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Allochthonous Groundwater Microorganisms Affect Coastal Seawater Microbial Abundance, Activity and Diversity

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Key Points:

- Groundwater discharge into the coastal zone delivers both nutrients and allochthonous microbes
- Groundwater microbes interact with seawater populations, by which affecting the delicate autotroph-heterotroph balance
- Subterranean microbial processes are key drivers of food webs, potentially affecting biogenic carbon fluxes in the ocean

Supporting Information:

Supporting Information may be found in the online version of this article.

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Abstract Submarine groundwater discharge (SGD) is a globally important process supplying nutrients and trace elements to the coastal environment, thus playing a pivotal role in sustaining marine primary productivity. Along with nutrients, groundwater also contains allochthonous microbes that are discharged from the terrestrial subsurface into the sea. Currently, little is known about the interactions between groundwater-borne and coastal seawater microbial populations, and groundwater microbes' role upon introduction to coastal seawater populations. Here, we investigated seawater microbial abundance, activity and diversity in a site strongly influenced by SGD. In addition, through laboratory-controlled bottle incubations, we mimicked different mixing scenarios between groundwater and seawater. Our results demonstrate that the addition of 0.1 μm filtered groundwater stimulated heterotrophic activity and increased microbial abundance compared to control coastal seawater, whereas 0.22 μm filtration treatments induced primary productivity and *Synechococcus* growth. 16S rRNA gene sequencing showed a strong shift from a SAR11-rich community in the control samples to *Rhodobacteraceae* dominance in the <0.1 μm treatment, in agreement with *Rhodobacteraceae* enrichment in the SGD field site. These results suggest that microbes delivered by SGD may affect the abundance, activity and diversity of intrinsic microbes in coastal seawater, highlighting the cryptic interplay between groundwater and seawater microbes in coastal environments, which has important implications for carbon cycling.

Plain Language Summary Submarine groundwater discharge (SGD) is an important process where groundwater flows into the ocean along the coast. When the groundwater mixes with seawater, the microbes from both sources interact with each other, which can impact the diversity, activity, and amount of microbes in the coastal environment. Currently, little is known about how groundwater-borne microbes affect marine microbial populations. Our research shows that when groundwater microbes are removed before mixing groundwater with seawater, the abundance and activity of certain microbes that consume organic matter significantly increase. Additionally, we noticed a significant difference in the types of microbes present between the sites where SGD occurs versus background (uninfluenced) coastal water, especially in terms of the microbes that consume organic matter. Overall, this study suggests that there is a connection between groundwater and seawater microbes, which can influence the delicate balance between organisms that produce carbon and those that consume it. This has important implications for how carbon cycles globally.

1. Introduction

Submarine groundwater discharge (SGD) is a globally important process, involving the intrusion and interaction of density-driven circulated seawater with sediment, soils and rocks while mixing with groundwater until discharging at the seabed (Burnett et al., 2003). This process of water exchange occurs at the subsurface land–ocean interface, termed subterranean estuary, STE (Moore, 1999; Rocha et al., 2021). Various dynamic driving forces are known to impact the chemical solute composition of SGD flux into the coastal ocean (e.g., Robinson et al., 2018). Thus, SGD acts as a major pathway for delivering terrestrial solutes across the land-ocean interface, and STEs as important hotspots for biogeochemical reactions (e.g., Moore, 2010). This is especially true for ultra-oligotrophic coastal regions such as the southeastern Mediterranean Sea (SEMS), where the effects of the SGD are potentially most prominent (Rahav et al., 2020; Rodellas et al., 2015).

The terrestrial subsurface is one of the largest habitats for microorganisms on Earth (Bar-On et al., 2018; Griebler & Lueders, 2009; Magnabosco et al., 2018), where biogeochemical processes, including the turnover of carbon

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and other nutrients, mineral cycling or pollutant degradation are primarily driven by diverse microbial populations (Probst et al., 2018). Recently, in situ dark inorganic carbon fixation rates in a carbonate aquifer were found similar to those in oligotrophic marine systems, indicating chemolithoautotrophs, play an important role in shallow groundwater aquifers (Overholt et al., 2022). Moreover, microbiological and geochemical data revealed considerable amounts of energy catalyzed through chemolithotrophic reactions, where sulfide oxidation coupled to methanogenesis, iron and ammonium oxidation were highly exergonic in both the shallow and deep subsurface (Jewell et al., 2016; Momper et al., 2017; Osburn et al., 2014). Contrary to the relatively stable conditions maintained in inland aquifers, coastal aquifers entail rapid fluctuations and steep physiochemical gradients to which STE microbes need to quickly adapt. This results in a highly diverse microbial community composition along the hydrological continuum of SGD sites, reflecting a mixture of seawater- and groundwater-associated taxa (Adyasari et al., 2019; Chen et al., 2020; Degenhardt et al., 2020), as well as microbial functional groups that follow redox gradients (McAllister et al., 2015; Purkamo et al., 2022). Therefore, diverse prokaryotic groups in coastal ecosystems potentially affect bacterioplankton communities upon discharge into the marine environment (Lee et al., 2017; Ruiz-González et al., 2021).

Bacterioplankton community variation across estuarine gradients were shown to be driven primarily by salinity (Fortunato & Crump, 2011; Herlemann et al., 2011). Adyasari et al. (2020) found that salinity was the most decisive variable that shaped the microbial community composition across surface water samples, while dissolved nitrogen and phosphorus were the major predictor of community shift within subsurface water samples. At a different study (Adyasari et al., 2019), brackish porewater samples were more similar to freshwater samples than to saline samples, comprising mainly taxonomic groups associated with nitrogen transformation (nitrification and denitrification) in natural water systems. Given that nitrogen cycling is a key pathway in the subterranean estuary (Erler et al., 2014; Hays & Ullman, 2007; Slomp & Van Cappellen, 2004), these findings indicate the possibility of active biological transformation of dissolved nitrogen along the land-water interface.

SGD's nutrients composition often alleviate co-limitation of N and Si in marine environments (Santos et al., 2021), stimulating the growth of bloom-forming phytoplankton (Garcés et al., 2011; Lecher et al., 2015, 2017). However, supplement of groundwater to seawater is not always straightforward and behaves in a dose-depended response. For instance, Lecher et al. (2015) observed through incubation experiments that 10% groundwater addition rather than 20%–50% (volumetric) yields the highest positive effect on chlorophyll A concentrations and phytoplankton abundance, despite the fewer nutrients introduced. Contrary, lower addition volume ratios (1%–10%) did respond at a dose-dependent manner (Lecher et al., 2015). The authors concluded that these responses were attributed to the elevated nutrients supplied by the SGD, but this was achieved only to a certain level. This implies a biological effect of SGD through the introduction of subsurface bacterial cells into the coastal environment, which could potentially affect the native coastal microbiome. However, investigations mainly focused on health implications of the marine environment due to the transport of pathogenic or fecal indicator bacteria through coastal aquifers or sediments (Adyasari et al., 2019), neglecting the ecological implications. Currently, the role of coastal aquifers as sources of microbial diversity and the responses of marine microbial communities to groundwater-derived microorganisms is still unknown.

The occurrence of specific heterotrophic bacterial groups associated with cyanobacteria is dynamic and highly influenced by biotic and chemical factors. For example, Garcés et al. (2011) found in the Mediterranean Sea that the addition of groundwater to marine communities resulted in *Synechococcus* fast growth, due to the nutrient addition. But, this observation was not consistent in other coastal settings, where other phytoplankton groups dominated (Chamberlain et al., 2014). Synergistic interactions between *Synechococcus* and alphaproteobacterial heterotrophs (*Rhodobacteraceae*), gammaproteobacteria (*Alteromonadaceae*) (Dang & Lovell, 2016; Wang et al., 2021; Zheng et al., 2018), and the phylum Bacteroidetes are fundamental in the marine food web (Bae et al., 2022; Buchan et al., 2014). Given the delicate autotrophic-heterotrophic metabolic balance in coastal marine ecosystems, SGD is an important factor that should be accounted for with respect to its chemical and biological effects on the ocean.

We recently compared a site strongly influenced by SGD (Achziv, northern Israel) and a nearby nutrient-poor reference site (Shikmona) at the oligotrophic Israeli shallow rocky coast (Rahav et al., 2020). At Achziv, we showed that SGD contributes high concentrations of dissolved nitrate and silica in comparison to the Shikmona coastal seawater, resulting in elevated in situ phytoplankton biomass and primary productivity. The main objective of this study is to further elucidate marine heterotrophic taxonomic and functional responses to SGD by

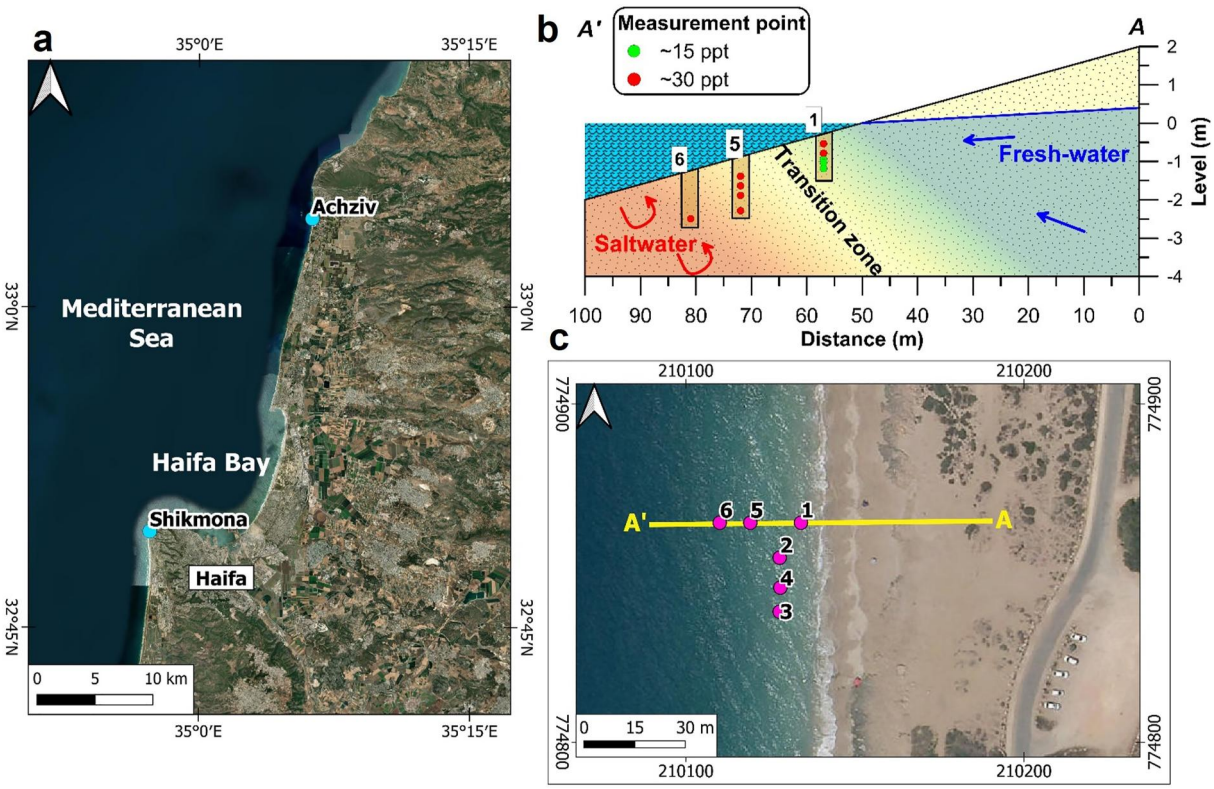

Figure 1. (a) Map showing the location of the two sampling areas along the Israeli coastline in the SE Mediterranean Sea: Achziv, the SGD-site (33°3′52N, 35°6′14.94E) and Shikmona (32°49′34N, 34°57′20E) within the Haifa Bay (sampled as the reference non-SGD site during the incubation experiments). (b) Schematic representation of the field site in Achziv showing the location of the piezometers (brown horizontal rectangle with numbers indicating piezometer ID). Piezometer samples are referred to in the text as either "Low or High salinity porewater" samples (n = 12 and 17, respectively) and are distinguished from the surface seawater samples (n = 3). Accordingly, red dots indicate high salinity (~30 ppt) porewater and green dots indicate low salinity porewater samples (~15 ppt). (c) Aerial photography with the piezometers setup for Achziv sampling campaigns. The numbers indicate piezometer ID along a perpendicular (1, 5–6) and parallel (2–4) to the shoreline cross sections to collect a wide range of samples around the discharge area.

addressing the following questions: i) Do coastal microbial communities respond to the nutrient-rich source of SGD in a dose-dependent manner (i.e., higher SGD-enriched mixtures correspond linearly with higher microbial activity and abundance)? ii) How do discharged groundwater microbes shape the activity and composition of the coastal microbiota? To explore these questions, three complementary incubation experiments were designed to illustrate the taxonomic and physiological response upon introducing groundwater and coastal communities. Additionally, porewater samples during three field campaigns were collected to follow composition, abundance and activity of the natural microbial communities at the Achziv SGD site. We show that groundwater prokaryotes transported through SGD influence coastal microbial activity and diversity, resulting in different heterotrophic composition patterns. We were able to mimic the SGD-site microbial community pattern in bottle incubation experiments by filtering the groundwater prior to mixing with coastal seawater. This points to potential competitive interactions for available nutrients in oligotrophic marine environments.

2. Materials and Methods

2.1. Field Site, Sampling Procedures, and Sample Analyses

2.1.1. Field Site

Groundwater and seawater samples were collected from the shore sediments and surface seawater at Achziv (Lat. 33°3′52N, Lon. 35°6′14.94E), located along the Northern Israeli Mediterranean shoreline (Figure 1). Achziv coastal aquifer structure is comprised of Pleistocene calcareous sandstone, beach rocks, and carbonate sand, providing a complex groundwater flow system. About five km east of the coastline, the carbonate Galilee Mountains (Judea Group) are elevated. The Quaternary permeable filling of the coastal aquifer is recharged

Table 1
Summary of the Three-Bottle Incubation Experimental Designs

	Experiment aim	Groundwater source	Controls	Treatments			
			No addition	% Of groundwater in seawater			
1	Investigate dose-dependent responses of seawater microbial community to groundwater additions	Brackish groundwater from Achziv; salinity = 7.9 ppt	Ambient seawater (Shikmona)	1%	5%	10%	20%
2	Compare the responses of seawater microbial community to ambient groundwater (as a source of both nutrients and microorganisms) and filtered groundwater (as a supply of dissolved inorganic nutrients only)	Inland fresh groundwater; salinity = 0 ppt	Ambient seawater (Shikmona) / Groundwater	Non-filtered 5%	5% filtered 0.1 μm		Non-filtered 20%
3	Investigate the responses of seawater microbial community to sequentially filtered groundwater as a source of nutrients with or without bacteria.	Inland fresh groundwater; salinity = 0 ppt	Ambient seawater (Shikmona)	Non-filtered 5%	5% filtered 0.1 μm	5% filtered 0.22 μm	5% Double distilled water (DDW)[a]

[a]The double distilled water (DDW) treatment was added to test the effect of mixing ambient seawater with distilled water that does not contain nutrients or microbes.

mainly from the east, by precipitation on these carbonate mountains (Kafri & Kessler, 2001; Paldor et al., 2020). Two fresh SGD components have been shown in previous studies: one shallow along the shoreline (Weinstein et al., 2006) and another deeper on the continental shelf, via preferential flow paths from the Judea Group aquifer (Paldor et al., 2019, 2020). The exact SGD sampling locations were selected based on the density and electric conductivity to identify the fresh groundwater and fresh-saline transition zone and to track changes in the salinity distribution compared to previous seasons.

2.1.2. Sample Collection

Three sampling campaigns (August 2020, February 2021 and July 2021) were conducted at a field site, highly influenced by SGD (Achziv, northern Israel), which we recently compared to a reference site (Shikmona) at the oligotrophic Israeli shallow rocky coast (Rahav et al., 2020). Each field campaign lasted 2–5 days and covered at least 2 tidal cycles. Physical parameters were measured, and porewater samples were collected on the shoreline using piezometers (AMS piezometers that reach depths of <2 m) and a portable peristaltic pump (Masterflex®, Cole-Parmer, Germany). The density (g cm^{-3}), electric conductivity (mS/cm), temperature (°C) and pH, of surface seawater, porewater and groundwater were measured on-site at the time of the sampling. Based on average temperature and water density measurements, salinity (parts per thousand, ppt) was converted.

Porewater samples were collected from the piezometers directly into a closed filter holder, keeping the filter unexposed during the entire sampling procedure. For microbial community analysis, samples were immediately filtered through polycarbonate 0.2 μm pore size filters (Merck, Israel), which were kept on ice and transported to the laboratory on the same day. Filter samples were stored frozen (−20°C) until DNA extraction (filtered porewater were kept for dissolved nutrient measurements, as described below). For Pico-/nano-phytoplankton and heterotrophic prokaryotic abundance, non-filtered samples were chilled on ice, transported to the laboratory on the same day, fixed with glutaraldehyde (final concentration 0.02% v:v, Sigma-Aldrich G7651), frozen in liquid nitrogen, and later stored at −80°C until analysis (See 2.3 for further analysis details).

2.2. Bottle Incubation Experiments

Three incubation experiments were conducted to determine the influence of SGD on the coastal microbial community. The experiments were conducted with five different treatments (including ambient seawater not exposed to SGD) in triplicates as described in Table 1. The reference site (Shikmona) was considered as the control ambient coastal seawater (salinity ~39.5 ppt), and was collected at the Israel Oceanographic and Limnological Research Institute (IOLR) into acid-cleaned carboys. For the first experiment (hereafter Exp. 1), discharged brackish groundwater (salinity = 7.9 ppt) was collected into acid-cleaned containers on the day the experiment initiated near Achziv Nature Reserve (33° 3′52N, 35° 6′14.94E). At this sampling site, a significant

groundwater discharge was reported (Rahav et al., 2020; Weinstein et al., 2006). For experiments 2 and 3 (hereafter Exp. 2 and 3), fresh groundwater (FGW, salinity = 0 ppt) was collected from drilling wells and pumped into 20 L acid-cleaned sample-rinsed carboys the same day the experiment was initiated. At the laboratory, fresh groundwater was either filtered through a 0.1 μm polycarbonate filter (Exp. 2) or serially filtered through 0.22 and then 0.1 μm polycarbonate filter (Exp. 3). The filtrates were added to ambient seawater at different mixing scenarios as described in Table 1. Each mixture was randomly distributed into acid-washed transparent polycarbonate Nalgene incubation bottles (4.5 L for Exp. 1 and 2; 250 ml bottles for Exp. 3) and incubated in ambient temperature and light.

The first experiment (Exp. 1) was designed to test the impact of discharged brackish groundwater on the microbial productivity and abundance in the coastal seawater. This was achieved by mixing discharged groundwater in the following fractions: 1%, 5%, 10% and 20% v:v with ambient coastal seawater. For each treatment, the dilution factor was calculated to account for the volume of discharged groundwater added to ambient seawater. Essentially, normalizing the data to the relative amount of seawater allows us to test the effect on the seawater population by removing the effect of dilution. Since the fresh groundwater contain an order of magnitude less cells compared with seawater, non-normalized data would highlight mainly the dilution effect. Temporal measurements indicated that a ∼40 hr incubation period yielded maximal production rates, while longer times resulted in saturation of heterotrophic and primary production rates (Figure S2 in Supporting Information S1). Thus, results of Exp. 1 are presented for the 42 hr incubation time point. The second and third experiments (Exp. 2; Exp.3) were designed to extend Exp. 1 and aimed to specifically investigate how groundwater-derived microorganisms affect the activity and abundance of marine organisms once discharged into the sea. For these experiments, we used several filtration fractions (non-filtered and 0.1 μm for Exp. 2; non-filtered, 0.22 and 0.1 μm for Exp.3, Table 1) of fresh groundwater that were added as a treatment of 5% addition. Non-filtered treatment tested the addition of both nutrients and bacteria from groundwater origin to the seawater microbial community. The filtrate of 0.22 μm, tested the addition of nutrients and very small cells that could have been miniaturized due to carbon scarcity and pass through 0.22 μm filter (Ruiz-González et al., 2021). The filtrate of 0.1 μm (representing "sterile" groundwater) tested the effect of nutrients only on the coastal ambient seawater microbial community. A treatment of 5% double distilled water (DDW) mixed with ambient sweater was added in Exp. 3 to test the effect of seawater dilution, neglecting nutrient and microbial supplements (Table 1). Temporal measurements during Exp. 2 indicated that a ∼67 hr incubation period yielded maximal production rates (Figure S3 in Supporting Information S1). Thus, the results of Exp. 2 are presented for the 67 hr incubation time point.

The duration of the experiments was 3–5 days, and samples were taken for the following analyses: chlorophyll A (Exp. 1 and 2, every 24 hr), dissolved nutrient concentrations (Exp. 2 and 3 initial and final time points), flow cytometry (bacterial and phytoplankton abundance, every 24 hr), primary and heterotrophic production rates (Exp. 1 and 2, every 24 hr; Exp. 3 initial and final time points), methods are specified below. In order to remove the dilution impact and test the dose response only, all results were normalized to the dilution factor (e.g. in the 20% treatment group, all measurement values were multiplied by 1.2). All bottles were incubated in a flow-through tank at the Israel Oceanographic and Limnological Research (IOLR) through which ambient Eastern Mediterranean water was continuously pumped to maintain surface seawater temperature. The incubation tanks were covered with an illumination net to maintain natural light (representing a full-day cycle).

2.3. Pico-/nano-phytoplankton and Heterotrophic Prokaryotic Abundance

Samples (1.8 mL) were fixed with glutaraldehyde (final concentration 0.02% v:v, Sigma-Aldrich G7651), frozen in liquid nitrogen, and later stored at −80°C until analysis. The abundance of autotrophic pico- and nano-eukaryotes, *Synechococcus* and *Prochlorococcus* was determined using an Attune® Acoustic Focusing Flow Cytometer (Applied Biosystems) equipped with a syringe based fluidic system and 488 and 405 nm lasers. To measure bacterial and archeal abundance (termed hereafter heterotrophic prokaryotic abundance), a sample aliquot was stained with SYBR Green (Applied Biosystems) and determined using the same flow cytometer. We used the term heterotrophic prokaryotic abundance to simplify and differentiate between primary producing organisms (autotrophic pico- and nano-eukaryotes, *Synechococcus* and *Prochlorococcus*) to heterotrophic organisms that do not contain a nucleus.

2.4. Heterotrophic Productivity

Prokaryotic (bacteria and archaea) heterotrophic production was estimated using the ^{3}H-leucine incorporation method (Perkin Elmer, specific activity 100 Ci mmol^{-1}). Water samples (1.7 mL in triplicate) were incubated in the dark with ∼100 nmol leucine L^{-1} for 4 hr (Rahav et al., 2019). For blanks, additional samples were immediately added with 100 µL of 100% trichloroacetic acid (TCA, 4°C) along with ^{3}H-leucine, and were processed as the other samples. The incubations were terminated with TCA and were later processed following the micro-centrifugation technique (Smith & Azam, 1992) and added with 1 mL of scintillation cocktail (Ultima-Gold). The samples were counted using a TRI-CARB 2100 TR (Packard) liquid scintillation counter.

2.5. Primary Productivity

Photosynthetic carbon fixation rates were estimated using the ^{14}C incorporation method (Nielsen, 1952). Briefly, water samples (50 mL in triplicate) were immediately spiked with 5 µCi of $NaH^{14}CO_3$ (Perkin Elmer, specific activity 56 mCi mmol^{-1}). The samples were incubated for 24 hr under in situ natural illumination. The incubations were terminated by filtering the spiked seawater through GF/F filters (Whatman, 0.7 µm pore size) at low pressure (∼50 mmHg). The filters were placed overnight in 5 mL scintillation vials containing 50 µL of 32% hydrochloric acid to remove excess ^{14}C, after which 5 mL of scintillation cocktail (Ultima-Gold) was added. Additional 50 ml sample was incubated in the dark and processed the same. The dark incorporation read was subtracted from the light sample reads. Radioactivity was measured using a TRI-CARB 2100TR (Packard) liquid scintillation counter.

2.6. Inorganic Nutrients Determination

Samples from the field were immediately filtered through 0.2 µm pore size filters into acid-washed plastic vials, chilled on ice and transported to the laboratory on the same day. Samples were stored frozen until analysis. Experimental samples at initial and final time points were filtered through 0.2 µm pore size filters into acid-washed plastic scintillation vials and immediately frozen (−20°C) until analysis. Dissolved nutrients ($NO_2 + NO_3 = NOx$, PO_4, and $Si(OH)_4$) were analyzed in the Interuniversity Institute for Marine Sciences (IUI, Eilat) using QuikChem 8000 flow injection analyzer (Lachat Instruments, Milwaukee, USA). The measurement is based on a color reaction created by each of the nutrients with its unique reagent to create a color complex with a wavelength in the visible light range, which is absorbed by the device's spectrophotometer.

2.7. DNA Extraction, Amplicon Sequencing, and Analysis of Bacterial Community

Seawater or groundwater (4–5 L) were filtered using a peristaltic pump onto polycarbonate membrane filters (0.22 µm, 47 mm, Merck, Israel) and stored at −20°C until extraction. After thawing, each filter was cut into small pieces using a sterile scalpel blade, which were placed immediately into PowerSoil DNA bead tubes and extracted with the dNeasy PowerSoil Kit (Qiagen, USA) following the standard protocol. To disrupt the cells, the FastPrep-24™ Classic (MP Biomedicals, USA) bead-beating was used (2 cycles at 5.5 m/s, with a 5 min interval).

To generate 16S rRNA gene libraries, the V3–V4 hypervariable region of the 16S gene was amplified (∼465 bp) using the 341F/806R primers containing CS1 and CS2 linkers: 5′-ACACTGACGACATGGTTCTACA**CCTACGGGNGGCWGCAG**-3′ and 5′-TACGGTAGCAGAGACTTGGTCT**GGACTACHVGGGTWTCTAAT**-3′, where the underlined sequences are common adapter sequences CS1 and CS2, and bold sequences denote the universal 16S rRNA primers 341F and 806R (Naqib et al., 2018). PCR conditions were as follows: initial denaturation at 95°C for 5 min, 28 cycles of denaturation (95°C for 30 s), annealing (50°C for 30 s) and extension (72°C for 60 s). Sequences were obtained on the Illumina MiSeq platform in a 2 × 250-bp paired-end run (HyLabs Israel). The two 250-bp paired-end sequences were merged to obtain a single read (approximately 416.27 bp, mean length). Quality-filtered reads were imported into QIIME 2 platform (v. 2020.2) (Bolyen et al., 2019), denoised, dereplicated, clustered and trimmed using the DADA2 plugin (Callahan et al., 2016). Sequences were filtered with DADA2 by trimming 17 nucleotides on the left-forward primer and 20 nucleotides on the left-reverse primer. Truncating forward and reverse reads were obtained to a length of 249 bases. Taxonomic assignment of the ASVs was achieved with the q2_feature_classifier (Bokulich et al., 2018), against the full Silva database (release 138-99) for 16S rRNA gene sequencing (Quast et al., 2012). A total of 1,163,791 sequences from 53 samples corresponding to 1632 (with ≥2 counts) unique ASVs were recovered.

Table 2
Summary of Environmental Parameters Averaged by Sample Groups

Sample source	Group	# Samples (n)	Salinity (ppt)	pH	N:P	Si (μmol L^{-1})
Environmental **Achziv study site.** Samples from 3 sampling campaigns.	Low (porewater)	12	14.8 ***a***	7.3 ± 0.2 ***a***	268.4 ± 84.1 ***a***	201.3 ± 77.9 ***a***
	High (porewater)	17	29.9 ***b***	7.5 ± 0.1 ***b***	266.9 ± 181.4 ***a***	110.4 ± 66.3 ***b***
	Achziv surface seawater	3	37.7 ***c***	7.9 ± 0.2 ***c***	49.6 ± 38.7 ***a***	10.2 ± 1.2 ***c***
Fresh groundwater inland well	GW	4	0.0 ***d***	7.3 ± 0.2 ***a***	232.2 ± 292.1 ***a***	194.9 ± 133.9 ***a***
National monitoring, SEMS,2020-2021 (surface seawater)	Reference site (Shikmona)	24	39.16 ***c***	8.1 ± 0.04 ***c***	23.1 ± 27.3 *b*	1.5 ± 0.8 ***c***
	Achziv	24	38.97 ***c***	8.0 ± 0.03 ***c***	281.8 ± 223.2 ***a***	2.7 ± 1.1 ***c***

Note. Lowercase letters indicate significant differences between groups (using ANOVA followed by Tukey post-hoc tests, $p \leq 0.05$).

3. Data Analysis

Data filtration and normalization were applied using marker-gene data profiling in the MicrobiomeAnalyst platform (Xia Lab, McGill University, Quebec, Canada) with default parameters (Dhariwal et al., 2017). A low count filter was used to filter all features with <4 counts in at least 20% of values. Features with <10% variance, based on the inter-quartile rank, between experimental conditions were filtered using a low variance filter. For Alpha diversity, all samples were rarefied to even sequencing depth using the minimum library size (12,817 reads). Diversity indices included Shannon and Chao1. For data scaling, total sum scaling was applied. The TSS normalized ASV dataset was used to generate an ordination plot by non-metric multidimensional scaling (NMDS) based on Bray–Curtis dissimilarity, for assessing differences in microbial community composition (beta diversity). Permutational multivariate analysis of variance (PERMANOVA) was used to make pairwise comparisons between the different sample types (Field: Low and high-salinity porewater, Achziv surface seawater and experimental treatment groups: control ambient coastal seawater (SGD-non influenced), non-filtered, and 5% filtered 0.1 μm pore size) treatments. Differential abundance analysis and biomarker identification were performed using the DESeq2 and linear discriminant analysis effect size (LEfSe) methods. Both methods were applied using the MicrobiomeAnalyst platform with default parameters (Dhariwal et al., 2017) to determine the genomic features most likely to explain differences between groups of samples (biomarkers). Biomarkers (ASVs with significant effect size) were determined according to LDA score >3.0 (Table S2) and an adjusted p-value cutoff of alpha <0.05.

Statistical analysis was performed in GraphPad Prism 5.03 (GraphPad, La Jolla, CA, USA). One-way analysis of variance (ANOVA) was used to determine statistical differences between the control and different mixing treatments for the three-bottle incubation experiments, and the three field campaign sample types. Multiple pairwise comparisons were achieved using Tukey 95% confidence intervals.

4. Results and Discussion

4.1. Physicochemical Parameters and SGD Characteristics

Physicochemical parameters of water samples collected during the three sampling campaigns are listed in Table 2. Porewater samples were collected on the shoreline using piezometers at different depths (0.25–1 m, Figure 1). According to the recorded porewater salinity values, the porewater samples were divided into two groups: low (<20 ppt) and high salinity (~30 ppt). In addition, we sampled surface seawater. The measured parameters of these samples were compared to values obtained for both Achziv and the reference site (Shikmona) during the routine monitoring program by Israel Oceanographic and Limnological Research, IOLR (Table 2).

The measured parameters reported for Achziv surface seawater during the three field campaigns were comparable to the ongoing monitoring results obtained during the years 2020–2021 (salinity, pH, and $Si(OH)_4$, ANOVA, $p > 0.05$) with no significant seasonal or annual differences. The low-salinity porewater samples showed significantly lower salinity/pH values compared to high-salinity porewater and surface water (Table 2), further supporting the contribution of freshwater inputs (groundwater and low-salinity porewater had similar pH values, ANOVA and Tukey post-hoc tests, $p > 0.05$). The mean N:P ratio of Achziv surface seawater, high- and low-salinity porewater samples were comparable to values obtained during routine monitoring of the Achziv site.

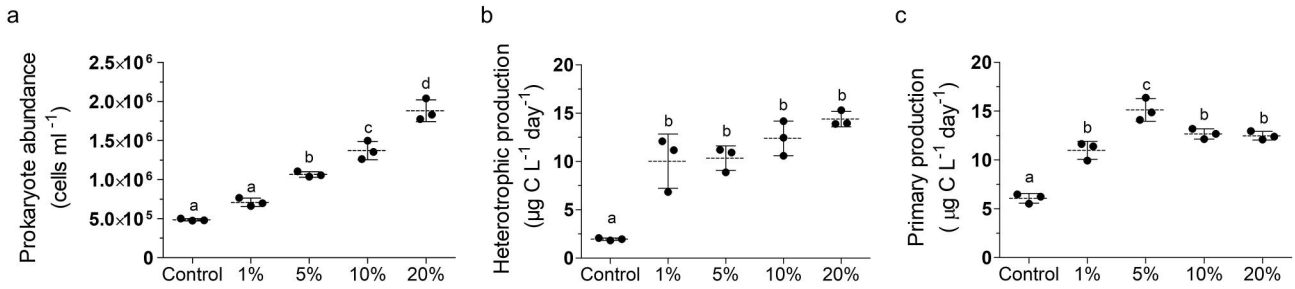

Figure 2. Scatter dot plots of surface seawater (control) prokaryote abundance (a), heterotrophic production rate (b) and primary production rate (c) following dilution with discharged brackish groundwater (1%–20% v:v) or un-amended seawater (control). Results are shown for the second time point of the experiment (42 hr) as described in the text. Lowercase letters indicate significant differences between treatments (using ANOVA followed by Tukey Post hoc tests, $p \leq 0.05$). The temporal variability of all time points is shown in Figure S2 in Supporting Information S1. The dilution factor was calculated for each treatment to account for the volume of discharged groundwater added to ambient seawater. Temporal variability of all time points without calculating the dilution factor is presented in Figure S1 in Supporting Information S1.

The N:P values obtained at all Achziv samples were significantly higher than in the Shikmona reference site, signifying the influence of SGD as an important source of N. Notably, $Si(OH)_4$, PO_4^{3-} and NO_3^- concentrations of porewater samples exceeded the surface seawater samples by an order of magnitude (Figure S5 in Supporting Information S1), and lie on a conservative mixing line between freshwater and seawater end members (Figure S6 in Supporting Information S1). Higher PO_4^{3-} concentrations were observed in all Achziv porewater samples compared to the reference site, ranging from 0.4 to 3.7 µmol L^{-1}, but generally peaked at the lower-salinity samples (3.1 ± 0.6 µmol L^{-1}). It is highly likely that the high N in the inland groundwater originated from fertilizers in the western Galilee basins due to the intensive agricultural use in this area (Furman & Abbo, 2013).

4.2. Groundwater Discharge Enhances Coastal Microbial Abundance But Constrains Activity

Three bottle incubation experiments were designed to independently study the microbial response of groundwater additions (brackish or fresh) to ambient coastal seawater. Specifically, the aim of the first experiment (hereafter, Exp. 1, Table 1) was to investigate the response of ambient coastal microorganisms (unexposed to SGD and corresponding microbes) to SGD by additions of discharged groundwater at pre-determined volumetric percentages (1%, 5%, 10%, 20%) relative to ambient seawater (Figure 2). The chosen volumetric range aimed to demonstrate naturally occurring conditions that could occur in a SGD site concerning point-source discharge flux. Results showed that heterotrophic prokaryotic abundance increased in a dose-dependent manner, ranging from $4.8 \times 10^5 \pm 0.1 \times 10^5$ cells ml^{-1} in the control to $1.9 \times 10^6 \pm 1.4 \times 10^5$ cells ml^{-1} in the 20% GW treatment (Figure 2a). Contrary, heterotrophic production rates reached a constant value in our tested mixing ratios already at 1% GW enrichment; 11.8 ± 2.4 µg C L^{-1} day^{-1}, while the control values were ~6 fold lower (Figure 2b). Primary production rates (Figure 2c) and chlorophyll A concentrations (Figure S2 in Supporting Information S1), reached a plateau at 5% treatment, in agreement with an earlier study from the intertidal area affected by SGD in Monterey Bay (Lecher et al., 2015).

These observations infer groundwater-microbes introduction to seawater populations potentially limits activity, but not abundance, despite the addition of nutrients by the groundwater discharge (Table 2).

4.3. Groundwater Microbes Transported Through SGD May Limit Coastal Production Rates

To assess the microbial contribution of SGD to heterotrophic production, a second experiment was designed (hereafter, Exp. 2, Table 1), in which filtered (<0.1 µm 5%) and non-filtered (5% and 20%) groundwater samples were mixed with the reference ambient coastal seawater (Shikmona). Natural coastal measurements from Achziv-site were also evaluated (Figure 3, Table 2). Compared to non-filtered groundwater and the control treatments, the filtered treatment showed the highest change for prokaryote abundance (Figure 3a) and heterotrophic production rates (Figure 3b). While prokaryote abundance of Achziv surface seawater was similar to the filtered treatment, heterotrophic production was significantly lower (similar to non-filtered treatments and control, ANOVA and Tukey Post hoc test, $p \leq 0.05$). Contrary, no changes in primary production rates were observed between the

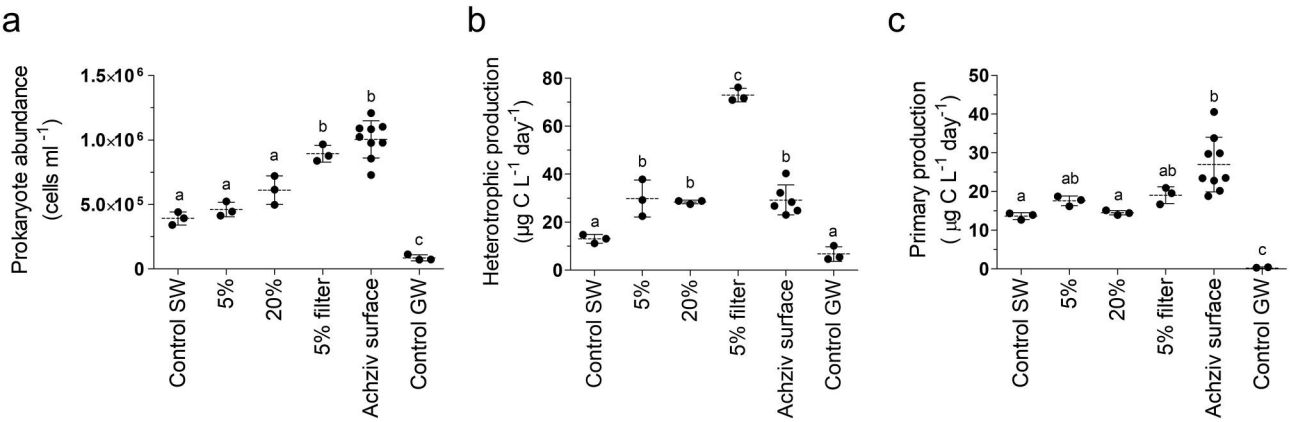

Figure 3. Scatter dot plots of surface seawater prokaryote abundance (a), heterotrophic production (b), and primary production (c) following dilution with fresh groundwater (5% and 5% filtered 0.1 µm v:v), un-amended seawater (control SW) or fresh groundwater (Control GW) during Experiment 2. Results are shown for the third time point of the experiment (67 hr) as described in the text. Surface seawater measurements from Achziv (SGD-site) were also added to this analysis. Lowercase letters indicate significant differences between treatments (using ANOVA followed by Tukey Post hoc tests, $p \leq 0.05$). The temporal variability of all time points is shown in the Figure S3 in Supporting Information S1. The dilution factor was calculated for each treatment to account for the volume of groundwater added to ambient seawater.

different groundwater amendment treatments (Figure 3c). The 5% filtered and non-filtered treatments were significantly higher than the control (ANOVA and Tukey Post hoc tests, $p \leq 0.05$), but slightly lower than Achziv surface seawater (ANOVA and Tukey Post hoc tests, $p > 0.05$). Moreover, the 20% non-filtered treatment had rates similar to the control. These findings are in agreement with the results of Exp. 1, where prokaryote abundance is dose dependent and activity reaches a stable threshold, supported by the natural seawater Achziv measurements (Figure 3).

Furthermore, changes in concentration of phosphate (PO_4^{3-}), silica ($Si(OH)_4$), nitrate (NO_3^-), and ammonium (NH_4^+) over the incubation experiment period indicated that mainly PO_4^{3-} and $Si(OH)_4$ were taken up by the microbial community provided by groundwater amendments (Figure S4 in Supporting Information S1). The 20% mixing ratio non-filtered treatment utilized significantly more of these nutrients than the other filtered and non-filtered treatments (Figure S4 in Supporting Information S1), as observed in a previous study (Lecher et al., 2015). Although prokaryotic productivity was higher in seawater amended with 5% filtered groundwater than with unfiltered groundwater, inorganic nitrogen did not change significantly between the different treatments during the experimental period (Figure S4 in Supporting Information S1). Inorganic nitrogen is not considered a limiting factor for heterotrophic prokaryotic activity in the eastern Mediterranean coast (e.g., Rahav et al., 2016, 2018), while orthophosphate is (e.g., Kress et al., 2005; Krom et al., 2010). Moreover, nitrate could be involved in other biogeochemical cycles that are highly affected by the microbial composition (e.g., nitrification/denitrification), compared with phosphate that is mostly assimilated by microbial cells. Therefore, it is not surprising that orthophosphate was consumed throughout the experiment, while the nitrogen levels remained overall unchanged.

The higher increase in prokaryote abundance or heterotrophic production in the filtered versus the non-filtered addition suggests that groundwater microbes affect the seawater communities, highlighting the intricate interactions between autotrophic and heterotrophic microbes in oligotrophic realms such as the Mediterranean coast.

The control fresh groundwater (collected from an inland aquifer), incubated without mixing, showed the lowest values of abundance and productivity (Figure 3). While lower phototrophic abundance and activity were negligible, as expected in subsurface environments, the lower prokaryotic abundance ($0.8 \times 10^5 \pm 0.2 \times 10^5$ cells ml^{-1}) and heterotrophic productivity (6.74 ± 3.0 µg C L^{-1} day^{-1}) are in agreement with the values reported for pristine inland fresh groundwater (Griebler & Lueders, 2009). These observations show that a large fraction of communities from inland aquifers are locally less active. Despite their lower numbers and activity rates, incubation experiments of groundwater communities showed that groundwater bacteria can respond rapidly to environmental changes as implied from our data, and grow even when mixed with marine waters. This could ultimately shape coastal microbial diversity and activity.

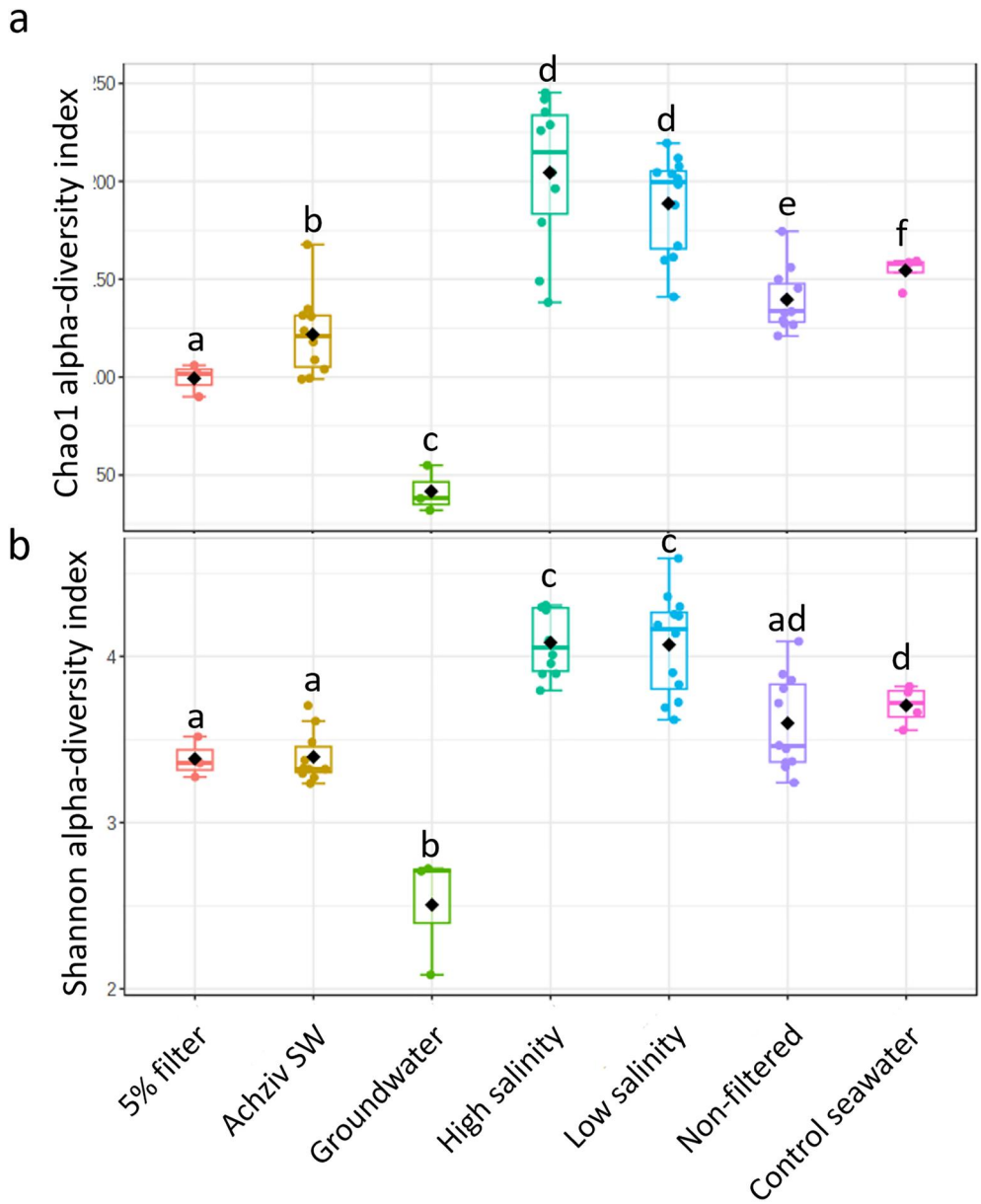

Figure 4. Box-Whisker plots showing the interquartile range (25th to 75th percentile), the median and mean values (horizontal line and black circle within the box, respectively) of the microbial communities' alpha diversity grouped according to field and experimental samples assessed by the Chao1 richness estimating the number of ASVs (a) and Shannon (b). Lowercase letters indicate significant differences between treatments (using ANOVA followed by post-hoc pairwise comparisons, $p \leq 0.05$).

4.4. Groundwater Microbes Transported Through SGD May Shape Coastal Community Composition

Sequencing of the 16S rRNA gene resulted in 1,163,791 quality sequences, which clustered into 1632 ASVs, including field campaigns and bottle incubation experiments. Microbial community diversity of samples collected in the Achziv field site (low-/high-salinity porewater and surface seawater) was compared with those obtained from the experimental treatments, namely control seawater (ambient seawater, not influenced by SGD), non-filtered treatments (1%, 5%, 20%), filtered (5% 0.1 μm pore size) and fresh groundwater from inland wells. Among these, the high and low-salinity porewater groups were the most diverse for both alpha diversity indices investigated (Figure 4), indicating high heterogeneity of the porewater microbial community, and a relatively even community inferred from the higher Shannon index (Figure 4b).

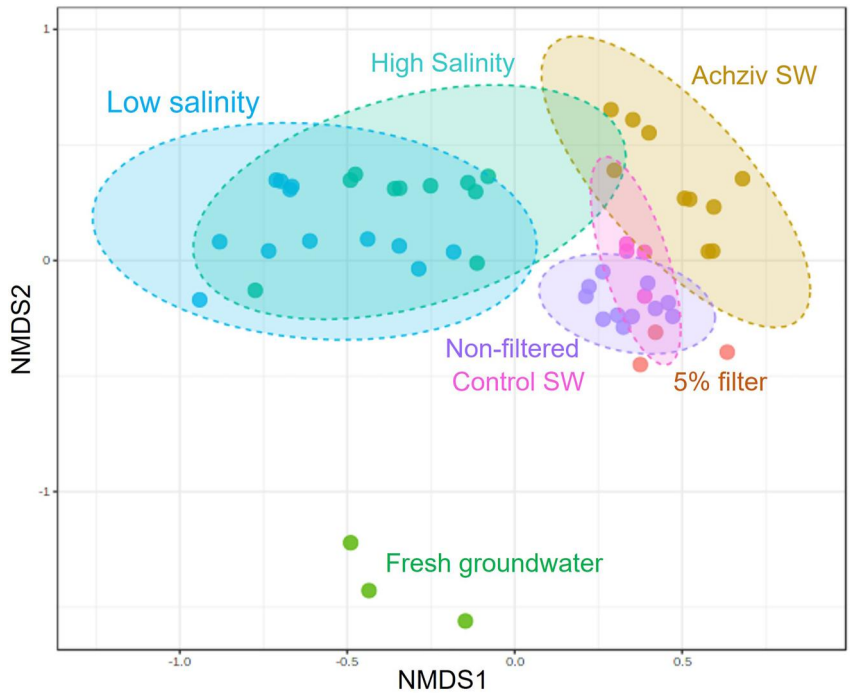

Figure 5. Overview of the prokaryotic community structure. Non-metric multidimensional scaling (NMDS) analysis, based on Bray–Curtis dissimilarities of samples collected during three sampling campaigns at Achziv site, grouped according to porewater salinity (Low/High) and Surface seawater (Achziv SW). Additionally, bottle incubation experimental treatment samples (control S, non-filtered, 5% filtered and fresh groundwater) are plotted. Stress = 0.12.

Non-metric multidimensional scaling (NMDS) analyses performed on the entire microbial community data (based on the Bray-Curtis distance) showed that samples separated into groups according to field groups and experimental treatments (Figure 5; PERMANOVA $p < 0.01$), where negligible dispersal appeared between sampling days (sampling days are not indicated here). This implies that the microbial community in Achziv porewater is relatively stable annually and diversity is primarily influenced by water source (fresh groundwater, porewater and surface seawater). As shown in Figure 5, the fresh groundwater communities significantly differed from all other sample types (along NMDS axis 2; Table S2 in Supporting Information S1). Communities from the different locations did not visually cluster in the NMDS analysis, but were statistically different (Table S2 in Supporting Information S1).

For one of the field sampling campaign, we measured surface seawater and porewater microbial activity (primary and heterotrophic production) and prokaryotic abundance. We observed a substantial decrease in all parameters between the conservative mixing line and seawater (Figure S7 in Supporting Information S1). These findings illustrate the change in the environment between open seawater and groundwater, which both prevents day light (may limit productivity) and introduces new communities.

Achziv bacterial community composition (surface seawater) was co-dominated by four marine family-level taxonomic groups (relative abundance ~55%), including the autotrophic *Synechococcus* and heterotrophs (Table S1, Figures S8, S9 in Supporting Information S1). The three heterotrophic bacterial families were *Rhodobacteraceae*, *Flavobacteriaceae*, *Actinomarinaceae* and SAR11. While *Actinomarinaceae* was the most abundant bacterial plankton in Achziv surface seawater samples and the experimental control seawater (ambient seawater, not influenced by SGD), the SAR11 clade was primarily abundant in the control seawater and non-filtered experimental treatments, Table S1 in Supporting Information S1. On the other hand, the lowest relative abundance of *Rhodobacteraceae* was detected in experimental control seawater, which was comparable with fresh groundwater samples. Thus, from ambient coastal seawater to SGD-coastal samples (Achziv), the community composition shifted from a SAR11-dominated to a *Rhodobacteraceae*-dominated community. Notably, the relative abundance of *Flavobacteriaceae*, *Alteromonadaceae* and *Rhodobacteraceae* were the highest in the filtered treatment (Figure 6). Previous studies have linked the presence of these marine microbial taxa in coastal

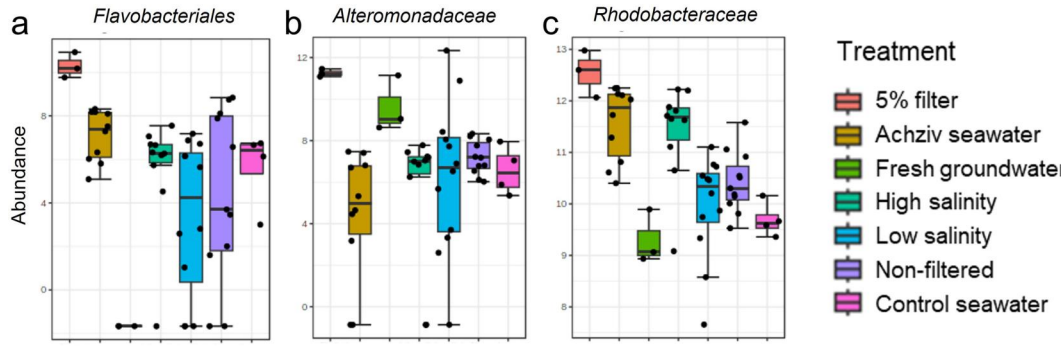

Figure 6. Normalized abundances of three families identified by DESeq2. Boxplots represent normalized count abundances of individual families in each group. Analysis was performed using marker-gene data profiling in MicrobiomeAnalyst (Xia Lab, McGill University, Quebec, Canada).

aquifers as potential indicators of seawater intrusion, indicating an active land-ocean interface (Chen et al., 2019; Unno et al., 2015).

Based on the SAR11 to *Rhodobacteraceae* shift and the substantially higher relative abundance of *Rhodobacteraceae* in the filtered treatment, we suggest that applying the filtration resulted in a unique scenario, where the typical-oligotrophic coastal microbial community, growing under constant nutrient-limiting conditions was exposed to a nutrient-rich solution, almost without potential competitors.

Our results suggest that mixing non-filtered groundwater with ambient coastal seawater induced competitive interactions between the two different communities, eventually affecting primary and heterotrophic production rates. This competitive effect was essentially eliminated through filtration because most microbes were removed from the water, relieving the nutrient limitations of the oligotrophic coastal seawater. Although the transport of microbial diversity into the coastal ocean through SGD has been addressed mostly with regard to pathogenic or fecal indicator bacteria (Vollberg et al., 2019; Yau et al., 2014), our results emphasize that marine heterotrophic bacterioplankton respond taxonomically and functionally to SGD-derived microbes.

Typically, low-salinity porewater and fresh groundwater samples were similar to each other in terms of low relative abundance of the marine bacteria (*Rhodobacteraceae*, *Flavobacteriaceae*) found in the other samples (Figures 6a and 6c). Moreover, the higher alpha diversity values in the porewater samples (Figure 4) are explained by a much more heterogeneous microbial community, which consisted of a diverse mixture of freshwater and marine microbes. These include the prevalent archeal *Woesearchaeotal* lineage, widely distributed in inland anoxic environments (Liu et al., 2018). *Woesearchaeotal* was found in the fresh groundwater samples (0.3%) and in very low abundances in Achziv and control surface seawater (Figure S9 in Supporting Information S1). Interestingly, this archeon was most abundant in low- and high-salinity porewater samples (2.73% and 1.13%, respectively) Overall, our results show that salinity was a major factor controlling community composition throughout the sample groups, where some ASVs occupied only a portion of the salinity spectrum (e.g., SAR11 was abundant in saline samples), while others (*Flavobacteriaceae*, *Alteromonadaceae* and *Rhodobacteraceae*) were mostly abundant in the mixed treatments, and *Woesearchaeotal*, *Sphingomonadaceae* were associated with fresh-brackish. This hints at the potential role of connectivity between terrestrial and marine hydrospheres driven by SGD in the Achziv site, as demonstrated previously (Herlemann et al., 2011).

Synechococcus was the dominant cyanobacterium detected in all surface seawater and high-salinity porewater samples (Table S1 in Supporting Information S1). The lowest abundance was associated with the low-salinity porewater and groundwater samples, probably due to the different environmental conditions, primarily the lack of light in the aquifer.

Given previous observations that prokaryotes can miniaturize in response to stress or starvation conditions in subsurface ecosystems (Hahn et al., 2003; Hood & MacDonell, 1987; Luef et al., 2015; MacDonell & Hood, 1982; Nakai, 2020; Velimirov, 2001), the third experiment (hereafter Exp. 3, Table 1) was designed to extend on the previous two experiments and further examine the response of coastal communities to different microbial cell size fractions by mixing sequentially pre-filtered groundwater (non-filtered; 0.22-μm pore size

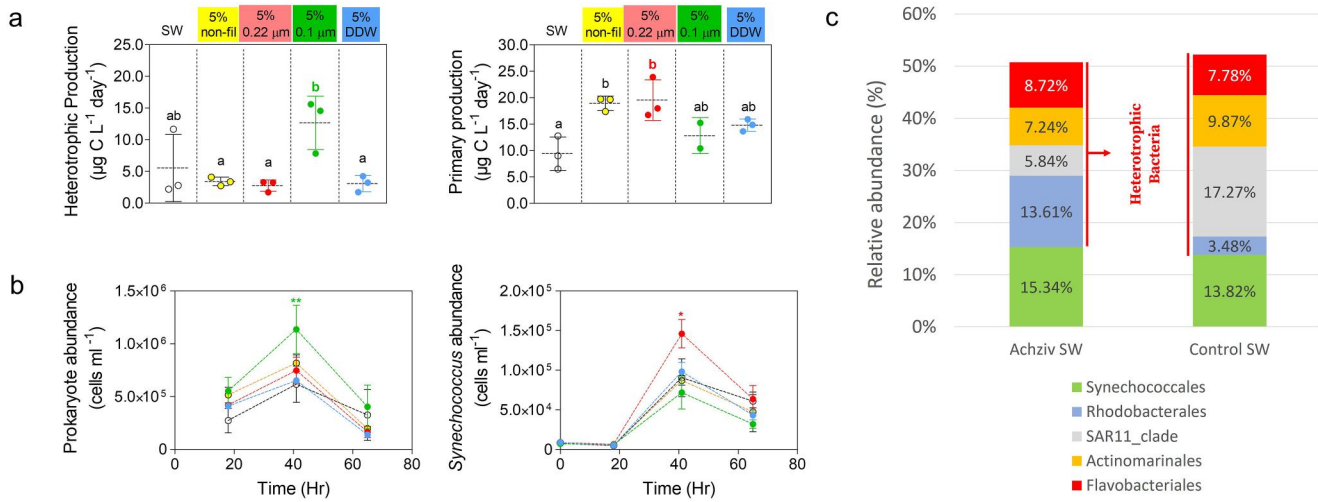

Figure 7. (a) Scatter dot plots of heterotrophic and primary production rates following dilution with fresh groundwater (5%, 5% filtered 0.22 µm and 5% filtered 0.1 µm v: v), un-amended seawater (SW) or diluted with 5% distilled water (5% DDW) during Experiment 3. Results are shown for the final time point of the experiment (only initial and final measurements were conducted for this experiment). Lowercase letters indicate significant differences between treatments (using ANOVA followed by Tukey Post hoc tests, $p \leq 0.05$). (b) Temporal variability of prokaryote and *Synechococcus* abundance detected (data derived throughflow cytometry); */** indicate significant difference between two treatments ($p < 0.05$ or $p < 0.01$, respectively), where the rest are non-significant. (c) Bar plot showing relative sequence abundance of dominant microbial taxa at order level for both coastal sites.

resulting in <0.22 bacteria µm; and 0.1-µm pore size filters resulting in "sterile" groundwater) with the control ambient seawater, Figure 7. A similar experimental setup reported that a 94 hr incubation period of 0.2-µm pre-filtered groundwater from a coastal Mediterranean aquifer resulted in increased abundance, cell size and heterotrophic production of the bacteria passing through the 0.2-µm filter, indicating that some cells had been miniaturized in situ due to carbon scarcity (Ruiz-González et al., 2021). Also here, heterotrophic production and prokaryote abundance increased when 0.1 µm filtered groundwater was mixed with the control seawater (as observed in Exp. 2, Figure 3). However, the 0.22 µm filtration treatment did not exert such a response on prokaryote abundance and heterotrophic production, rather induced primary productivity rates, specifically associated with *Synechococcus* abundance, after a 40 hr incubation (Figures 7a and 7b). Cell abundances dropped for all treatments after a 65 hr incubation period, but even the delayed production rates measured only for the last time period (65 hr) were still showing a significant response, which supported the abundance trend observed after a 40 hr incubation period. The DDW treatment (Table 1) was included to demonstrate that groundwater mixing effects occur as a result of terrestrial-coastal microbial interactions, and not due to simple volumetric dilution of the seawater with fresh water.

4.5. SGD Affects the Interplay Between *Synechococcus* and Heterotrophic Bacteria

In our two investigated sites (SGD and control), *Synechococcus* was the dominant cyanobacterium based on 16S rRNA gene data. Although *Synechococcus* relative abundance was similar for both sites, we observed marked differences in the composition of heterotrophic bacteria, specifically SAR11 and *Rhodobacteraceae* groups (Figure 7c). The strict oligotrophic SAR11 clade are highly abundant in the open sea, and were found to associate with Cyanobacteria primarily under poor dissolved organic carbon and nutrient conditions (Kearney et al., 2021). Alternatively, *Rhodobacteraceae* is much more metabolically flexible (Xia et al., 2021), allowing this group to outcompete and dominate other heterotrophs upon groundwater discharge.

Therefore, heterotrophic bacterioplankton largely determine carbon biogeochemical cycling in the ocean through respiration and biomass production, and their composition appears to be affected by SGD (Kaile'a & Wiegner, 2016). These results suggest that *Synechococcus* abundance can increase similarly in the two sites (SGD-enriched and oligotrophic), but primary production activity was constant. Currently, the mechanistic role SGD plays in maintaining ecosystem stability remains unknown, and how the groundwater microbiota drives this effect, particularly concerning different microbial cell size fractions.

Our study suggests that this relationship may be more complex through changes in SGD fluxes due to sea level changes. An increase in sea level would reduce SGD fluxes (Kiro et al., 2008), and thus affect the heterotrophic community in seawater, as concluded in this study. This may have direct implications on the delicate balance between heterotrophs and autotrophs, and the biogenic carbon fluxes.

5. Conclusions

SGD is an important source of nutrients in coastal environments, especially in oligotrophic environments. In this study we shed light on the complex contribution of SGD on the ecology of coastal microbial communities through the transport of groundwater-borne prokaryotes. Diverse microbial communities, naturally inhabiting the subterranean estuary, shape coastal microbial abundance, taxonomic diversity and activity. This ultimately affects photoautotrophic-heterotrophic specific interactions, marine food webs and biogeochemical cycles of the entire coastal ecological system. In our current changing climate world, these interactions are crucial for understanding the possible effects and feedback mechanisms on carbon cycling. Further research is needed to integrate the global chemical-biological effect of SGD on marine environments, focusing, for example, on STE sediment microbial dynamics along redox gradients at the terrestrial–marine interface.

Data Availability Statement

Environmental, experimental datasets, and the ASV taxonomic table were deposited in an open-access data archiving and publication repository (Pangaea, a member of the ICSU World Data System) and are available at: Yanuka-Golub et al. (2024): Groundwater microorganisms affect coastal seawater microbial abundance, activity and diversity. PANGAEA, https://doi.org/10.1594/PANGAEA.962218. Raw amplicon sequencing accession numbers: Raw data from Illumina MiSeq sequencing are deposited to the National Center for Biotechnology Information (NCBI) Sequence Read Archive (SRA) under BioProject number PRJNA973031.

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Erratum

In the originally published version of this article the authors inadvertently omitted the contribution information for Maxim Rubin-Blum (Software, Validation, Formal analysis, Writing - review & editing) and Itay J. Reznik (Conceptualization, Methodology, Supervision, Writing - review & editing). The contributions have been updated, and this may be considered the authoritative version of record.