, gracias al experimento de mezcla observamos aumentos rápidos pero transitorios en la actividad de algunos grupos bacterianos marinos oportunistas pocas horas después de la adición de agua subterránea. Además, a pesar de la baja abundancia y actividad de las bacterias del agua subterránea, observamos que algunos grupos bacterianos de aguas subterráneas tales como Gammaproteobacteria, *Roseobacter* and Bacteroidetes son capaces de crecer rápidamente una vez expuestos a condiciones marinas. Este trabajo representa el primer intento de describir la

variación espacial en las comunidades de bacterioplancton presentes en acuíferos subterráneos mediterráneos. Además, nuestros resultados proporcionan evidencia de que la descarga de aguas subterráneas al océano podría proporcionar, además de nutrientes y otros compuestos, algunos taxones bacterianos capaces de crecer en el océano. Estos resultados amplian nuestro conocimiento sobre la influencia del SGD en las comunidades de bacterioplancton marino, así como sobre el potencial de estos microorganismos para persistir en condiciones desfavorables y su capacidad de crecer en diferentes ambientes.

1 INTRODUCTION

1.1 The Earth, a microbial planet

At the beginning of the evolution of the Earth, nearly all metabolic processes were performed by microorganisms. They were able to influence all elements speciation and created the environment we have nowadays (Falkowski et al., 2008). Microbes (and in particular prokaryotic microorganisms such as bacteria and Archaea) can be found everywhere, in all the environments on our planet. Five out of six main biological fluxes of the major elements such as H, C, N, O, S (Falkowski et al., 2008) are driven by microorganisms, and together with some geochemical processes they create the average redox conditions of all the systems we have today (for example atmosphere and hydrosphere). Environmental changes during the evolution of the Earth accounted for huge animal and plant extinctions, but not microorganisms, which remained the main biological core of the planet (Falkowski et al., 2008). Microbes are small in size, have special adaptations and are able to evolve fast, all which allowed them to survive in such drastic environmental changes (such as global glaciations and volcanic eruptions) and massive life extinctions.

In the present, prokaryotes are an enormously diverse group of organisms that represents a enormous amount of genetic and metabolic diversity on our planet (Whitman et al., 1998). Prokaryotes can be found in all imaginable environments, even in the most extreme. Besides being major players in biogeochemical cycles, prokaryotes also play a very important role in the structure and dynamics of all food webs, especially in aquatic ecosystems (Andrade et al., 2003). Initially they were considered to be the main organisms to decompose organic matter (Cole et al., 1988), but their heterotrophic production can be quite high in the environment and their biomass can even exceed biomass of autotrophs. Indeed, the role that bacteria play in the aquatic carbon cycle was only unveiled after the postulation of the so-called 'microbial-loop' hypothesis (Azam et al. 1983), since they were initially thought to be predominantly inactive or in a dormant state. The microbial loop concept is based on the fact that bacterial communities comprise an important trophic link in aquatic food webs, given that they can take up a large fraction of the dissolved organic matter (DOM) and can either respire it to carbon dioxide (CO_2), or transform it into particulate organic matter (POM, bacterial biomass) making it available for higher trophic levels (i.e., small flagellates or ciliates that feed on bacteria, and that can be in turn eaten by larger organisms). This DOM used by aquatic bacteria is usually released by phytoplankton

communities into the environment, but it can also derive from the surrounding terrestrial environments. For example, bacterioplankton respiration of terrestrial dissolved organic carbon (DOC) has recently been shown to be one of the major pathways fuelling CO_2 emissions from inland waters (Jonsson et al., 2003, McCallister and Giorgio, 2008). Similarly, since approximately 0.5 Pg of OC are annually discharged by rivers to the ocean (Bianchi, 2011), there is also a widespread dependence of marine communities on allochthonous DOC (Duarte and Prairie, 2005). Moreover, bacteria also demand inorganic nutrients that are usually in deficiency, in particular in oligotrophic systems, where the surrounding coast is also a major source of nutrients for bacteria.

In the last decades, the use of high throughput sequencing technologies has largely advanced our knowledge of prokaryotic communities ecology and biogeography by enabling an extremely detailed characterization of the taxonomic composition of such communities (e.g. Pedrós-Alió 2012). We know now that microbial communities are extremely diverse, and that different bacterial groups can perform different functions in the environment. For example, the SAR11 clade is one of the most abundant microorganisms in the surface waters and in some regions accounts for 50% of the total surface bacterial community (Morris et al., 2002). Together with their freshwater sister clade (LD12), they are highly competitive in environments with low nutrient concentrations (Giovannoni, 2017). On the other hand, groups like the *Roseobacter* clade and Gammaproteobacteria represent very diverse opportunistic groups that can respond fast to nutrient inputs (Luo and Moran, 2014, Eilers et al., 2000a). The distribution of certain phototrophic taxa, like the cyanobateria *Synechococcus* is mainly influenced by physical factors such as temperature and light (Flombaum et al., 2013), while marine Bacteroidetes are usually present attached to particles (Nold and Zwart, 1998) or algae (Newton et al., 2011) due to their ability to degrade high molecular weight compounds. All these specific physical and biological conditions influence the structure of communities, which in turn determines community functioning. Moreover, even small changes in these conditions affect the composition of bacterial communities, causing changes in the dominant species and the activity of bacteria in the environment. As a result, there are plenty of studies showing that different bacterial taxa dominate along environmental gradients and across different aquatic and terrestrial ecosystems. Understanding how the taxonomic composition and functioning of these communities vary upon changes in environmental conditions is thus essential to predict how communities (and thus

ecosystem functioning) will respond to climate or environmental variations related to global changes.

In addition, prokaryotic communities harbour a huge number of rare taxa (i.e., taxa present at very low abundances) that has been referred to as the 'rare biosphere' (Sogin et al., 2006). Multiple studies have attempted to understand the ecological implications of these rare taxa. For example, it has been suggested that due to their ability to stay in a dormant state with low metabolic activity for long periods of time, many of these rare taxa comprise a 'microbial seed bank' (Lennon and Jones, 2011) that can grow as soon as environmental conditions change or if microorganisms are transported to more appropriate conditions, like for example when being washed from soils to rivers and lakes (Comte et al., 2014, Ruiz−González et al., 2015). However, most microbial ecology studies are restricted to single ecosystems (e.g., only soils, only lakes, only the ocean), but in view of the abovementioned ability of bacteria to persist, ecosystems interconnections should be studied to get an overview on microbial migrations through different ecosystems and their ability to grow in new environments and to impact local species composition (Ruiz−González et al., 2015, Crump et al., 2012). Whereas this kind of approaches have been mostly applied in terrestrial or freshwater ecosystems, oceanographic studies have rarely considered linkages with surrounding ecosystems in terms of the dispersal of microorganisms.

1.2 **Submarine groundwater discharge: an important connection pathway between terrestrial, freshwater and marine ecosystems**

Groundwater is defined as water that is located in soils and pores between grains under the surface, and it can be found in subsurface layers called aquifers. Aquifers can be located at different depths and contain different water types and quality depending on the surrounding rocks and substrate. Usually, aquifers that are located deep in the soil are used as a source of potable water. Groundwater usually has deficit of organic carbon, as there is no light reaching these ecosystems and therefore there is a lack of photosynthesis of fresh and labile organic carbon. These characteristics influence the microbial communities inhabiting these harsh ecosystems with a preference for heterotrophic organisms (and mostly microorganisms) that are well adapted to these dark oligotrophic environments (Griebler and Lueders, 2009).

Among groundwater aquifers, those located in coastal zones are essential water sources for humanity, given that population density is significantly higher in coastal than in non-coastal

areas (Small et al. 2003). This, together with the longer drought conditions in many coastal areas, is dramatically increasing reliance on groundwater resources, leading to enhanced contamination and salinization of aquifers globally (Werner et al. 2013, Zhou 2015). Moreover, groundwater aquifers draining to coastal waters have been recently shown to be much more important than previously believed in terms of nutrient inputs to the ocean (Rodellas et al., 2015, Burnett et al., 2006, Kwon et al., 2014), and in particular for semi-enclosed oligotrophic seas like the Mediterranean, where submarine groundwater discharge (SGD) has been shown to account for as much nutrient inputs as rivers or atmospheric deposition (Rodellas et al., 2015). Coastal aquifers draining to the ocean can be divided in two types: fresh meteoric waters from karst aquifers that go straight to the sea; or saline water that is recirculated through sediments and mixed with freshwater (Rodellas et al., 2015). The first type is the one that is often used as "drinkable" water. In this environment, biological activity can only be driven by specialized and highly adapted microbes (Flynn et al., 2013). All the oxidation and reduction reactions of these microorganisms will affect the composition of the groundwater and weathering processes in the aquifers. Although usually only this fresh groundwater discharge is taken into account while estimating total submarine groundwater discharge flow, but in terms of water flow, recirculated SGD tends to dominate (Rodellas et al., 2015). Recirculated brackish groundwater is present in the so-called detrital aquifers, where saline water seeps to the sand and soil, mixes with freshwater creating also a salinity gradient and dissolves components from the ground. Then this water goes back to the sea enriched in nutrients, metals and carbon, which have been shown to influence the structure and functioning of the receiving marine communities (Berdalet et al., 1996, Garcés et al., 2011).

The influence of the groundwater is extremely important in oligotrophic regions, where as soon as new nutrients reach the marine environment they promote an increase in microbiological activity and their diversity. A good example of this is the semi-enclosed Mediterranean Sea, which due to its geology and oceanography, is considered as one of the most oligotrophic seas (Redfield, 1963, Rodellas et al., 2015). As a result, the Mediterranean Sea is characterized by low primary production and phytoplankton biomass over the year. Nevertheless, some areas are known to be highly productive with high nutrients availability (Garcés et al., 2011). These areas are nearshore coastal zones that are mainly influenced by the continental runoff, groundwater discharge as well as aerosols and anthropogenic impacts. Although submarine groundwater discharge (SGD) was not usually taken into consideration, recent studies

have shown that it plays an important role in nutrient delivery (Rodellas et al., 2015). Interestingly, the addition of groundwater was experimentally shown to promote the growth and activity of bacteria and phytoplankton, as well as changes in the taxonomic composition of phytoplankton communities from Mediterranean coastal waters (Garcés et al., 2011). All this suggests that groundwater inputs are very important for coastal microbial communities and their activity. Thus, understanding how coastal communities react to SGD is essential to eventually predict the consequences of hydrologic changes related to global changes.

1.3 Are coastal aquifers sources of microbial diversity to the sea?

Besides the potential effects on coastal communities due to nutrient or carbon inputs, it is also possible that groundwater transport a vast diversity of microorganisms to the sea, but this has never been explored. According to several studies done in surface and subsurface habitats, it was estimated that the total number of bacteria in groundwater could vary from 10^2 to 10^8 cells per cm^3. Nevertheless, comparing prokaryotic biomass in groundwater with marine ecosystems it is clear that there are often more microorganisms in marine environments (22-215 Pg of C compared to 305 Pg of C, respectively), (Whitman et al., 1998). In terms of diversity, however, recent studies have revealed that groundwater systems are huge reservoirs of prokaryotic taxa, much of which are still unknown (Anantharaman et al. 2016). For example, according to Schloss et al. (2016) only 2% of all 16S rRNA sequences in public databases derive from groundwater organisms. As a result we have a quite limited knowledge about the prokaryotic species inhabiting these systems, their functioning and their potential ability to persist and to thrive when transported to other ecosystems such as the sea. In this same line, a few recent studies have shown that groundwaters and soils harbour a large amount of rare and dormant taxa that become active when they get to freshwater ecosystems, and that can even dominate lake and river communities (Ruiz−González et al., 2015, Crump et al., 2012). Thus, if some of these groundwater taxa can thrive in marine waters this would mean that coastal aquifers comprise a reservoir of diversity for marine microbial communities, but this has never been tested. Moreover, understanding bacterial potential to be active in different environments or stay dormant for a long time opens the door for potential applications in fields such as biotechnology and bioremediation.

In addition, so far no study has explored how bacterial communities respond in terms of their composition or single-cell activity to groundwater inputs, since the abovementioned studies

considered bacterial communities as a whole (Garcés et al. 2011). A recent study using 16S rRNA sequencing showed that coastal microbial communities can be shaped by flushing of groundwater taxa through SGD (Lee et al, 2017), but they did not explore how these changes in bacterial communities affected the functioning of communities or whether some of these groundwater taxa were actually able to grow. It is thus important to explore more about the potential of particular groups of bacteria to grow upon groundwater inputs, as it will answer many questions about group diversity and their role in the ecosystem. In this regard, techniques that allow coupling the taxonomic identity of taxa with their single-cell activity are valuable tools for understanding how communities react to changes in environmental conditions. For example, catalyzed reporter deposition-fluorescence in situ hybridization (CARD-FISH) (Pernthaler et al., 2002), which allows visually identifying target prokaryotic groups, coupled to bioorthogonal non-canonical aminoacid tagging (BONCAT) (Hatzenpichler et al., 2014), that identifies individual cells actively synthesizing proteins, can provide an extremely useful information about specific responses of particular bacterial taxa to changes in conditions. Previous studies that used a similar combination of techniques (CARD-FISH coupled to microautoradiography) have found that different groups of bacteria tend to response differently to such factors as ultraviolet radiation (UVR) in terms of their single-cell activity (Alonso-Sáez et al., 2006), but so far no study has explored whether different groups and their activity are differentially affected by SGD. Moreover, compared to microutoradiography, BONCAT is a much faster approach, but it has been only rarely used for aquatic bacterial communities (Leizeaga et al. 2017). Applying this technique in experimental assays has a large potential for determining which bacteria are actively contributing to processes taking place in the environment or stay in a dormant state.

1.4 Importance and novelty of the study

This project is going to provide the first description of the bacterial communities inhabiting coastal groundwater aquifers draining to the NW Mediterranean Sea, since so far no study has explored how these communities look like in terms of taxonomic composition and function. Moreover, it will integrate two different ecosystem types (fresh vs. marine water) that are often studied separately even though they are highly connected naturally. Finally, in order to explore how bacterial communities respond to groundwater additions in the coastal ocean, an experiment was performed mixing groundwater and seawater in different combinations (see

Methods). This will let us follow changes in bacterial communities that also occur in nature under groundwaters input to the sea. Finally, a highly novel technique, Bioorthogonal non-canonical amino acid tagging (BONCAT), will be applied to study the single cell activity of different bacterial groups; this will help us to identify the bacterial taxa that have the potential to grow in a new environment, or that can quickly respond to groundwater additions. All this will enlarge our knowledge on the influence of SGD on marine communities, as well as prokaryotic potential to grow in different environments.

2 HYPOTHESES AND OBJECTIVES

In this work we will evaluate the hypothesis that groundwater inputs influence marine bacterioplankton communities through the stimulation of particular taxa as a result of nutrient additions, but also through the inoculation of some groundwater bacteria with potential to grow in marine conditions. To do so, this project will be divided into two parts that correspond to the two main objectives (see below). First, we will characterize the bacterial communities from several aquifers draining to the Mediterranean as well as those from the adjacent marine sites. Second, we will perform a mixing experiment where groundwater and seawater from one of those sites will be mixed in different combinations (see Materials and Methods). Using a combination of techniques such as flow cytometry, ^{3}H-leucine assays, CARD-FISH and BONCAT, we will characterize the abundance, activity, taxonomic composition and single-cell activity of specific bacterial groups across the studied communities.

Objectives

1. To characterize the composition and function of bacterial communities that inhabit groundwater aquifers draining to the Mediterranean Spanish coast in order to identify the main bacterial groups present in these unexplored groundwater ecosystems. For comparison, the adjacent marine coastal communities and those from the main river in the area (the Ebro river) will be also characterized.

2. Explore how groundwater inputs influence the composition and activity of coastal bacterial communities. This will be done by means of a mixing experiment where groundwater and seawater will be mixed in different combinations (see Materials and Methods) that will

allow addressing 1) the responses of marine bacteria to groundwater inputs, as well as 2) the capacity of groundwater bacteria to grow in marine conditions.

3 MATERIALS AND METHODS

3.1 Study area and sampling design

The NW Mediterranean Sea is a semi-enclosed oligotrophic sea that is highly dependant on the riverine, atmospheric as well as groundwater nutrient inputs. The salinity of the sea is quite high around the whole basin with the average salinity around 38. Water temperature varies according to the season and is the highest in summer. All these flows and climatic conditions influence the composition of marine communities, especially the highly abundant and fast responding fraction of the organisms such as bacterioplankton, which has been shown to change seasonally in the NW Mediterranean (Alonso-Sáez et al., 2007).

In order to gain knowledge on groundwater communities and the responses of marine communities to groundwater inputs, our sampling design was divided into two parts: i) a spatial study of bacterial communities inhabiting several aquifers of the NW Mediterranean coast, and ii) a manipulation experiment to explore the responses of both marine and groundwater bacteria upon groundwater and marine water mixing.

3.1.1 Spatial survey

In march 2017, several groundwater aquifers were sampled from the NW Mediterranean coast along 300 km approximately (Fig.1), as well as the adjacent marine sites and the most important river in the area, the Ebro river. All sites were sampled during the same day. Additionally, in July 2017, an additional aquifer and the adjacent sea shore were also sampled for the mixing experiment (see below). In total, 16 sites were sampled in order to capture a large diversity of environments present along the coast. Seawater was manually collected directly from the shore or from a pier. Depending on the type of aquifer, groundwater was collected either by pumping it through an acid-clean Teflon tubing from installed piezometers (GR3_3 and GR6), or directly at the surface spring as most of the aquifers reach the surface right next to the sea shore (Supplementary Table 1).

At each site, temperature and salinity were measured in situ with an YSI probe. Activity assays (bacterial production and BONCAT) were performed *in situ* in coolers filled with ambient water (see below). Samples for flow cytometry were fixed at the sampling site and kept refrigerated until arrival to the lab. Water for DNA analyses (not included in this project) was pre-filtered *in situ* through a 200 µm mesh to remove large plankton and kept in 4L HCl-rinsed carboys, refrigerated and in the dark until processing in the lab.

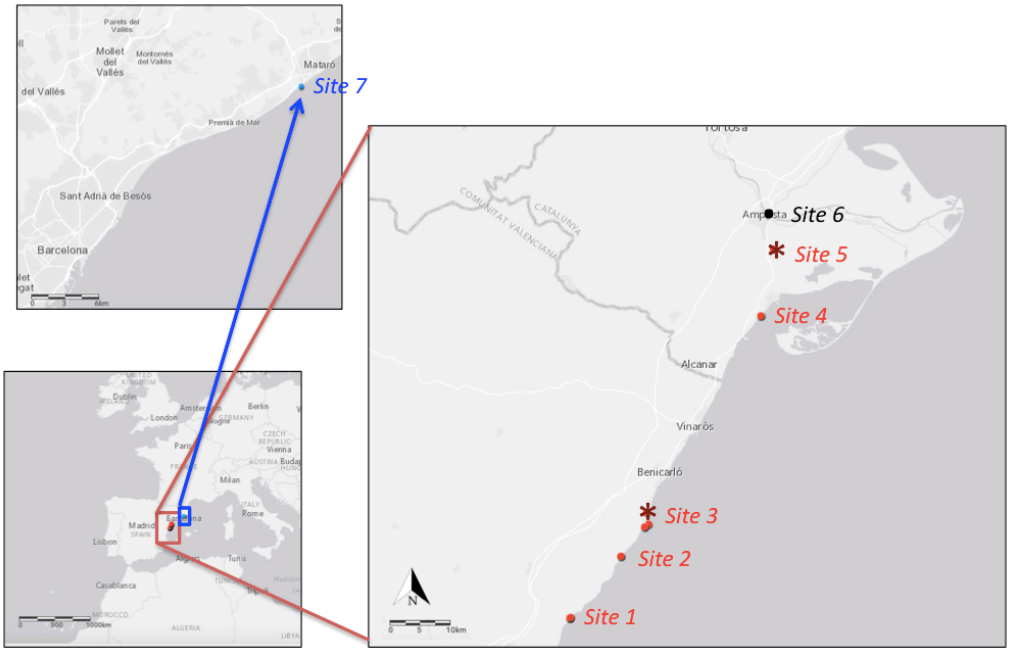

Figure 1. *Map showing the location of sampling sites along the Spanish coast. At most sites marine samples from the adjacent shore were also taken for comparison, as well as one sample from the most important river in the area (the Ebro river, indicated with a black dot). Asterisks indicate two surface freshwater bodies fed directly by groundwater, see details below; the blue arrow shows the aquifer and sea shore sites from which water was taken for the mixing experiment.*

The location of the sampling sites and their basic characteristics are included in a Supplementary Table 1. A more detailed description of the sampling sites is provided below:

- **Site 1** included four sampling points at Platja de Les Fonts, Alcossebre. Two of them are groundwater sites (GR1_1 and GR1_2) that constantly flow into the sea, reaching the surface right at the beach. The other two sampling points were either located right at the

seashore (SW1_1), showing low salinity due to the high groundwater flow, or sampled from a pier (SW1_2), representing seawater less influenced by the groundwater discharge.

- **Site 2** (Torre Badun, Sierra de Irta, Peñíscola) was located just below a high cliff, where a groundwater aquifer reaches the surface between rocks right at the shore line (GR2). The adjacent shore site (SW2) was sampled next to the freshwater input.
- At **Site 3** (Peñíscola) four sites were sampled, two different groundwater aquifers (GR3_1,GR3_3), a surface stream at the beach, directly fed by three different groundwater sources (GR3_2), and a seawater sample collected from the pier (SW3). Font de Dins (GR3_1) is an aquifer in the village with clean drinkable water that is channelled and reaches the surface as a fountain. The sample from Aquifero Inferno (GR3_3), located nearby, was collected from 25 m depth from an installed piezometer; water from this site is brown, brackish, and much warmer than in any other aquifer.
- **Site 4** was located in the region of St. Carles with two sampling points: a groundwater aquifer reaching the surface at the beach (GR4) and a seashore sample (SW4), respectively.
- At **Site 5**, only one sample was taken from a small pond (Ojals de Baltasar, GR5) fed entirely by a groundwater entrance (GR5)
- **Site 6** was at the region of Amposta where the sample from River Ebro (R6) was taken. This portion of the river is relatively close to the river mouth.
- **Site 7** in the region of Mataró includes two sampling points, one from a detrital groundwater aquifer (GR6), sampled from a depth of 20 m with an installed piezometer, and the corresponding seashore sample (SW6). Water from these two sampling sites was also used for the mixing experiment.

3.1.2 Mixing experiment

For the mixing experiment, 60 L of submarine groundwater was taken from the aquifer in Site 7 (Suppl. Table 1). The water well is located close to Riera de Argentona, in Mataró, at around 20 meters from the coast and is controlled by the University of Barcelona (UAB). Water from 20 m depth was collected as explained above. In addition, 120 L of seawater were collected

directly from the shore next to the well. The physicochemical conditions of both water types at the sampling time are shown in Supplementary Table 1.

Both, groundwater and marine water were used to create three different mixes for the experiment. By performing some previous manipulations, four types of water were prepared for the experiment:

- Whole unfiltered seawater (whole marine bacterial community present)
- "Bacteria-free" seawater (filtered through 0.2 μm filters)
- Whole unfiltered groundwater (whole groundwater bacterial community present)
- "Bacteria-free" groundwater (filtered through 0.2 μm filters).

To obtain 'bacteria-free' seawater and groundwater, water was sequentially filtered through 1μm filter for removing big particles and predators, and through a 0.2 μm filter. Afterwards, the three mixes were prepared in 30L carboys in three different combinations: i) 75% of unfiltered seawater with addition of 25% of "bacteria-free" groundwater (Mix 1); ii) 75% of whole seawater with 25% of whole groundwater (Mix 2), and iii) 75% of "bacteria-free" seawater with whole groundwater (Mix 3) (Fig. 2). Mix1 was aimed at exploring the responses of marine communities upon changes in the environmental parameters due to groundwater addition, whereas Mix 2 was aimed at analysing the response of both types of bacterial communities when mixed, as it happens naturally. Mix 3 was performed to address whether there are groundwater bacteria that can thrive in a seawater conditions.

Right after mixing, samples for initial time (T0) measurements were taken from the overall mixes in the 30L carboys. Each Mix was then subdivided into three 8 L Nalgene bottles, obtaining three replicates for every Mix, which were named Mix1 A, Mix1 B, Mix1 C, and so on. All the replicates were incubated in two 300 L tanks that were placed on the roof of the laboratory. The pools were filled with running seawater proceeding directly from the coast of Barcelona in order to keep a constant temperature. To avoid mortality due to UV, incubators were covered with two layers of Ultraphan filter and with a mesh to reduce the light intensity and mimic the light conditions of the depth at which seawater samples were taken. Bottles were incubated for 4 days, during which samples were taken to examine changes in bacterial communities and their activity throughout the experiment. Samples were collected at four different times: 0h, 23h, 67h and 90h for flow cytometry and bacterial production, and only three times (0h, 23h, 90h) for CARD-FISH and BONCAT analyses (see below).

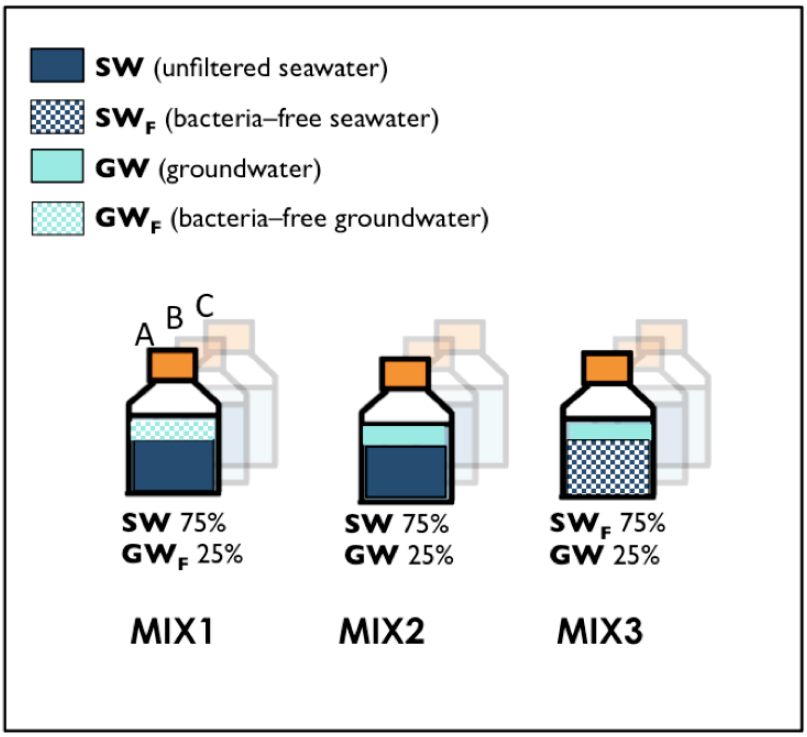

Figure 2. *Graphical representation of the experimental design. Three mixes (mix 1, mix 2, mix 3) with different water types (75% of whole seawater with 25% of filtered ("bacteria-free") groundwater; 75% of whole seawater with 25% of whole groundwater; 75% of filtered ("bacteria-free") seawater with 25% of whole groundwater respectively) were divided in three replicates each (A, B, C) and incubated for 4 days under natural light and temperature conditions.*

3.2 Bacterial abundances

Among the most used techniques to estimate bacterial abundance from aquatic samples are flow cytometry and microscopic counts after staining with a fluorescent dye. For this project we used both techniques, but only microscopic count data (explained below as part of the CARD-FISH analyses) are presented for simplification.

3.2.1 Flow cytometry

Water samples of 1.8 mL were preserved with 1% paraformaldehyde and 0.05% glutaraldehyde (final concentrations) and kept frozen at 80°C until analysis with a Becton-

Dickinson FACSCalibur flow cytometer. When processing, samples were thawed and stained with Sybr green, mixed and left in the dark for 10 min. Before running samples through the flow cytometer, 10 μL of yellow-green Polysciences latex beads were added as an internal standard. All the samples were run at low speed for 2 minutes or max 10 000 events traced (Gasol et al., 1999). Results were plotted in a plot with green fluorescence versus size scatter that allowed us to differentiate high DNA (HDNA) bacteria that are more active from low DNA (LDNA) bacteria (Giorgio et al., 1996).

3.3 Bacterial heterotrophic production

Bacterial protein synthesis rates were measured by means of ^{3}H-leucine incorporation, following the standard procedure developed by (Kirchman et al., 1985). Four replicates and two killed controls (5% of trichloroacetic acid, TCA) were incubated in the dark with ^{3}H-leucine for 2 hours at *in situ* temperature. Then, samples were kept frozen at -20°C until further processing. Afterwards, the centrifugation method was used to get the bacterial protein synthesis by means of the uptake rate of ^{3}H-leucine.

3.4 Single-cell bacterial activity through BONCAT coupled with CARD-FISH

3.4.1 Bioorthogonal non-canonical amino acid tagging (*BONCAT)*

BONCAT is a relatively new approach that allows the estimation of single-cell activity in a natural sample as well as changes in individual cells activity by changes in fluorescence intensity. It is mainly based on the ability of active cells to incorporate synthetic aminoacids during proteins synthesis (Leizeaga et al., 2017, Azam et al., 1983). As a result, active cells that have incorporated synthetic aminoacids in their proteins can be later detected through epifluorescence microscopy. Combined with CARD-FISH (explained below), this approach allows identifying the contribution of particular target bacterial groups to the total community activity (Hatzenpichler et al., 2014).

Samples were analysed following the standard protocol performed by (Sekar et al., 2003) with corrections made by Leizeaga et al. (2017). Three aliquots (two life and one killed control) per site (10-50 mL depending on *in situ* cell abundances) were incubated with L-homopropargylglycine, HPG (methionine analogue, 100 nM final concentration) for 3 hours in the dark at room temperature. Killed control samples were fixed with paraformaldehyde (PFA,

1% final conc.) before incubation with the substrate in order to prevent bacterial activity and amino acid incorporation, , as it is required in order to correct the background fluorescence from natural azides (Leizeaga et al., 2017). Incubations of life samples were stopped adding PFA (final concentration 1% [v/v]), and were fixed overnight at 4ºC. Then, samples were gently filtered through a 0.2 µm pore size polycarbonate filter, washed three times with milliQ water and kept frozen at -80 °C.

Before the permeabilization procedure, all filters were dipped in 0.1% low-gelling-point agarose, dried at 37°C and dehydrated with 95% ethanol, in order to attach cells to the filter and prevent their loss. Permeabilization of cells was done with 10 ml of fresh lysozyme solution and achromopeptidase at 37°C following standard procedures (Sekar et al., 2003). Each filter was cut into several portions using a sterile razor blade, and the remaining portion was kept frozen.

For catalysed click-reaction dye-premix was prepared by mixing 15 µl of 20 mM of filter-sterilized $CuSO_4$, 30 µl of 50 mM of filter-sterilized Tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine (THPTA, www.clickchemistrytools.com) and 12 µl of 1 mM of the Alexa 594 azide dye and left to react 3 minutes in the dark at room temperature.

At the same time 100 µl of a freshly prepared 100 mM solution of sodium ascorbate and 100 µl of 100 mM solution of aminoguanidine hydrochloride were added to 1.7 mL of phosphate buffered saline (PBS) solution. For the click reaction mix, the dye-premix was added to the PBS-ascorbate-aminoguanidine mix and the tube was inverted twice. Filter portions were transferred to a 1.5 mL eppendorf tube and dye-mix was pipetted to the tube and the cap to avoid the presence of the air. Filters were incubated at room temperature for 30 min in the dark. Afterwards all filters were washed three times for three minutes in PBS and then in ethanol with different concentrations (50 %, 80 % and 96 % EtOH respectively).

3.4.2 Catalyzed Reported Deposition Fluorescence in situ hybridization (CARD-FISH)

The taxonomic composition of the studied communities was assessed by CARD-FISH, which was performed on the same filters used for BONCAT. Hybridization and signal amplification steps were performed following the standard protocol performed by Pernthaler et al. (2002). For hybridization, horseradish peroxidase (HRP) labelled oligonucleotide probes targeting several bacterial groups were mixed with previously prepared hybridization buffer (HB). For our study we used the following HRP probes: from Alphaproteobacteria SAR11-441R

to target SAR11 group (Morris et al., 2002), ROS537 for *Roseobacter* group (Eilers et al., 2000b), LD12-121 for LD12 group (Salcher, 2013), Gam42a to target Gammaproteobacteria and Beta42a for Betaproteobacteria (Manz et al., 1992), CF319 for the Bacteroidetes (Manz et al., 1992), HGC96a for Actinobacteria (Roller et al., 1994), CYA339 for the photosynthetic Cyanobacteria (Nübel et al., 1997) and Syn405 for the cyanobacterial genus *Synechococcus* (West et al., 2001).

Specific hybridization conditions were established by addition of different amounts of formamide to the hybridization buffers (45% for SAR11, 35% for LD12, and 55% for the rest of probes). Filters were hybridized overnight at 35 °C. In order to stop the hybridization process, the filters were washed for 15 min in washing buffer at 37ºC and then placed in PBS at room temperature in the dark for another 15 min in order to remove the excess of washing buffer.

The amplification step was performed mixing amplification buffer (2 mL PBS, 0.4 mL blocking reagent, 16 mL NaCl, milliQ water to a final volume of 40 mL, 4 g dextran sulphate) with H_2O_2 stock (200 µL PBS, 1 µL H_2O_2)) and 4 µL of Alexa488 labeled tyramide. Filter sections were placed in an eppendorf with this mix and left for 15-20 min at 46ºC. Then they were washed with PBS and left in absolute ethanol for 1 hour in order to effloresce natural pigments like chlorophyll that can interfere with the BONCAT signal.

After drying the filters, they were mounted on slides and stained with 4`6-diamidino-2-phenylindole (DAPI) in order to quantify the abundance of the different phylogenetic groups in relation to total prokaryotic counts. DAPI is a fluorescent dye that binds to DNA and RNA structures emitting light at a certain wavelength, allowing to differentiate bacterial cells from non-living particles under the epifluorescence microscope (Porter and Feig, 1980).

3.5 Image analysis and cell counts

Total bacteria, total active bacteria, the abundances of the targeted bacterial groups and the single-cell activity associated to each group were estimated with an automated epifluorescence microscope Axio Imager.Z2m connected to a Zeiss camera (AxioCam MRm, Carl Zeiss MicroImaging, S.L., Barcelona, Spain) at 63x magnification. Between 55 and 66 fields of view were analysed per filter. At each field of view, three pictures were taken using different fluorescence filters that allow counting total bacteria (DAPI – UV channel), total active bacteria (BONCAT – red channel) and the number of the hybridized cells (FISH – blue channel). Images were acquired using AxioVision and SamLoc-1.6 softwares as detailed in Leizeaga et al. (2017),

with exposure times ranging between 20 – 75 ms for the different channels (DAPI, BONCAT and FISH) depending on the quality of the sample and the target bacterial group characteristics. Afterwards, all the pictures were analysed using the automated image analysis software ACMEtool3 (ACMEtool3, version 2013-04-07, M. Zeder, technobiology GmbH2014). The percentage of translationally active cells was estimated from the total DAPI counts. The intensity of active cells (a proxy of the individual activity of a cell) was estimated using the mean gray value (MGV, sum of the gray values of all pixels in one cell divided by the total number of pixels). These intensities were ranked and divided into three groups: high intensity (top third of fluorescence activity), intermediate (middle third) and low intensity (bottom third). Then the percentage of the target bacterial groups was calculated from total cell counts, and the contribution of each group to total activity was estimated as the number of active cells within a particular group with respect to the total active cells. The number of active cells detected in killed BONCAT controls was very low (avg. 1.31% of active cells), and those values were subtracted from the life count data.

3.6 Statistical analysis

Statistical analysis of the results was performed with Microsoft Excel (Excel 2012) and R software (RStudio, version 0.99.903.). Paired *t*-test ($p < 0.05$) was utilized for comparing bacterial communities between different treatments. Analysis of variance (ANOVA) was used to conclude if the mean differences between treatments over time were statistically significant. Afterwards, the multiple pairwise-comparison between groups was computed using Tukey Honest Significant Differences (Tukey HSD) in order to define statistically different treatments ($p < 0.05$). Correlations between variables were evaluated using Pearson's correlation coefficient. To explore the ordination of different communities based on the contribution of the different bacterial groups or their activity, principal component analysis (PCA) was performed and compared to environmental parameters.

4 RESULTS

4.1 Spatial patterns in composition and function of coastal groundwater and marine bacterioplankton communities

4.1.1 Spatial variability in bacterial abundances, active cells and bacterial heterotrophic production

Bacterial abundance, activity, and the abundance of specific bacterial groups varied between different sampling sites. Overall, the total prokaryotic abundance in groundwater aquifers was much lower that that from surface waters, ranging from 1.03×10^4 to 2.2×10^5 cells mL^{-1} (mean of $9.2\times10^4 \pm 0.9\times10^4$ cells mL^{-1} (Fig. 3). The two surface water bodies that were fed directly from groundwater aquifers (GR3_2 and GR5, see asterisks in Fig. 3) showed ten times higher bacterial abundances, reaching up to $1.2\times10^6 \pm 0.05\times10^6$ cells mL^{-1}. Contrary to the groundwater, seawater samples show on average 10 times higher abundance than for groundwater and was similar on all sampling sites. Bacterial abundances from seawater samples varied between 8×10^5 and 17×10^5 cells mL^{-1} (mean $13\times10^5 \pm 0.7\times10^5$ cells mL^{-1}). Finally, the highest number of cells was found in the river Ebro, reaching up to 4.5×10^6 cells mL^{-1}.

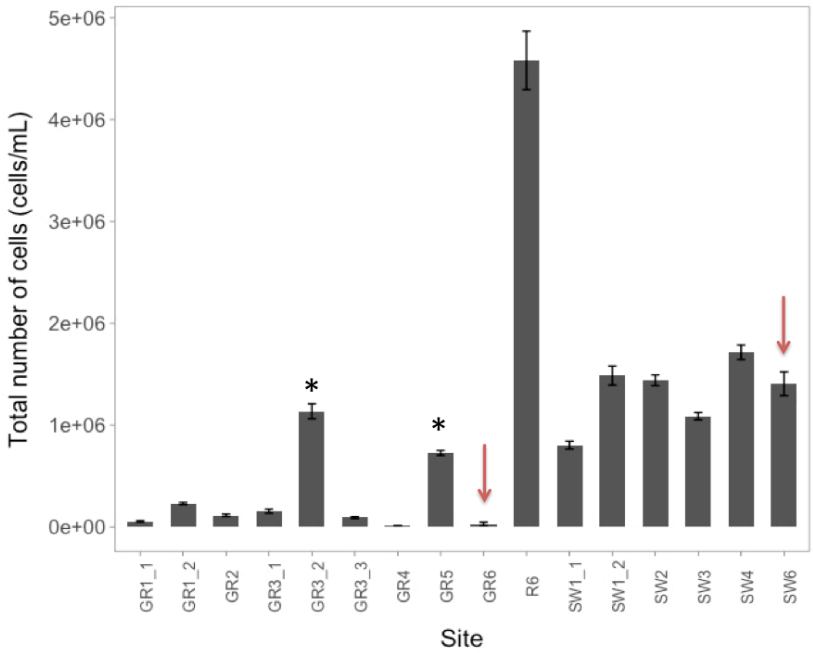

Figure 3. *Total number of prokaryotic cells at the different study sites estimated by DAPI cell counts. Asterisks indicate surface water bodies fed directly by the groundwater, red arrows show sampling sites that were used for the mixing experiment. Bars represent means and error bars represent standard deviations of DAPI counts among three filters per sample.*

The number of total active cells estimated with BONCAT was highly correlated with total cell counts, showing a pattern similar to that shown in Figure 3. The highest number of active cells was found in the Ebro river, accounting for 42 ± 5 % of the total cells (fig. 4 (A)). In all groundwater aquifers, the number of active cells was quite low and did not exceed 5 % of the total cell counts. The two surface water systems fed with groundwater (marked with asterisks in Fig. 4A) had higher numbers of active cells which accounted for around 50 % of total cell counts. Total active cells counts in seawater communities was higher than in groundwater systems but varied largely between sites (range 10 % - 45 % of total cell counts) and were the highest in Argentona, Mataró (SW6), while the lowest were for two sites located in les Fonts (GR1_1, GR1_2).

Bacterial heterotrophic production, estimated as ^{3}H-leucine incorporation rates, also followed a pattern similar to that of bacterial abundance and number of active cells (Figure 4B). Again, most groundwater sites showed the lowest bacterial production rates (range 0.2 to 34 pmol ^{3}H-leucine L^{-1} h^{-1}), and those from marine sites ranged from 23 in site SW1_1 to 400 pmol

^{3}H-leucine L^{-1} h^{-1} in sample from SW6. Interestingly, although in terms of total bacterial abundance and active cells the Ebro river showed the highest values (Figs. 3, 4A), bacterial production rates were 1.5 times higher in the two systems fed by groundwater than in the river, suggesting that these bacteria were on average much more active (Figure 4B).

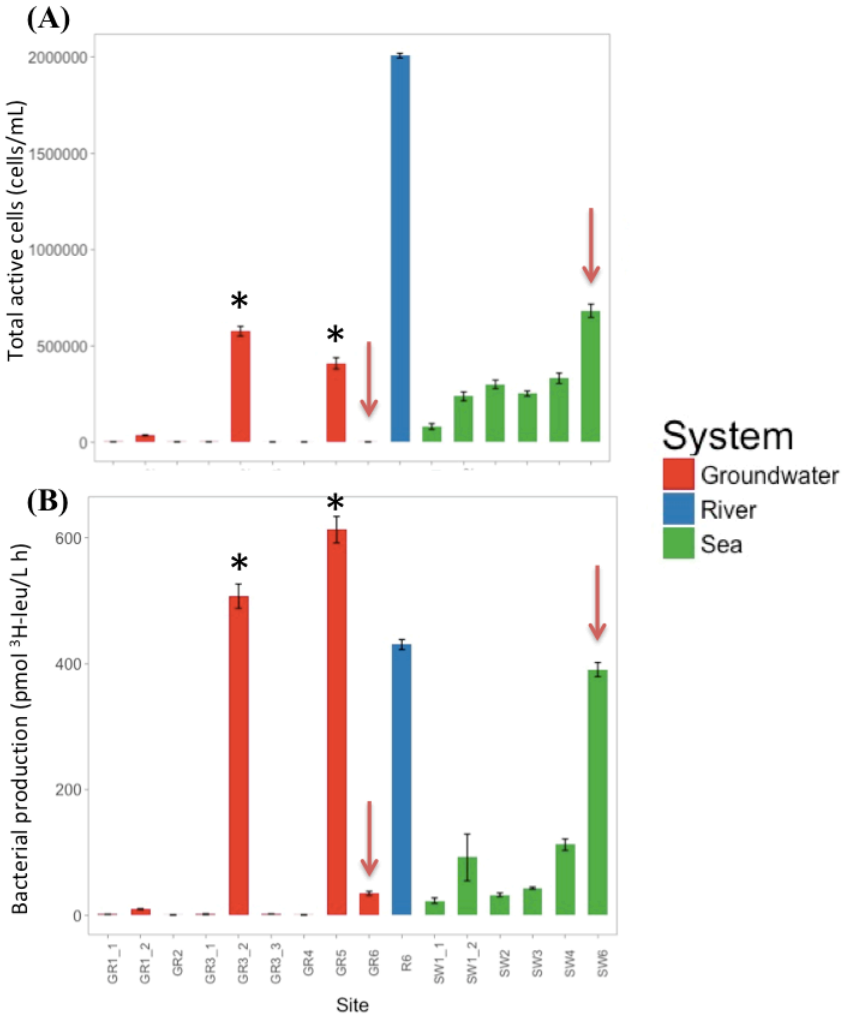

Figure 4. *Total number of active cells at each of the sampled bacterial communities measured with BONCAT technique (A), bacterial heterotrophic production measured as the rate of radioactive leucine incorporation (B). Bars are color-coded depending on the ecosystem types (red bars – Groundwater, blue bar –River and green bars – Sea), asterisks indicate surface water bodies fed directly by groundwater, red arrows show sampling sites that were used for the mixing experiment. Bars represent means and error bars represent standard deviations of BONCAT counts calculated from two filters per sample (A) and of ^{3}H-leucine incorporation rates calculated from four analytical replicates (B)*

As previously mentioned, the abundance of bacterial cells was estimated using two different techniques. Although they showed similar patterns, we observed that DAPI counts overestimated the total cell counts in the case of most groundwater samples due to the presence of sediment particles (details not shown). Thus, flow cytometry data were used for calculations of the relative abundances of the different CARD-FISH bacterial group in all groundwater aquifers except GR6, for which DAPI counts appeared correct.

4.1.2 Spatial changes in bacterial composition

In general, hybridization with the eight chosen CARD-FISH probes captured over 50 % of total bacterial cells, with the exception of two groundwater aquifers where the target groups accounted for less than 25% of the communities (Fig. 5A). We observed that the contribution of different bacterial taxa varied according to the type of ecosystem. First, groundwater aquifers were characterized by the prevalence of Betaproteobacteria, Gammaproteobacteria and in some aquifers also Bacteroidetes group that together account for over half of all bacteria present in community. Conversely, seawater samples were characterized by higher percentages of bacteria from the SAR11 clade that alone contributed over 20% of the total bacteria counts. Bacteria associated with the Bacteroidetes were also abundant in the seawater accounting from 10% (Site 4) up to 20% (Site 7) of total cells. Finally, the community from the river Ebro was remarkably different due to the dominance of Actinobacteria, which comprised 34% of the total bacterial cells (Fig. 5A).

The single-cell activity of the different bacterial groups was assessed by means of CARD-FISH coupled to BONCAT (Fig. 5B). We found that, in seawater samples, the most active bacteria (30%) belonged to SAR11 group, followed by Gammaproteobacteria (27% of total active cells). Interestingly, bacteria from the *Roseobacter* group, that represented only 3.5% from total abundance, showed very high activity in the community accounting for 13% from total active cells. Conversely, the relatively abundant Bacteroidetes were mostly inactive in seawater samples and did not represent more than 5% of total active cells in the community (Fig. 5B).

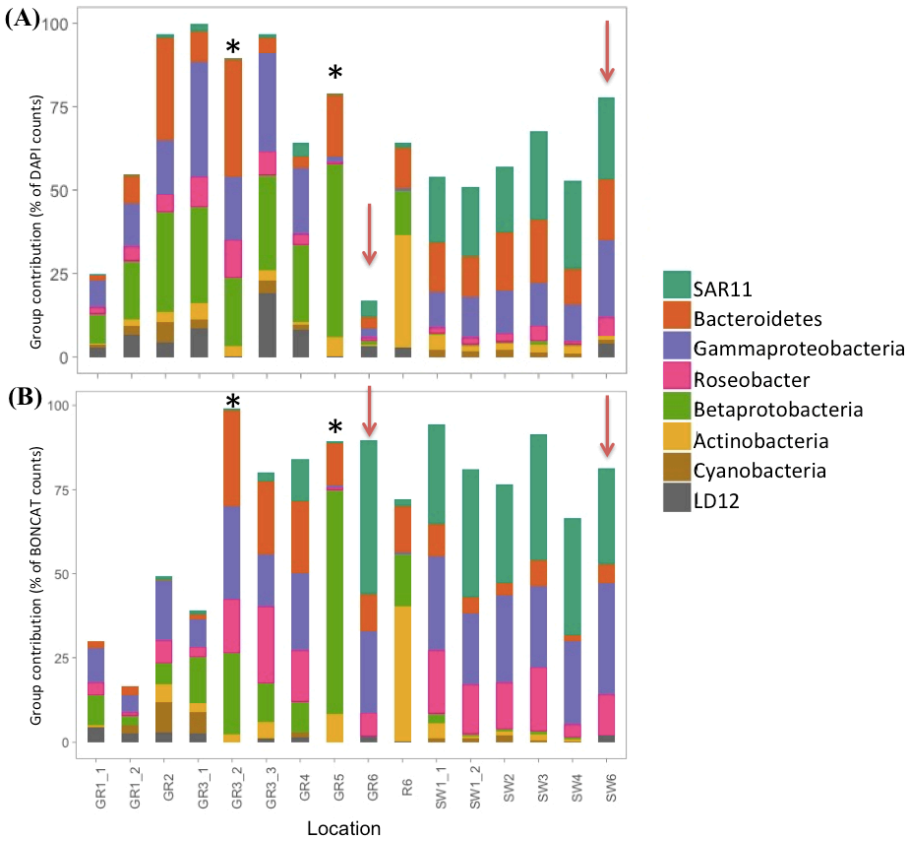

Figure 5. *Contribution of different bacterial groups to the total cell abundance (A) and to the total number of active cells (B) across the sampled communities. Data are presented as percentage of each targeted group with respect to the total bacterial counts (A), or percentage of active cells within each bacterial group with respect to total active cells. Each colour indicates different taxonomic groups of bacteria identified through hybridization with specific CARD-FISH probes, asterisks indicate surface water bodies fed directly by groundwater, red arrows show sampling sites that were used for the mixing experiment.*

The composition of active bacteria from groundwater communities showed a larger heterogeneity between the sampling sites. At most sites, Gammaproteobacteria showed the largest contribution to the total active cells. Bacterioplankton from sites GR3_3 and GR4 together with superficial sources (GR3_2, GR5, marked with asterisks on Figure 5B) were quite active in terms of percentages of bacteria that were recovered with high contribution of Bacteroidetes, Gammaproteobacteria and Roseobacter groups with an average around 20% from total number of active cells in each group. Other aquifers (GR1_1, GR1_2, GR2, GR3_1)

showed very low percentage of activity of all target groups that in total did not exceed 50%. Unexpectedly, the groundwater aquifer in Mataró (GR6) was characterized by very high number of active cells from marine SAR11group, which we believe was due to unspecific hybridization of some groundwater bacteria with this CARD-FISH probe (see Discussion). Finally, in the Ebro river, the most abundant group was Actinobacteria, which comprised almost half of the total active cells, followed by Bacteroidetes and Betaproteobacteria that accounted for 13% and 15% of the total active cells respectively (Fig. 5B).

In order to summarize the observed differences in terms of the different bacterial group contribution in composition and single-cell activity described above, we performed principal component analyses (Fig. 6A, B). This analysis confirms that seawater sites cluster separately from freshwater sites, and comprise a distinct and more spatially homogenous group. This distinction between freshwater and marine sites, as expected, was mainly due to higher abundances of the SAR11 group followed by Gammaproteobacteria, that are the most abundant groups in all marine samples. Conversely, all groundwater sources were much more spatially heterogeneous in terms of bacterial composition and could not be grouped together, even for sites that were located close to each other (Fig. 6A). These compositional differences in groundwater aquifers were mostly due to differences in the abundances of Betaproteobacteria and Gammaproteobacteria. Finally, surface freshwater systems were also quite different, but clustered within groundwater communities (Fig. 6A). A similar pattern was also observed when the single-cell activity of the different groups was considered, with seawater communities clustering closely together and freshwater sites being more heterogeneous. This time the two surface freshwater sites were separated from the rest of the groundwater sites due to the much higher activity of groups like Betaproteobacteria and Bacteroidetes (Fig. 6B).

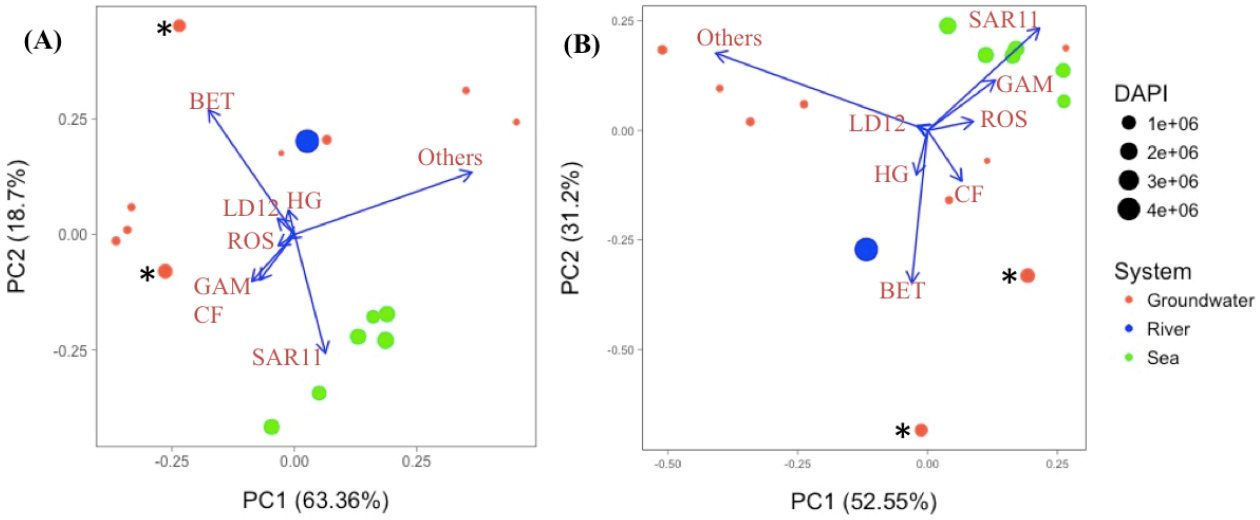

Figure 6. *Results of the principal component analysis (PCA) for the spatial study based on bacterial composition assessed by CARD-FISH probes (A), and total active cell counts of these different groups (B). The data included are expressed as percentages of total bacterial abundance (A) and percentage of total active cells (B). Each colour indicates different water systems (blue – river, red – groundwater, green – sea), asterisks indicate surface water bodies fed directly by groundwater and size of the dots is proportional to total bacterial abundance in each community. Vectors indicate various bacterial groups used for analysis.*

Overall, this spatial exploration of the communities inhabiting different groundwater and surface ecosystems along the coast suggests that groundwater aquifers are very heterogeneous in terms of bacterial communities and their activity. Interestingly, besides the very low cell abundances and activity levels in all groundwater communities (Figs. 3, 4), the fact that in the two surface freshwater systems fed by groundwater we found highly active communities suggests that as soon as groundwater bacteria encounter suitable environmental conditions they can grow fast (Figs. 3, 4). Since all these different aquifers drain to the sea, it is also possible that some of these inactive bacteria have the potential to grow when they reach marine waters. We found evidence for this using water from one of the aquifers (GR3_1), since we were able to grow groundwater bacterial isolates in marine agar (Fig. 7). Then, our next step was to perform a mixing experiment in order to see how seawater and groundwater bacterial communities behave when groundwater is delivered to the coastal ocean.

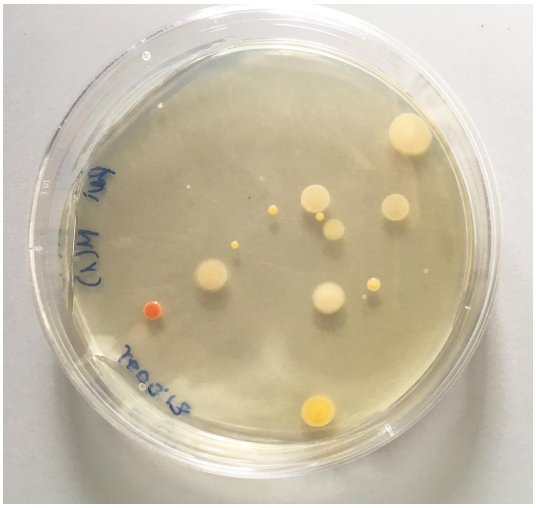

Figure 7. *Example of groundwater bacterial isolates growing on marine Zobell agar (1.0 g yeast extract, 5 g peptone, 15 g agar, 250 ml MQ water and 750 ml ultra-filtrated seawater) to show that groundwater communities harbour bacterial taxa that are able to grow when exposed to marine conditions.*

4.2 Mixing experiment

During the experiment, we observed that bacterial communities behaved differently depending on the experimental treatment as well as on the incubation time. Overall, mixes 1 and 2 allowed exploring the responses of marine bacteria upon groundwater inputs, either without or with natural groundwater bacteria (mix1 and mix 2, respectively). Conversely, mixes 2 and 3 allowed exploring how groundwater communities respond when exposed to marine conditions with (mix 2) or without (mix 3) native seawater communities.

4.2.1 Changes in bacterial abundances and total activity between experimental treatments

The total number of cells at the onset of the experiment varied largely between mixes 1-2 and mix 3 (ANOVA and Tukey-Kramer, $p \leq 0.05$). Cell abundance was much higher in seawater with 1.5×10^6 cells mL^{-1}, whereas original groundwater showed much lower cell concentrations with only 3×10^4 cells mL^{-1} (Fig. 8A). A rapid increase in cell abundance was found during the first 23 hours for mixes 1 and 2 (ANOVA, $p < 0.05$ and $p < 0.01$, respectively, Fig. 8A) that where composed of only marine taxa, and both marine and groundwater taxa, respectively,

reaching maximum abundances of around 1.8×10^6 cells mL^{-1}. After that, cell abundances decreased again and at the end of the incubation time (90h) was close to the initial levels (Fig. 8A). A comparably 2-fold increase and subsequent decrease was also observed for the total active cells in mixes 1 and 2 (Fig. 8B). In contrast, the total number of cells in mix 3 increased slowlier, showing no significant differences during the first 23 hours of incubation with respect to the initial time (ANOVA, $p > 0.05$, Fig. 8A), while the total number of active cells in the same mix showed a significant 14-fold increase during the same period. Nevertheless, total number of active cells in this mix decreased after 23h, it was still significantly higher at the end of the experiment than at time zero (Fig. 8B). Thus, the percentage contribution of active bacteria from mix 3 (i.e., which theoretically comprised only groundwater bacteria) increased from 5% to almost 60 % in 23h, and then drop again to 20 % (Supplementary figure 1), while the total number of cells continuously increased during the whole incubation time (Fig. 8A).

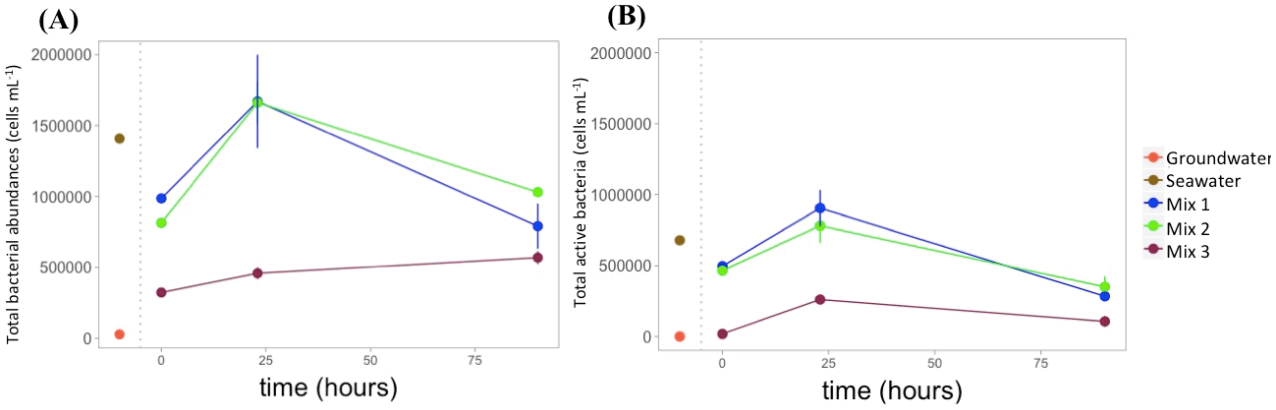

Figure 8. *Changes in total cell abundance* ***(A)*** *and in the number of active cells* ***(B)*** *during the experiment in different treatments, indicated by different colours (blue – mix 1 – 75 % seawater with 25 % bacteria-free groundwater; green – mix 2 – 75 % seawater with 25 % groundwater; purple – mix 3 – 75 % bacteria-free seawater with 25 % groundwater). Single dots represent seawater and groundwater original communities in situ, and are separated by the dashed line.*

A very similar pattern in changes of total bacterial abundance was also observed with the flow cytometry technique (data not represented), that shows the consistency between both techniques. Overall, the highest cells abundance was observed in mix 1 after 23 hours of

incubation, and almost reached 2×10^6 cells mL^{-1}. Mix 3 showed lower abundances than mixes 1 and 2 during the whole experiment, but by the end of incubations the total cell number was quite similar in all three mixes (Fig. 8A). Interestingly, the bacterial community from mix 3, that were composed only with groundwater taxa, constantly increased their abundance along the experiment. Conversely, mixes 1 and 2, that had marine and mix of marine and groundwater taxa, respectively, showed a quick response during the first hours of experiment, after which their abundances started to decrease.

As for the spatial survey, total bacterial abundance and the number of active cells in the mixing experiment behaved similarly along the whole incubation time, showing a strong significant correlation between them (Fig. 9, R=0.936, p< 0.001, n=29).

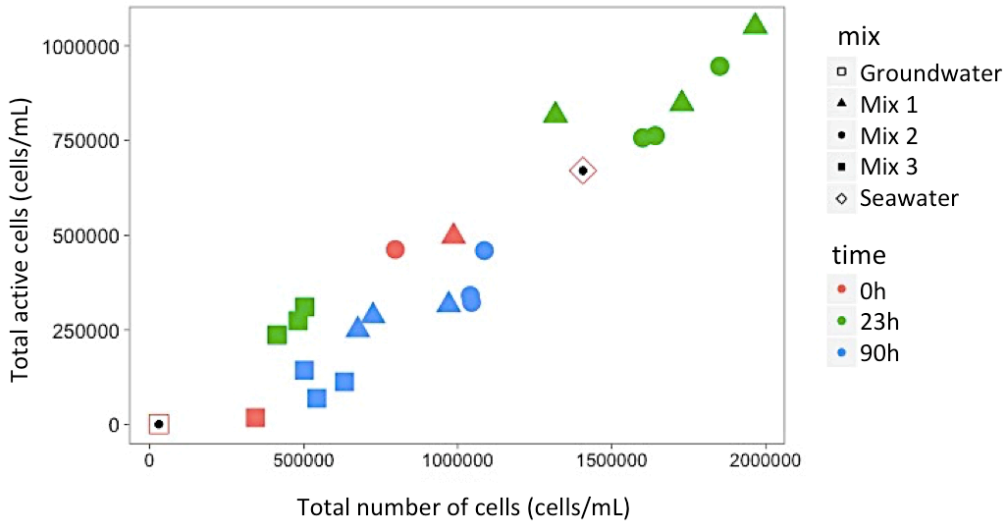

Figure 9. *Correlation between cells activity and total number of cells in different treatments during the incubation time. Shapes of the points represent treatments that are separated by colours for different incubation time. Single open squares represent seawater and groundwater original communities in situ.*

In addition to the general decrease in number of active cells in all treatments after 23 h, the cell fluorescence intensity (i.e., the intensity of the BONCAT fluorescence signal per each active cell, a proxy of the individual activity of cells) also noticeably changed over time. The two mixes with seawater bacteria (mix 1 and mix 2) showed a remarkable decrease in the number of high translational active cells during the first 23 hours of incubation with an increase afterwards. Thus, the pool of active cells there at T1 (23h) was mainly composed of low activity cells

(bottom third of fluorescence intensity, see Methods). Conversely, groundwater cells growing in mix 3 were highly active during the first hours of incubation, with a slight decrease in intensity signal at the end of the experiment (Fig. 10).

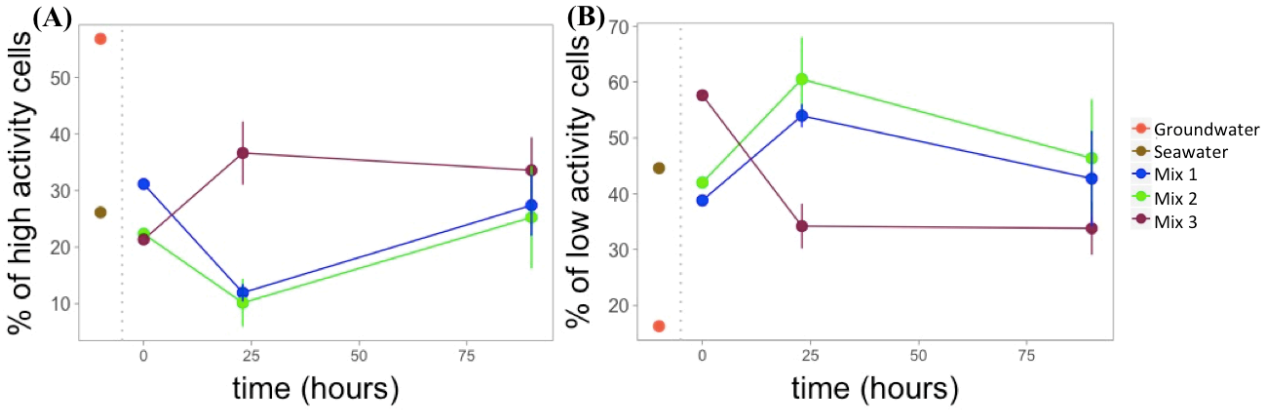

***Figure 10**. Average percentage of high activity cells **(A)** (top-third of fluorescence intensity, see "Materials and Methods" for details) and low activity cells (B) (bottom-third of fluorescence intensity) during the experiment in different treatments, indicated by different colours. Single dots represent seawater and groundwater original communities in situ, and are separated by the dashed line.*

4.2.2 Bacterial groups response to groundwater and seawater mixing

Studying the composition of bacterial communities developing in three different water treatments over time allowed us to explore the responses of marine bacteria upon groundwater additions (mix 1) as well as to assess the potential of groundwater taxa to grow when exposed to seawater conditions (mix 3). In addition to that, the experimental treatment in mix 2 allowed us to understand any potential interactions and changes when both marine and groundwater communities are exposed to each other, which is what actually happens in the natural environment.

4.2.3 Responses of seawater bacteria to the groundwater inputs (mix 1 and mix 2)

Mixes 1 (whole seawater with filtered ("bacteria-free") groundwater) and 2 (whole seawater mixed with whole groundwater) behaved similarly in terms of changes in taxonomic composition and activity of the different groups (Figs. 11, 12).

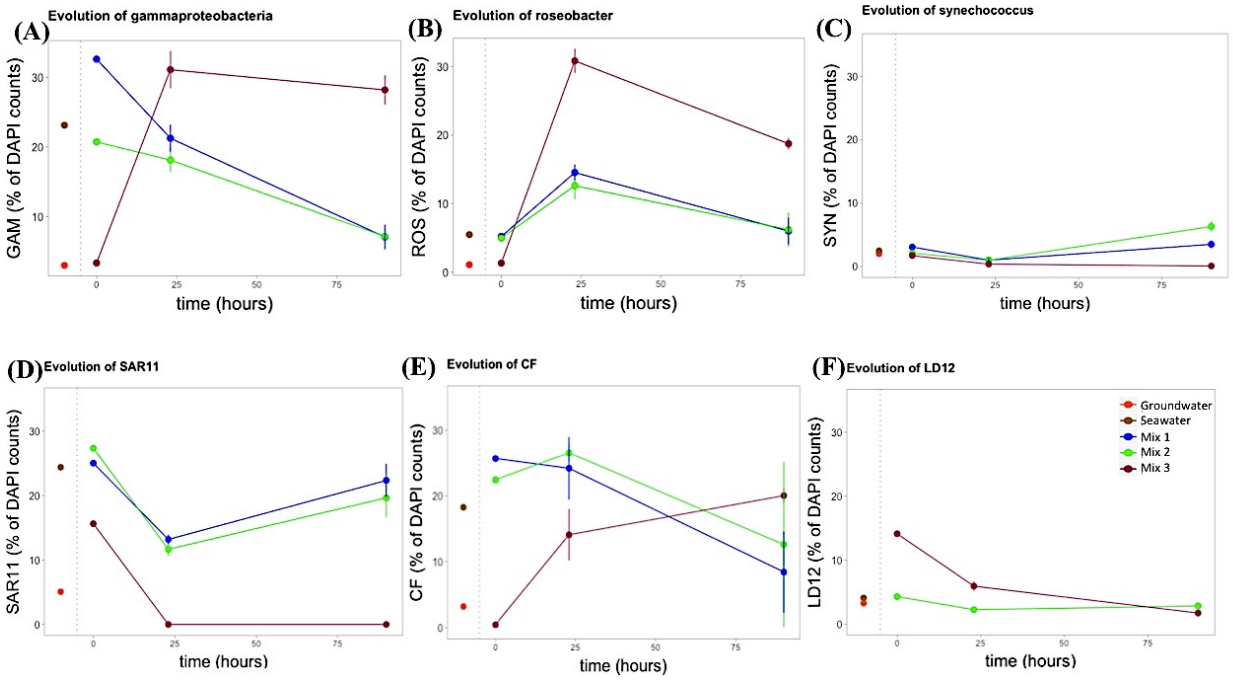

Figure 11. *Changes in relative cell abundance (percentages from the total cell counts) over incubation time of the different groups of bacteria hybridized with CARD-FISH probes in the different experimental treatments, indicated by the different colours: Gammaproteobacteria (A), Roseobacter (B), Synecoccocus (C), SAR11 (D), Bacteroidetes (E), LD12 (F). Single dots for seawater and groundwater represent the initial composition of in situ communities and are separated by the dashed line.*

The different bacterial taxa showed quite different response to the groundwater input by changing their total abundance. For example, the contribution of Gammaproteobacteria (Fig. 11A) to the total cell counts decreased by more than two-fold through the whole experiment (from 35% to 10% in mix 1 and from 20% to 10% in mix 2), while Bacteroidetes (Fig. 11E) tended to slightly increase their number of cells within the first 23 hours, but after that its abundance largely decreased and at the end of experiment comprised only 10% of the total

community composition. Cells belonging to *Roseobacter* showed the largest increase during the first 23 hours of incubation, changing from 5% to 14%, but decreased again to the initial values at the end of the experiment (fig. 11B). *Synechococcus* showed low abundances, contributing less that 6 % of the total cell counts, but they showed a 2-fold increase in abundance (from 1 % to ca. 4 % in mix 1 and ca. 6 % in mix 2) by the end of the experiment (Fig 11C). SAR11 cells were quite abundant at the beginning in both mixes 1 and 2 (30 % of totals), yet their abundances decreased to 15% during the first 23h and went back to the initial values at the end of the experiment (Fig. 11D). The freshwater LD12 group, as expected, was absent in mix 1, and showed very low abundances in mix 2 as well, remaining almost absent during the whole experiment (Fig. 11F)

The experimental addition of groundwater also promoted changes in the contribution of each group to total active cells (Fig. 12). For example, in mix 1, where only marine communities were present, SAR11 showed a decrease in number of active cells at the beginning of experiment, although by the end of the incubation it had recovered the initial activity values. Interestingly, whereas at the end of the incubation the abundance of SAR11 comprised less that 20% of the total cell counts, their activity (measured as the BONCAT signal fluorescence intensity, a proxy of the single-cell activity) increased more than three times (Suppl. Fig. 2) accounting for almost 40% of total community activity and outcompeting other groups. *Roseabacter* showed the opposite pattern, responding fast to the groundwater addition by increasing their contribution to active cells and their fluorescence intensity twice within the first hours of incubation (Fig. 11B, 12A, B), and then decreasing again. Although the number of active cells of *Roseobacter* was highly similar in mix 1 and mix 2, their overall activity was not the same in these mixes (Suppl. Fig. 2). Contrary to that, community cell activity in the mix 2 increased along the whole experiment. The number of active Gammaproteobacteria cells was almost twice higher in the mix 1 than in the mix 2 at the beginning of the experiment, yet they behaved similarly showing a large decrease in number of active cells and their total activity at the end of experiment (Fig. 12A, B, suppl. Fig. 2A, B). Finally, although the abundance of Bacteroidetes showed almost a 2-fold decrease during the experiment, their single cell activity did not change a lot (Figs. 11E, 12, suppl. Fig. 2).

Remarkably, the fact that in general most bacterial groups behaved very similar in mixes 1 and 2 suggests that the observed changes were mostly due to the physicochemical changes as a

result of groundwater addition rather than to the interaction with the much less numerous groundwater taxa.

4.2.4 Response of groundwater communities upon exposure to marine conditions (mix 3)

A different response was observed when groundwater communities were exposed to marine conditions ("bacteria-free" seawater) in mix 3 in the absence of resident marine communities.

Three out of six target prokaryotic groups (i.e., Gammaproteobacteria, *Roseobacter* and Bacteroidetes) showed a large increase in total cell number during the first 23 hours of experiment (Fig. 11A, B, E). However, whereas the abundance of Bacteroidetes increased throughout the whole experiment reaching almost 20% of total cells at the end, Gammaproteobacteria and *Roseobacter* showed a decrease in abundances after 23 hours (Fig. 11A, B). The freshwater LD12 group (Fig. 11F) did not grow under the seawater input showing a 5-fold decrease from 15% at the beginning of experiment to only 3% at the end of the incubation. Similarly to LD12, the marine SAR11 clade (that was present at the beginning possibly due to passing through the filter due to their small size (see Discussion)) did not show any further growth during the experiment (Fig. 11D). Finally, the marine cyanobacteria *Synechococcus* were rare throughout the whole experiment, as expected, accounting for less than 1% of the total cells.

The number of active cells and their activity in mix 3 changed similarly to the bacterial group abundance. At the beginning of experiment, mix 3 was characterized by high numbers of LD12 active cells (Fig. 12), even though they showed low activity levels (Suppl. Fig 2C) and were not very abundant (Fig. 11F). Over the incubation, both freshwater LD12 and marine SAR11 became almost inactive. Conversely, Gammaproteobacteria became very active and at the end of experiment they accounted for around 35% of the total active cells (Fig. 12C). Interestingly, the fluorescence intensity of Gammaproteobacteria was on average very high in the initial sea- and groundwater communities and contributed around 50 % of the total activity (Suppl. Fig. 2C). Although not very abundant at the beginning (Fig. 11B), *Roseobacter* showed high cell activity that together represented almost 10% of total community fluorescence intensity at initial time (0h). This likely allowed a subsequent rapid activity increase during the first hours of incubation, reaching up to 41% of the total fluorescence intensity (proxy of single cell

activity) and decreasing at the end of experiment to 23%. Bacteroidetes showed a 10-fold increase in number of active cells by the end of experiment compared to the initial conditions (2.9% to 21% of total active cells), and a 5-fold increase in their fluorescence intensity (3% to 16% from total BONCAT fluorescence intensity) throughout the experiment.

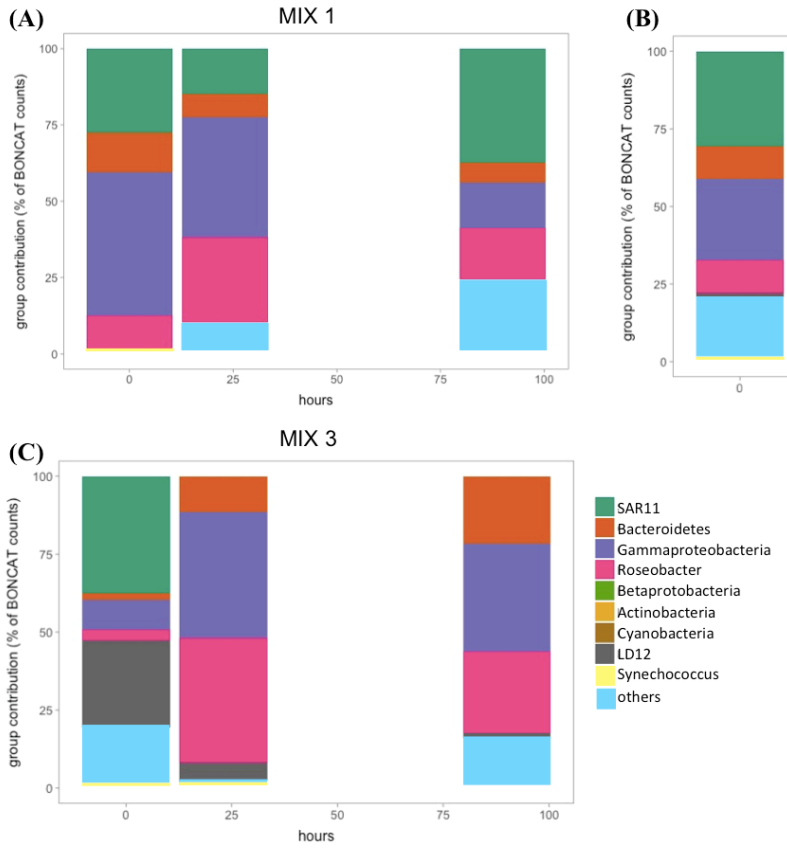

***Figure 12**. Activity contribution of each bacterial group represented in percentage from total number of active cells over time for the different treatments (see experimental design in Figure 8). Each colour indicates different bacterial groups.*

5 DISCUSSION

Despite the recent evidences that groundwater discharge to the coastal ocean is much more important than previously believed (Rodellas et al., 2015), we still know very little about the composition and functioning of the communities inhabiting groundwater aquifers draining to the ocean and how such groundwater inputs may influence coastal bacterioplankton communities. More importantly, nothing is known about whether groundwater microorganisms

can be a source of diversity to coastal bacterioplankton communities. Here, by combining field and experimental surveys, we provide the first spatial characterization of the bacterial communities inhabiting aquifers from the Spanish Mediterranean coast and we show that the mixing of groundwater and seawater differentially affects various bacterial groups. Furthermore, we show that certain groundwater bacterial taxa seem to have the potential to grow when reaching marine waters, in which case coastal groundwater aquifers would represent as a reservoir of microbial diversity for the coastal Mediterranean Sea.

5.1 Spatial patterns in groundwater and marine bacterioplankton communities

5.1.1 Spatial variability in bacterial abundance, active cell counts and heterotrophic production

Here we show that total bacterial abundance varied largely depending on the type of ecosystem (Fig. 3), being the lowest in groundwater aquifers (range 1×10^4 – 2×10^5 cells mL^{-1}) and the highest in the Ebro river (4.5×10^6 cells mL^{-1}, showing values in accordance to previous reports in this system, Ruiz-González et al. 2013). Marine communities had generally lower bacterial abundance than the river, ranging between 8×10^5 and 1.5×10^6 cells mL^{-1}, and in general they showed little variability between sites (Fig. 3). Although previous reports on bacterial abundance in NW Mediterranean have found lower cell concentrations (e.g, ranging from 5.27×10^5 in September to 7.08×10^5 cells mL^{-1} in June, Sala et al. 2002), great increases in bacterial abundance (exceeding our values) have been reported after experimental addition of nutrients (Sala et al. 2002) or following phytoplankton blooms (Cole et al., 1988, Ghiglione et al., 2005). Thus, the high bacterial numbers observed in our sampling marine sites, known to be subjected to groundwater discharge, might indicate that these groundwaters are providing marine communities with nutrients, as reported elsewhere, and thus allow higher abundances of bacteria (e.g. Garcés et al. 2011).

We observed that when estimating bacterial abundance with different techniques (microscopic counts using DAPI staining versus flow cytometry), we obtained higher bacterial cell counts with DAPI stain in the case of most groundwater samples. This was likely due to the presence of sediment particles that can produce fluorescence at a certain wave length (Porter and Feig, 1980) and were thus captured by the image analysis. In order to avoid this overestimation, for further calculations (e.g., the relative abundances of the different CARD-FISH bacterial groups, see below) we used the flow cytometry cell abundance data, which ranged from 3.7 to

14×10^3 cells mL^{-1} and are in an agreement with previously reported data for groundwater samples (Griebler and Lueders, 2009, Stein et al., 2010).

Regarding the activity, a similar pattern was observed for both the total number of active cells measured through BONCAT (Fig. 4A) or the bulk heterotrophic production of the community estimated by the incorporation of radioactive leucine (Fig. 4B): groundwater communities showed much lower activity than river or seawater samples, suggesting that these communities are mostly inactive likely due to the lack of organic carbon in these systems (Griebler and Lueders, 2009).

The exception for the low abundance and single cell activity in the groundwater aquifers were sites GR3.2 and GR5, which are actually surface water bodies directly fed by the groundwater. Bacterial abundance in these water bodies was significantly higher than in the actual aquifers with a mean of 5.6×10^5 cells mL^{-1}, on average two orders of magnitude higher than the bacterial abundance from the true groundwater sites (Fig. 3). Moreover, these sampling sites had high numbers of active cells, and remarkably, the highest bulk activity rates, even higher than those measured in the river (Fig. 4). Indeed, comparing those two surface water bodies with the river sample, we found that although river had the highest number of total and active cells, river bacteria showed 1.5 times lower productivity rates (Figs. 4B). This means that the bacteria from these two groundwater-fed surface sites were extremely active, and might indicate a large growing potential of groundwater bacteria when reaching the surface, likely due to the availability of organic carbon derived from phytoplankton or the surrounding terrestrial environment. Usually, all bacterial communities harbour few groups that are abundant while at the same time there is a huge amount of taxa with low abundance or being dormant (Finlay, 2002, Lennon and Jones 2011). Due to their small size, bacteria can be easily distributed everywhere and, as soon as conditions are suitable, these rare or dormant cells can activate (Pedrós-Alió, 2012) thus increasing their cell abundance in the environment. Although the correlation between organic carbon and bacterial activity was questioned by some authors (Stein et al., 2010), this large bacterial abundance increase in sites GR3.2 and GR5 might be related to the presence in these surface environments higher amount of carbon or nutrients (Steube et al., 2009), but unfortunately these factors were not estimated in our work.

5.1.2 Bacterial community composition in groundwater and adjacent marine coastal sites

The previously mentioned spatial heterogeneity of all the studied sites was also reflected in the relative contribution of different bacterial groups structuring the various communities. Overall, the eight different oligonucleotide CARD-FISH probes used could identify over 50 % of bacterial cells in the samples, with the exception of two groundwater aquifers where less than 25% of the total cells were captured with these probes. This might be due to the low activity of groundwater cells shown above, since the hybridization only targets active cells (i.e., cells with ribosomes), or to the fact that other bacterial groups not considered here dominated in those samples. In order to check this assumption, an additional hybridization of these groundwater samples with the probe for Eubacteria will be done, that binds to all bacterial groups present in the aquifers with ribosomes, and will thus help us to understand the causes underlying the low percentages of CARD-FISH targeted cells recovered. In any case, our results provide the first characterization of bacterioplankton composition in the groundwater aquifers draining to the NW Mediterranean (Fig. 5(A)).

We detected large differences in composition between the studied groundwater, river, and seawater communities (Fig. 5A). Our river sample was mainly dominated by Actinobacteria, accounting for 33% of total DAPI counts (Fig. 5A), followed by Betaproteobacteria (13%) and Bacteroidetes (12%), consistent with previous studies in Ebro river (Artigas et al., 2012, Ruiz-González et al., 2013) as well as from other riverine systems (Stepanauskas et al., 2003). Seawater communities from the different sites were quite similar and were characterized by the dominance of Alphaproteobacteria (21-33%), represented mostly by SAR11 (22%) and *Roseobacter* (3%). Gammaproteobacteria and Bacteroidetes groups represented around 20%, that is generally consistent with previous phylogenetic composition studies based on 16S rDNA (Rodríguez-Blanco et al., 2008) and other studies of bacterial communities in the NW Mediterranean (Ruiz-González et al., 2012, Alonso-Sáez et al., 2007). Small spatial variation between coastal bacterioplankton communities was also shown by Lee & Fuhrman, 1991 and Ghiglione et al., 2007. Indeed it was shown that the main differences in marine bacterioplankton communities at the horizontal scale can be observed when comparing coastal surface water and far offshore samples (over 10 km from the coast) (Ghiglione et al., 2005), while our offshore sampling sites were much closer than that. Finally, groundwater sites were more heterogeneous among each other, and whereas in some aquifers Bacteroidetes and Betaproteobacteria were the

dominant groups, in others Gammaproteobacteria showed the largest abundances (Fig. 5A). These results agree with the reported dominance of these groups in the groundwater aquifers (Héry et al., 2014, Lee et al., 2017).

High heterogeneity observed in groundwater aquifer communities (Fig. 5A, Fig. 6A) suggests that even small physico-chemical, hydrological and geological differences lead to the establishment of specific bacterial communities. In addition to that, salt-water intrusions could even promote vertical gradients in aquifers that with time promote development of autochthonous bacterial communities. Moreover, different residence time of the water as well as amount of seawater seeping to the aquifer can promote various but at the same time stable physico-chemical conditions and as a result distinct bacterial communities (Garing et al., 2013, Hery et al., 2014). As a result, Grieber et al. (2009) claims that all groundwater aquifers are very different in their physical and chemical composition and that even small changes in grain size, organic content or mineralogical variations strongly influence microbiological diversity from pore to ecosystem scale (Brockman and Murray, 1997, Griebler and Lueders, 2009). Our results from bacterial composition in various aquifers also support this large heterogeneity between all the studied groundwater sources, even those located close to each other (Fig. 5A, 6A), likely due to the lack of connectivity between them and higher isolation of communities.

Moreover, our results also suggest a certain marine influence in some aquifers, since some of our groundwater sites (GR6), known to be subjected to seawater intrusions, showed higher numbers of the marine clade SAR11 than other groundwater sites with a high water flow towards the sea and surface systems with minimum seawater influence. Lee et al. (2017) further suggested that not only seawater can influence aquifers bacterial composition, but also *vice versa.* Thus, levels of groundwater and its velocity, as well as tidal phase may impact microbial composition of coastal ecosystems. Interestingly, some of our seashore samples contained low numbers of typical freshwater groups like Betaproteobacteria (SW1.1) and LD12 (SW6), indicating that SGD can influence coastal microbial community composition through the inoculation of groundwater bacteria. Indeed, higher abundance of groundwater bacterial groups was present in the seashore sites often subjected to high groundwater flow.

Regarding the contribution of these bacterial groups to the cell activity (Fig. 5B) that is highly correlated between each other, we also observed large differences between groundwater, river and seawater communities. Seawater active cells were dominated by SAR11. The predominance of this group has also been shown in various marine surveys around the world

(Morris et al., 2002), making this group the most abundant marine bacteria that may play an important role in this environment. Interestingly, whereas some previous surveys pointed out that activity of SAR11 was lower than expected based on their abundance (Alonso-Sáez and Gasol, 2007, Rodríguez-Blanco et al., 2008, Hunt et al., 2013, Campbell et al., 2011), our results indicate on average higher activity contribution of this same group, which made up to 38% of the total active cells in marine samples (Fig. 5B). This can be related to the time of the year when these samples were taken, as seasonal fluctuations together with the type of substrate used could influence the uptake rate of bacteria. For example, it is known that the Bacteroidetes group does not take up low molecular weigh (LMW) compounds (e.g., the amino acids used for bacterial production or BONCAT), showing low cell activity with respect to their abundance (Cottrell and Kirchman, 2000, Alonso-Sáez et al., 2007), and indeed this agrees with the observed small contribution of seawater Bacteroidetes to total active cells (Fig. 5B). Conversely, Roseobacter and SAR11 lineages are able to incorporate low concentrations of available substrate more efficiently (Alonso and Pernthaler, 2006) being highly represented among active cells (Fig. 5B).

In the case of groundwater communities, we found that a large fraction of the active cells belonged to Betaproteobacteria, which agrees with the dominance of this group in groundwaters (Griebler and Lueders, 2009, Héry et al., 2014), although their contribution varied between aquifers (Fig. 5B). Betaproteobacteria are often also the most abundant surface fresh water bacterial group, and in some environments they can account up to 70% of the total number of cells (Newton et al., 2011). Such a high abundance of these bacteria is usually related to their opportunistic behaviour when nutrients are available, likely explaining their dominance and high activity in the groundwater-fed pond-like site (GR5), since lakes are considered the most typical environment for Betaproteobacteria (Newton et al., 2011).

Remarkably, we found unexpectedly high percentage of the marine SAR11 bacteria with extremely high cell activity at the groundwater site GR6. We believed that it is possible that this was due to unspecific probe binding during the hybridization step to other close representatives from Alphaproteobacteria class.

To summarize our results and support some of our assumptions we performed a PCA based on differences in bacterial composition and single cell activity in our samples (Fig. 6). In agreement to the results shown above, all marine samples were grouped together according to their similar bacterioplankton composition as well as activity mainly driven by the SAR11 group (Fig. 6A, B). Conversely, groundwater samples showed a higher dispersion on both PCA plots

suggesting larger differences in bacterial composition and activity among them. Some of the aquifers were more related to the riverine type of bacterioplankton communities, while others had more similarities with seawater samples, probably due to their close location to the coast and as a result stronger marine influence that is also shown by previously mentioned studies (Héry et al., 2014, Griebler and Lueders, 2009, Lee et al., 2017).

5.2 Influence of groundwater inputs on coastal bacterioplankton communities structure and functioning

Our results from the spatial study suggest that most of the subterranean groundwater systems are quite poor in bacterioplankton abundance, showing 3 to 5 times lower numbers than in the other surface aquatic environments studied, and low levels of bacterial heterotrophic activity. However, as mentioned above, the observation that the two groundwater-fed surface sites showed such high activity and abundance levels may suggest that some of these groundwater dormant bacteria can activate and grow when exposed to surface conditions. This agrees with recent reports showing that bacteria are able to persist inactive in soils or sediments but are able to grow and dominate communities once inoculated in the water (Ruiz‐González et al., 2015). Seeing this, we questioned ourselves if certain groundwater bacterial taxa have the potential to grow in marine environments as well, in which case such groundwater sources would comprise a reservoir of diversity for coastal ecosystems. Preliminary results support this idea, since we were able to grow groundwater bacteria on a typical marine agar medium, and we recovered several colonies of different colours and morphologies (see Fig. 7). In view of these results, we performed a manipulation experiment devoted not only to assess groundwater bacterial recruitment potential in marine environment, but also the responses of marine bacteria to freshwater inputs, as well as possible interactions between groundwater and seawater communities. To do so, seawater and groundwater were mixed to make three different experimental treatments. Changes in total cell number, number of active cells as well as bacterial composition were monitored throughout the experiment in order to address the issues mentioned above.

5.2.1 Influence of mixing of groundwater and seawater on bacterial abundance and activity

We observed that overall, the two treatments that included marine bacteria exposed to groundwater (mix 1 and mix 2) tended to behave similarly, whereas the treatment that included only groundwater bacteria exposed to marine water (mix 3) showed a different response. Although mix 2 in which groundwater bacteria were also present (Fig. 2) differed from mix 1, abundance of groundwater bacteria were much lower than those of marine bacteria, and thus likely they were outcompeted by seawater taxa. This suggests a low impact of groundwater bacteria on stable and abundant marine communities. Therefore, we consider that whereas mix 1 and mix 2 mostly represent the response of marine bacteria to groundwater inputs, mix 3 addresses whether groundwater bacteria are able to thrive when arriving to coastal waters.

Exposure of marine bacteria to groundwater (either with autochthonous bacteria absent or present, mix 1 and mix 2, respectively) resulted in a fast increase in bacterial numbers in the first 23 h and a subsequent decrease (Fig. 8A), which was mirrored by the number of active cells (Fig. 8B), and in general we found a good correlation between the number of total and active bacteria throughout the experiment (Fig. 9). On the other hand, groundwater bacteria, which were quite inactive *in situ*, responded fast to the seawater input (mix 3) largely increasing the number of total cells during the first day, and keeping a steady increase until the end of the experiment, reaching bacterial abundances and number of active cells similar to those in mixes 1 and 2 (Fig. 8). Although bacterial abundances in mixes 1 and 2 increased to higher levels than in mix 3, it is interesting to note that groundwater bacteria from mix 3 showed much higher activity levels (intense BONCAT signal in the micrographs) than marine bacteria from mixes 1 and 2 and remained high throughout the experiment (Fig. 10). This was also reflected in terms of the bacterial production, which in mix 3 showed a 100-fold increase, while mixes 1 and 2 showed only 1.5-fold increase after the first day of incubation (Suppl. Fig. 3). This suggests a large potential of some groundwater bacteria to grow under marine influence when no interactions with other communities are present (Salcher, 2013). Interestingly, the only other study experimentally assessing the effect of groundwater on marine bacteria (Garcés et al., 2011) had shown that groundwater additions promoted just a slight increase in abundance of marine bacterioplankton. However, these authors had measured the responses of bacteria after 3 days, and here we show that bacteria respond to groundwater inputs in a much shorter time, after which the communities recover again to the initial conditions.

Then, in order to explore which bacterial groups were responsible to the observed changes in cell numbers and single-cell activity, we used the CARD-FISH technique coupled to BONCAT in order to estimate the contribution of six different bacteria groups to the total abundance and community activity. We observed that different groups responded differently to the experimental mixes, and again, that in general mix 1 and mix 2 behaved similarly but different from mix 3.

5.2.2 Changes in marine bacterial composition and activity upon groundwater additions (mixes1 and 2)

Among the short-term responses of marine bacteria to groundwater inputs (23h), we observed that abundance of *Roseobacter* increased during the first 23h of incubation (Fig. 11B), Bacteroidetes remained at the same abundance (Fig. 11E), and SAR11 quickly decreased (Fig. 11D). Gammaproteobacteria seemed to be the first ones responding, at least in mix 1, since their levels at the beginning of the experiment were already higher that those *in situ*, suggesting that they probably responded quickly to manipulation due to sampling and experimental setup (Fig. 11A). But after 23 hours they started to decrease. By the end of the experiment, SAR11 and *Roseobacter* went back to their initial levels, whereas Gammaproteobacteria and Bacteroidetes had decreased with respect to their initial abundances. Temporal variation in terms of communities' succession was also reported by Ghiglione et al. (2005) in the NW Mediterranean but only at the large time scale (more than 2 weeks) that is usually related with phytoplankton blooms and organic matter input. Our mixing experiment showed much faster changes suggesting a very rapid response of bacteria due to the groundwater input that bring nutrients to the environment. Fast-growing copiotrophic groups of bacteria seemed to be responsible of these changes, after which some other processes, such as removal of nutrients or grazing, might allow the communities to go back to the typical oligotrophic conditions.

For example, the rapid decrease of Gammaproteobacteria abundance under groundwater input could occur as a response to salinity variations (Héry et al., 2014), but also as a result of intense grazing by different protozoa and heterotrophic nanoflagelates (HNF). These predators can change bacterial community structure by the direct grazing (Lekunberri et al., 2012) as well as through the release of DOM that can be used afterwards by other bacteria (e.g. Tada et al. 2011). As the experimental mixes 1 and 2 include whole seawater, we cannot exclude grazing as

a factor that promoted a decrease in Gammaproteobacteria abundance or other groups such as Bacteroidetes (Pernthaler et al. 1997, Kirchman 2002). Alphaproteobacteria, that in our study includes such groups as *Roseobacter*, SAR11 and the freshwater LD12 group are usually known to represent a wide range of behaviours. SAR11 is the most abundant surface marine bacterioplankton group (Giovannoni, 2017), which accounted for around 25% to the total number of cells in the initial seawater sample, and is known to be highly successful in low nutrient oligotrophic conditions typical of the open ocean. The abundance and activity of this bacterial group might have decreased as a response to salinity decrease or to the nutrient inputs from the groundwater, since nutrient concentrations are usually negatively correlated with SAR11 abundance (Newton et al., 2011, Giovannoni, 2017). SAR11 are also known to have quite low rRNA/rDNA ratio that makes them usually unable to adjust enzyme content when environmental conditions change, thus they do not grow rapidly under the changing environment. Although the nutrient concentrations over time in the treatments were not measured during our experiment, the decrease in nutrients caused by opportunistic bacterial taxa might have allowed the recovery of SAR11 abundances by the end of the experiment (Brinkhoff et al., 2008). In addition, Dadon-Pilosof et al. (2017) showed that that mostly all marine grazers did not retain low-nucleic-acid bacteria such as the SAR11 group. Thus, we can assume that by removing highly active opportunistic groups by grazing, it becomes possible for SAR11 to attain higher abundances again. Indeed, it was found that incubation of the seawater previously filtering it (removing all the grazers) showed an increase in abundance of such groups as Bacteroidetes and *Roseobacter* as well as Gammaproteobacteria suggesting a large impact of Protozoa and other grazers on structuring natural bacterial communities (Ferrera et al., 2011).

Representatives of LD12 were not detected in mix 1 (Fig. 11F) as they are considered as a freshwater bacteria (Salcher, 2013), whereas initial bacterial composition of the mix 2 included low amount of LD12 (<10% of total DAPI counts). However, further incubation did not promote the growth of this group, suggesting inhibition of their growth due to the salinity increase as well as the low competitive level of this group in the presence of other species (Salcher, 2013). Finally, the cyanobacterial group *Synechococcus* was at very low abundance and activity compared to other bacterial groups. However, by the end of the experiment they had started to grow (Fig. 11C), in accordance to the positive response of *Synechococcus* to groundwater additions reported after 3-day incubations (Garcés et al. 2011). This delayed response is in accordance to longer regeneration times of *Synechococcus* compared to heterotrophic bacteria,

and suggests that we might have observed a more important increase in their abundances if we had kept our experiment for a longer time.

As previously mentioned, during the experiment bacterial abundance was well correlated with number of active cells. However, some groups of bacteria, like for example Gammaproteobacteria, showed a much higher contribution to activity in comparison to their abundance, while Bacteroidetes group activity was lower (Suppl. Fig. 2). This might be due to the limiting nutrients, growing rates of each group, type of substrate and efficiency of bacteria to uptake these substrates that usually depends on bacterial growing strategy, taxonomic composition and environmental conditions (Leizeaga et al., 2017). Thus, bacterial groups with high substrate efficiency uptake and growing potential tend to have higher activity rates. Such opportunistic behaviour of several bacterial groups (i.e. Gammaproteobacteria and *Roseobacter*) shows that initially low abundant or dormant groups can become very numerous under sudden nutrient-rich environmental conditions. Moreover, few recent studies have shown that groundwaters could harbour a large amount of rare bacterial taxa that become active when they arrive to surface freshwater ecosystems (Crump et al., 2012, Ruiz−González et al., 2015), supporting that these systems may hide a microbial seed bank (Lennon and Jones, 2011) for other ecosystem types like the ocean. Instead, the low activity rates observed for Bacteroidetes agrees with the observations that they are usually underrepresented in 3H-leucine uptake (Zubkov et al., 2001) due to their low affinity for this compound and preference to the high molecular weight substrate.

5.2.3 Growth of groundwater bacterial groups exposed to marine conditions (mix 3)

Exposure of the initially inactive groundwater communities to marine conditions (mix 3) showed a rapid increase of Gammaproteobacteria, Bacteriodetes and *Roseobacter* in their activity and cell abundance (Fig. 11), suggesting a large potential of some representatives of these groups to bloom when reaching coastal waters. They were also the groups responsible for most of the increase in active cell counts as well as percentage of high translational active cells in the mix 3 (Fig. 12C, suppl. Fig. 2). Interestingly, however, comparing mix 3 with mix 2 (that also include groundwater taxa) it became obvious that these low-abundant opportunistic freshwater bacteria are not able to outcompete the more numerous and stable marine communities.

Hery et al. (2014) found that Gammaproteobacteria from a groundwater aquifer subjected to saline water intrusions showed a positive correlation with salinity, consistently with our results where we found a rapid increase in this group when seawater was added. Similarly to Gammaproteobacteria abundance, the representative of Alphaproteobacteria group *Roseobacter* showed a rapid increase in active cells and total cell number during the first hours of the experiment. Much larger abundance increases of these two groups compared to their marine counterparts growing in mixes 1 and 2 might be due to the fact that they represent different ecotypes of Gammaproteobacteria and *Roseobacter* adapted to fresh or marine waters. Alternatively, since seawater in mix 3 had been prefiltered, we did not have grazers that might have lowered the abundances of these highly active groups in mixes 1 and 2 (e.g. Ferrera et al. 2011).

It is important to mention that, although we assume that experimental mix 3 included only groundwater bacteria, some small or miniaturized marine bacteria could have passed the 0.2 um filter, since it is known that many natural bacteria can be smaller than that size (Luef et al., 2015, Brown et al., 2015, Ghai et al., 2013). Actually, when 0.2 um-filtered seawater was incubated for the same time in parallel to our experiment, we observed a large growth of groups like Bacteroidetes and *Roseobacter* (details not shown), which were probably miniaturized *in situ* due to nutrient limitation (Lever et al., 2015) but that grew upon experimental manipulation. Thus, we cannot discard that the observed bacterial growth in mix 3 was due to some previously dormant or inactive miniaturized marine bacteria. In addition, after mixing groundwater with prefiltered seawater, we found that the initial composition of mix 3 had on average high percentage of SAR11 cells, which could happen due to the small size of these bacteria, around 0.01 – 0.04 μm^3 (Giovannoni, 2017) that were able to pass the filter. We also suspect, as mentioned before, that some of these SAR11 were detected due to unspecific hybridization of some other groundwater Alphaproteobacteria with the SAR11 probe as well as possible transfer of SAR11 with marine water. Future studies assessing the diversity of these communities at the species level with sequencing technologies will allow us to confirm whether this presence of SAR11 was true or not.

A PCA analysis further indicated that mix 3 was very different from the other two experimental treatments in terms of bacterial composition and activity (Suppl. Fig. 4). These differences were mainly driven by the presence of the highly active *Roseobacter* and Gammaproteobacteria, and again highlights the potential of these presumably inactive or

dormant freshwater groups to grow when reaching the coastal zone of the sea. However, the fact that mix 2, that also included marine bacterial communities and some predators did not allow groundwater taxa to fully occupy this niche, suggests that such potential groundwater colonizers may only be successful when large inputs of groundwater are present, like for example in site SW1_1, where salinity in the bay dropped down to 15.1. The experimental mix 2 should be considered as the most realistic, as it includes interactions between the two natural communities that are naturally mixed upon submarine groundwater discharge as well as factors like predation or viral lysis.

6 CONCLUSIONS

In summary, here we show that coastal aquifers draining to the NW Mediterranean show consistently low bacterial abundances and highly inactive communities, which are much more spatially heterogeneous in terms of community composition and single-cell activity patterns than adjacent coastal sites. Moreover, by sampling two surface water bodies that were fed by continuous flow of the groundwater we were able to see the high potential of groundwater bacterioplankton become active when reaching suitable environment conditions giving us a large field of further research. In addition, we experimentally demonstrate that groundwater inputs promote pronounced but transient changes in the composition and functioning of the receiving marine communities, which are driven by the fast growth of some copiotrophic taxa. Finally, exposure of groundwater communities to marine conditions unveiled the presence of certain taxa that are able to grow in marine environment, suggesting that in situations of high groundwater discharge these aquifers may represent reservoirs of viable diversity for coastal bacterioplankton communities. Our results provide insight into how coastal communities respond to groundwater additions, which can give us information related to different bacterial groups' behaviour and environmental preferences, as well as to their ability to occupy other environments. Groundwater in the Mediterranean is known to be a large source of freshwater as well as a very important source of nutrients (Rodellas et al., 2015), but whether it also provides viable taxa was unknown until now. Moreover, due to the magnitude of the groundwater discharge in the Mediterranean, it is possible that although groundwater discharge results in dramatic but brief changes in composition and activity, these changes may have significant biogeochemical implications that are nonetheless difficult to quantify due to their transient nature. Finally, as freshwater is mixed

with marine in the coastal environments, salinity variations tend to impact bacterial abundance and community composition (Héry et al., 2014), so our results are also relevant in order to understand potential changes in aquifer biogeochemistry exposed to salinization, which is an increasingly recurrent concern in various Mediterranean areas (Custodio Gimena, 2017). Further longer experiments should be done in order to understand the long-term impacts of changes in hydrologic regimes on marine bacterial community structure and functioning.

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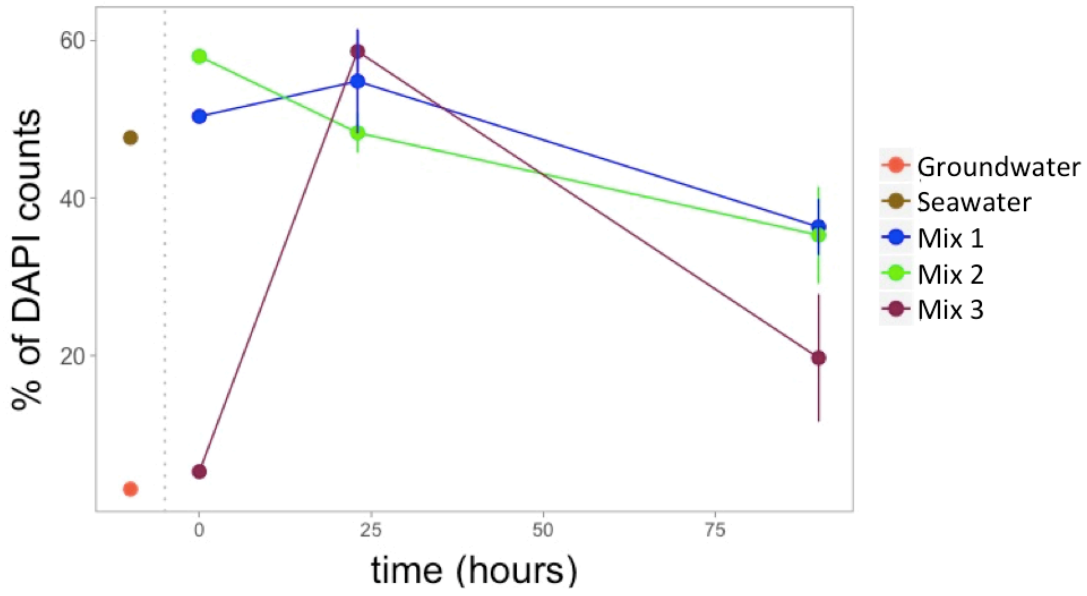
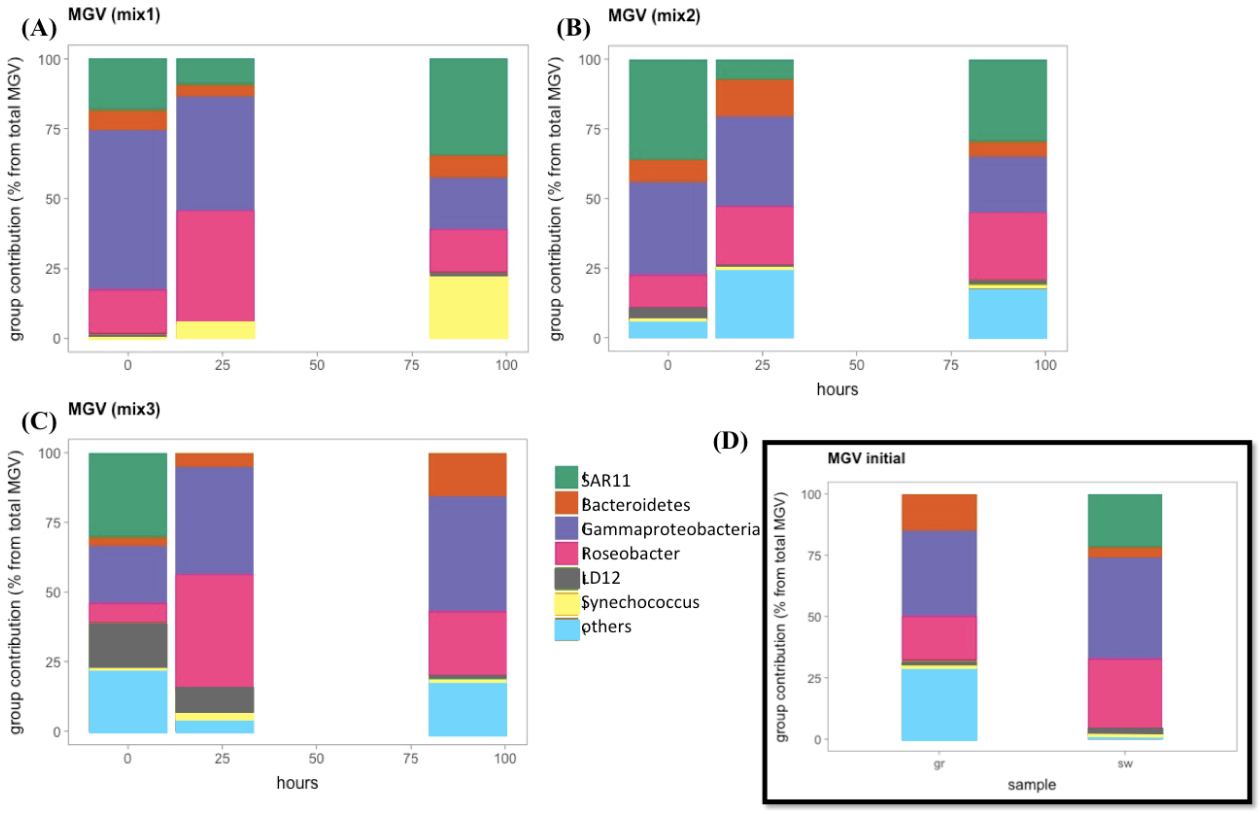
SUPPLEMENTARY MATERIALS

Sample ID	Site	Station	System	Latitude	Longitude	Sampling technique	Temperature	Conductivity	Salinity
GR1_1	Site 1	Fuente del centro, Les Fonts	Groundwater	40,252431	0,286538	Manually	18,39	13,6	7,89
GR1_2		Fuente Sur, Les Fonts	Groundwater	40,251197	0,286892	Manually	19,1	3,97	2,12
SW1_1		Sea Shore, Les Fonts	Sea shore	40,25185	0,287101	Manually	18,91	24,7	15,1
SW1_2		Espigon, Les Fonts	Sea	40,2524	0,288683	Manually	16,7	56,26	37,47
GR2	Site 2	Fuente, Torre Badun	Groundwater	40,321948	0,363714	Manually	18,86	14,02	8,15
SW2		Sea shore, Torre Badun	Sea shore	40,321868	0,363777	Manually	17,5	49,4	32,4
GR3_1	Site 3	Manantial, Peniscola	Groundwater	40,358912	0,405303	Manually	17,23	0,66	0,32
GR3_2		Marsall (river on the beach), Peniscola	Groundwater superficial	40,355957	0,399366	Manually	21,61	11,7	6,7
SW3		Espigon, Peñiscola	Sea	40,355474	0,40013	Manually	16,88	52,38	34,55
GR3_3		Acuifero Inferno, Peñiscola	Groundwater	40,372844	0,40234	Piezometer	45,46	37,48	23,16
GR4	Site 4	Font playa, St. Carles de la Rapita	Groundwater	40,597447	0,573591	Manually	19,36	11,44	6,5
SW4		Sea shore, St. Carles de la Rapita	Sea shore	40,597225	0,573846	Manually	16,8	52,83	34,88
GR5	Site 5	Ojals de Baltasar	Groundwater superficial	40,673065	0,593817	Manually	16,9	3,26	1,71
R6	Site 6	Ebro river, Amposta	River	40,71234	0,584749	Manually	14,33	1,18	0,6
SW6	Site 7	Argentona aquifer, Mataro	Sea shore	41,518677	2,424588	Manually	25,24	59,1	37,7
GR6		Argentona sea shore, Mataro	Groundwater	41,519867	2,423944	Piezometer	18,8	13	8,6

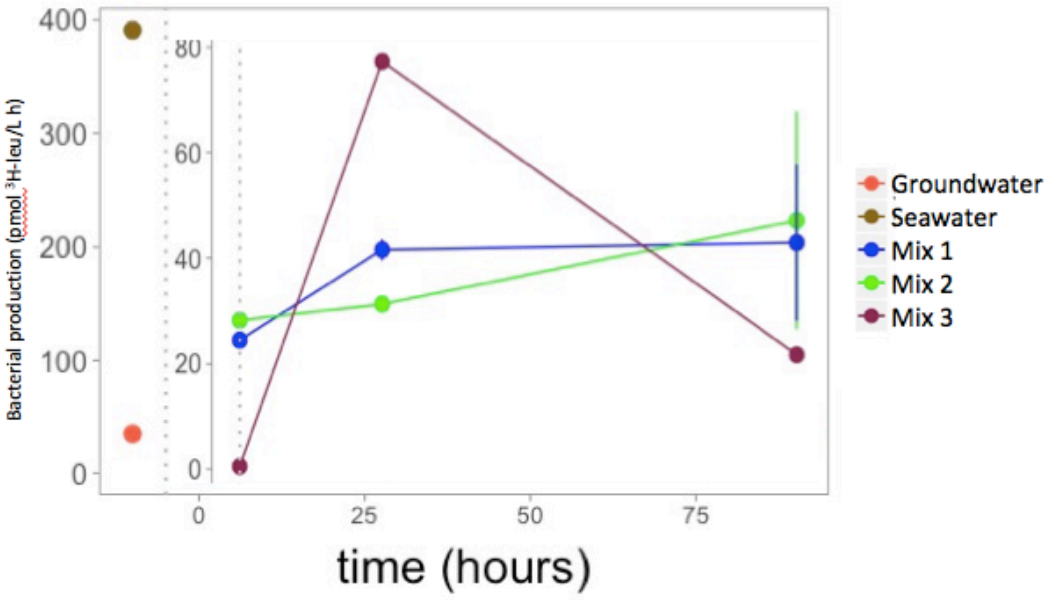
Supplementary table 1. *The location of sampling sites in the NW Mediterranean and their basic characteristics.*



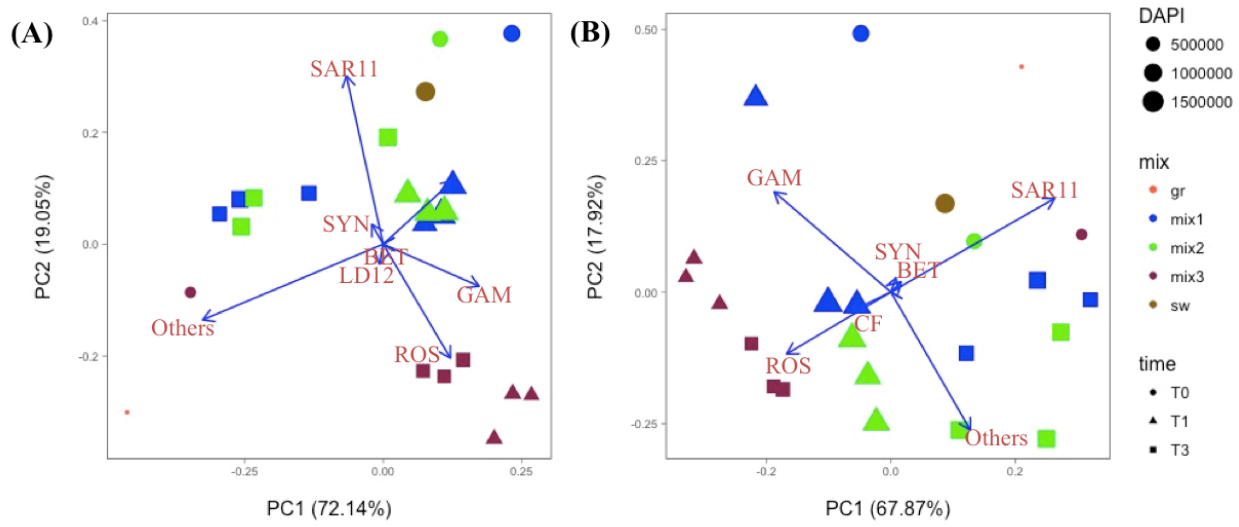
Supplementary Figure 1. *Percentage of active cells (from total cell counts) during the experiment. Different experimental treatments are indicated by different colours. The two single dots represent the in situ seawater and groundwater original communities used for preparing the experimental mixes, and are separated by the dashed line.*



Supplementary Figure 2. *Changes in the translational activity of cells (based on the single cells fluorescence intensity – Mean Gray Value) over time in different treatments: (A) mix 1 – 75 % seawater with 25 % bacteria free groundwater; (B) mix 2 – 75 % seawater with 25 % groundwater; (C) mix 3 – 75 % bacteria free seawater with 25 % groundwater. Each colour indicates the different bacterial groups.*



Supplementary Figure 3. *Changes in bacterial heterotrophic production measured as radiolabeled ^{3}H-leucine incorporation rates during the mixing experiment. Different experimental treatments are indicated by different colours: blue – mix 1, green – mix 2, purple – mix 3. The two single dots represent the in situ seawater and groundwater original communities used for preparing the experimental mixes, and are separated by the dashed line.*



Supplementary Figure 4. *Results of the principal component analysis (PCA) for the communities sampled during the mixing experiment, based on bacterial composition assessed by CARD-FISH (A), and on the single-cell activity of these different groups assessed by BONCAT (B). The data included are expressed as percentages of total bacterial abundance (A) and percentage of total active cells (B). Each colour indicates different mixes and the size of the dots is proportional to total bacterial abundance in each community. The vectors indicate different bacterial groups used for the analysis.*

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THE PRESENT RESEARCH WORK HAS BEEN (PREPARED TO BE) PUBLISHED IN:

As the research topic includes novel field of study this thesis will lead to a minimum of one publication in the “The ISME Journal”. The amount of the data collected is expected to provide insights for additional few other publications.