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Detection of Spatial and Temporal Influences on Bacterial Communities in an Urban Stream by Automated Ribosomal Intergenic Ribosomal Spacer Analysis

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The Yarqon is the largest urban river in Israel, and is a slow-flowing stream whose water originates mostly from wastewater treatment plants. Thus, its microbial community is expected to be heavily impacted both by anthropogenic factors and by seasonal temporal variation. In order to identify the main factors that influence the bacterial community, and their spatial-temporal variation, 50 samples were collected representing five different time points and eleven locations. Samples were analyzed for biotic and a-biotic parameters and the bacterial populations were analyzed by Automated Ribosomal Intergenic Spacer Analysis (ARISA). Bacterial richness and diversity were calculated and compared across samples. Canonical Correspondence Analysis (CCA) showed that ARISA clustered the samples according to temporal variation. Molecular fingerprinting analysis provided a snapshot of the microbial community and showed good correlation with geochemical parameters, despite the rapid changes of the Mediterranean environment and the anthropogenic impact. Molecular fingerprinting methods based on natural fragment length polymorphisms may therefore represent a supplementary approach for stream monitoring, alongside physico-chemical measurements.

Key words: bacteria, stream, spatial, temporal, ARISA

Lotic or riverine environments are often described as inherently unstable. Despite the unidirectional flow, rivers and streams are affected by the variable surrounding landscape, creating a high internal diversity of microhabitats and niches (23). Temporal changes, such as floods or fragmentation of the flow, may also occur in rivers and streams. Thus, streamwater characteristics often change very rapidly, both in terms of geochemical parameters and faunal and floral species' richness and diversity (11). In comparison, the microbial communities of streams, and especially Mediterranean streams, have been relatively little studied. The main obstacles for such microbial studies in rivers and streams are their complex and highly dynamic nature. Hence, describing and monitoring the microbial community in lotic environments is extremely challenging and requires appropriate approaches which should be rapid, simple and reproducible.

Several previous studies focused on the effects of spatial and temporal changes of bacterioplankton communities. One study evaluated the synchrony and seasonality of two geographically close but non-intersecting rivers (6), while another showed the longitudinal changes of the bacterial community at the Danube river (24). Both of these studies showed the same geochemical parameters, namely temperature and phosphate concentration, to be highly significant determinants of the river microbial composition, despite the differences in methodology. Yet the question of whether the major influence on the microbial community in a lotic environment is spatial-, temporal- or landscape-driven remains unanswered.

The Yarqon is the largest urban stream in Israel, and the southernmost perennial stream in Israel's coastal plain (32°06'N; 34°50'E). The Yarqon is a slow-flowing stream whose water mostly comes from three wastewater treatment plants (WWTPs). Based on the degree of perturbation and water quality, the Yarqon can be divided into three sections (Fig. 1): the upper unpolluted section, from Rosh Ha'Ayin springs to the confluence with the Qane tributary (7.5 km), which contains fresh water. The central section (17.5 km), severely impacted by pollution of municipal effluents at its upper reaches, with gradual recovery downstream, contains a mixture of freshwater and WWTP effluents. The lowermost 4 km section (downstream to the "7 Mills dam"—"7M"), is a partially polluted estuary that contains brackish water. Of the six major tributaries of the Yarqon stream, one "Qane" discharges into the upper section, four into the central section and one into the lower section (Fig. 1). The Qane tributary

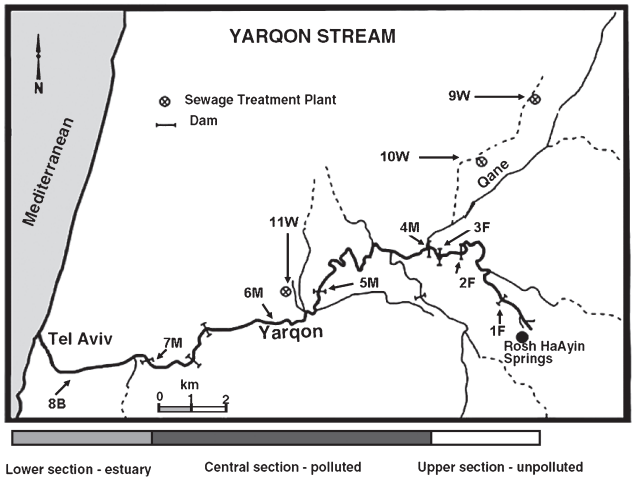

Fig. 1. Geography of the Yarqon stream (adapted from [10])

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collects two of the three effluents from "Nir-Eliyahu" WWTP (9W) and from "Kfar-Saba Hod-Hasharon" WWTP (10W), the former being a primary grade facility and the latter a secondary grade plant. At the "Hadar" tributary, effluent from the tertiary "Ramat-Hasharon" (11W) is also added to the stream. The "Seven mills" (7M) dam is a physical barrier which prevents the brackish water of the estuary from mixing with upstream water with the tide, and hence has highly different biological and geochemical parameters.

In recent years, there has been increased use of community fingerprinting methods which addressed the need for rapid, low cost and efficient means for assessing microbial diversity and species composition (7, 9, 15, 17). Automated Ribosomal Intergenic Spacer Analysis (ARISA, [8]) is often used to assess similarities in composition between different microbial communities. ARISA relies on a PCR amplification step, conducted with one fluorescence-tagged oligonucleotide primer and one unlabeled primer. The electrophoretic step is subsequently performed with an automated sequencing system, which provides separation. ARISA is based on the length heterogeneity of the bacterial rRNA operon 16S-23S intergenic spacer (also known as an internal transcribed spacer, or ITS). ITS regions are nearly ubiquitous in bacterial genomes (14) and are highly divergent in length (150–1,500 bp), a variation that allows discrimination between bacterial species, and sometimes between strains.

Unlike other fingerprinting methods, ARISA relies solely on the natural inherent length variability of regions within the bacterial ribosomal operon, and is therefore simple to carry out and standardize. Recently, length heterogeneity-based methods have been successfully incorporated into lab-on-a-chip systems (2), indicating that they may be applied for routine on-line diagnostics in the near future. If fully automated, these methods may serve for on-line monitoring of streams, providing a rapid and more detailed evaluation of a stream's contamination levels than traditional cultivation-based approaches, which are currently the standard in this field.

In this study we utilized ARISA in order to examine the microbial community structure and composition in a complex and rapidly changing lotic Mediterranean environment, and correlated them with geochemical measurements.

Materials and Methods

Environmental parameters

Several environmental parameters were measured while collecting the samples: dissolved oxygen and temperature were measured by a YSI 55 dissolved oxygen meter (YSI, Yellow Springs, OH, USA); pH was measured by an H1 9025 pH meter (Hanna Instruments, Woonsocket, RI, USA) and conductivity was measured by a H1 3733 conductivity meter (Hanna Instruments). Turbidity was measured by a HACHI 21000p turbidimeter (Mitsubishi Chemical Corporation, Tokyo, Japan). Biological oxygen demand (BOD) and chemical oxygen demand (COD) were analyzed by the USA EPA-approved standard procedures SM 5210 B and SM 5220 D, respectively. Cl^- was measured by SM 4500-Cl-B, and nutrient concentrations (total N, NO_3, NH_3-N, NO_2, and total P) were analyzed by SM 4500-NO_3^- B, SM 4500-NH3 C, SM 4500-NO_2 B and SM 4500-P B, C colorimetric EPA methods, respectively. All the environmental data were normalized by the Z-score, where the mean of the population was subtracted from each observation, and the difference was divided by the standard deviation of the population. Normal distributions were observed after this procedure. A Euclidian distance-based similarity matrix was then produced by the program Primer-E version 6 (4).

Sampling procedure

Sampling was conducted during 5 distinct temporal periods: Winter: 22/02/2007 (Wi), mid-summer: 07/08/2007 (MS), autumn: 23/10/2007 (Au), spring: 12/05/2008 (Sp) and late-summer: 10/09/2008 (LS). The 11 different sampling locations were chosen considering accessibility, tributary entry points and other potentially important landscape points such as dams and WWTPs entry points, as shown in Fig. 1. Fifty samples were collected where several sampling points were not always accessible. The samples were named using a 4-character designation, where the first two characters represent the season and the last 2 represent the location (see Table 1). To establish that on a finer temporal scale the stream communities are relatively stable, an additional sampling round was performed starting from 11:00 on 6 July, 2009 at 7-hour intervals, upstream of the "Seven Mills" weir. Seven consecutive sampling rounds were performed and were treated and analyzed in the same manner as in the other sampling rounds.

Water was collected by the Yarqon Stream Authority from 0.5 m deep surface water using a custom-made sterile 500 mL bottle with chlorine-absorbing sodium thiosulphate (Bactochem, Nes-Tziona, Israel) to prevent bacterial cell lysis due to residual chlorine from water treatment. The water samples were placed in a cooler to maintain ambient temperature at ±2°C until processed in the laboratory at ~3 hours after collection. Samples were pre-filtered

Table 1. Sampling sites, including WWTP entry points and distance from the origin of the stream

Number	Article marking	Water Quality	Location	Distance from origin (springs) in meters
1	1F	Fresh water	Nofarim	0
2	2F	Fresh water after contact with riverine flora and fauna	Abu Rabach	5,800
3	3F	Fresh water after contact with riverine flora and fauna	Qane joining upstream	6,600
4	4M	Mixed fresh water and treated waste water effluent	Qane joining downstream	6,800
5	5M	Mixed fresh water and treated waste water effluent	Agricultural Dam	13,900
6	6M	Mixed fresh water and treated waste water effluent	Geha Bridge	18,000
7	7M	Mixed fresh water and treated waste water effluent	Seven Mills	24,000
8	8B	Brackish water	Marine Sport center	26,000
9	9W	Waste water treatment plant effluent–primary grade	Nir Elyahu WWTP	WWTPs join the stream between 3 to 4 sampling points by the Qane tributary
10	10W	Waste water treatment plant effluent–secondary grade	Kfar-Saba and Hod-Hasharon WWTP	
11	11W	Waste water treatment plant effluent–tertiary	Ramat-Hasharon WWTP	Joining point with the stream between 5 to 6 by the Hadar tributary

through a 47-mm diameter Whatman glass microfiber GF/C filters (nominal pore size, 1.2 µm; Whatman, Maidstone, UK) to remove large particles and protists, then filtered through a 0.2-µm pore size ME-24 membrane filter (Schleicher & Schuell, Keene, NH, USA) to collect the majority of bacteria, which are retained on the filter. The filters were then placed in sterile sealed Petri dishes and stored at –20°C until DNA extraction, about one hour later.

DNA extraction and purification

Filters (see above) were shredded under sterile conditions. DNA was extracted from the samples using the PowerSoil DNA extraction kit (Mo Bio Laboratories, Carlsbad, CA, USA) according to the manufacturer's protocol. DNA concentrations of all samples were determined by a Thermo Scientific NanoDrop 1000 spectrophotometer (Waltham, MA, USA) and then stored at (–20°C).

Generation of ARISA Profiles

PCR—all reaction templates were normalized to the same DNA concentration, 20 ng template DNA were used for ARISA reactions. PCR was performed with 1.25 U *Taq* DNA polymerase (BIOLINE, London, UK), 3 mM $MgCl_2$, 2.5 µl of 10×PCR buffer, 0.1 mM of each dNTP, ultra pure water (Biological Industries, Beit Ha'emek, Israel) and 10 pmol primers. The PCR oligonucleotide primers used for the ARISA were 1392F- 5'-GYACACACCGCCCGT-3', 125R-Tet- 5'-GGGTTBCCCCATTCRG-3' (9). Reactions were prepared in duplicate in a dedicated PCR cabinet with filtered air laminar flow. Negative controls, containing no template, were also prepared to verify lack of contamination. The reaction was performed as follows: 3 min at 94°C; 32 cycles of 1 min at 94°C, 1 min at 52°C, 1.5 min at 72°C; and a final elongation step of 6 min at 72°C, using a T-personal BIOMETRA PCR Thermocycler. All PCR products were visualized by gel electrophoresis (1% TBE agarose gel) to verify successful amplification and to rule out contamination.

Fragment Analysis

PCR products were analyzed using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems, Foster city, CA, USA). The labeled fragments were separated on the capillary sequencer, while an internal size standard was used in each capillary. The ARISA size standard used was a custom made marker—CST ROX 250–1150 bases (Bioventures, Murfreesboro, TN, USA).

Analysis of fingerprinting data

Raw data generated by the ABI PRISM 3100 Genetic Analyzer were initially analyzed using GeneMarker (SoftGenetics, Palo Alto, CA, USA). After accurate size calling using the software, all data were exported to Microsoft Excel for further analysis. All peaks with relative fluorescence intensity of 40 or lower were excluded. Subsequently, all operational taxonomical units (OTUs) were binned as described previously (3) and intensities were summed for each bin. Next, the relative intensity of each binned OTU in a certain sample was calculated, and binned OTUs, which contributed less than 0.5% to the total intensity of the sample, were excluded. Duplicates were compared, and only OTUs that appeared in both duplicates were used and their new relative intensities calculated. CCA and diversity indices were generated by PAST, a statistical data analysis package (12). One-way ANOSIM (ANalysis Of SIMilarity) was performed (999 permutations) for all three datasets. SPSS version 14 was used to generate the box plots and Spearman correlations. Bray-Curtis similarity values were calculated for all the fine-scale temporal variation samples using PAST (Table S1).

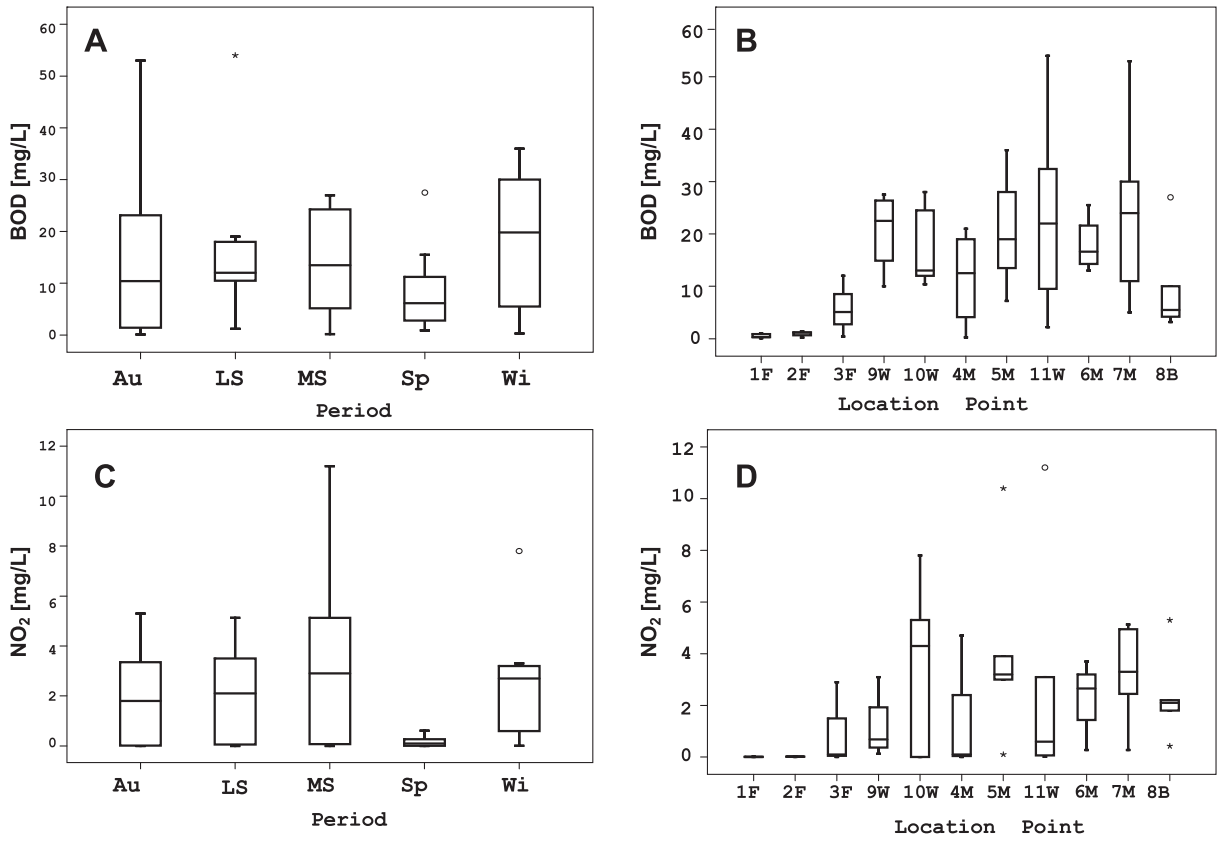

Fig. 2. Box plot diagrams of BOD, NO_2, by location and season. Temporal box plots represent the different sampling periods for all sites combined (Au: autumn, LS: late summer, MS: mid-summer, Sp: spring, Wi: winter). Location-based box plots represent geographical sampling points, marked by a number which represents the location of the sampling point and the water affiliation, as in Table 1 in the Materials and Methods section. The results are separated to panels where (A) BOD by period, (B) BOD by location, (C) NO_2 by period, and (D) NO_2 by location. Asterisk and small circles represents outliers when present.

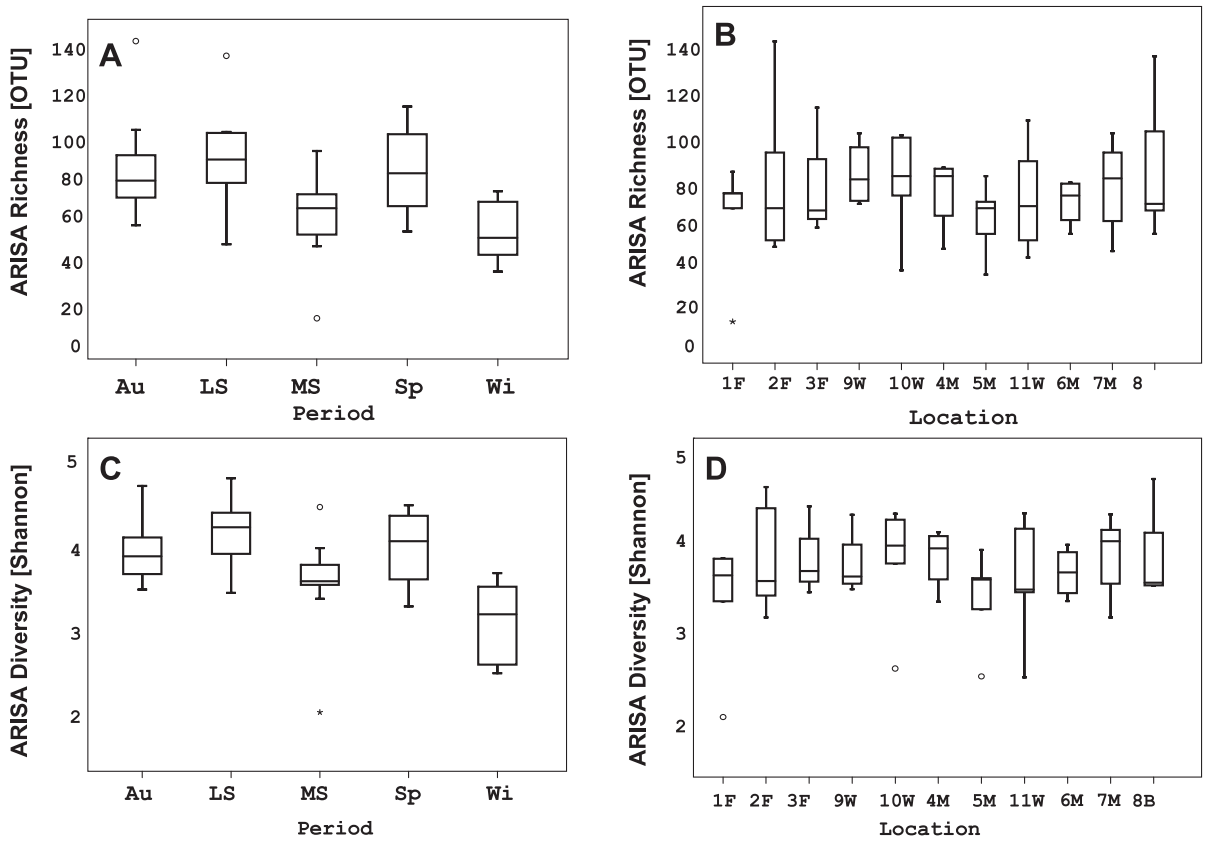

Fig. 3. Box plot diagrams of ARISA-based diversity measured by the Shannon index and richness, by location and season. Temporal box plots represent the different sampling periods for all sites combined (Au: autumn, LS: late summer, MS: mid-summer, Sp: spring, Wi: winter). Location-based box plots represent geographical sampling points, marked by a number which represents the location of the sampling point and the water affiliation, as in Table 2 in the Materials and Methods section. The results are separated to panels where (A) ARISA richness by period, (B) ARISA richness by location, (C) ARISA Shannon by period, and (D) ARISA Shannon by location. Asterisk and small circles represents outliers when present.

Results

Geochemical parameters, but not microbial diversity, are driven by the quality of treated wastewater

We obtained geochemical measurements and molecular fingerprints of the microbial community from different sampling sites in the Yarqon during five sampling seasons. The data were first analyzed either by season (averaging all locations) or sampling site (averaging all seasons). It should be noted that at a finer temporal scale, the average similarity between any two samples taken from the same site was high (average Bray-Curtis similarity value of 0.622, standard error=0.04, see Table S1), indicating some short-term stability of the bacterial community.

Obvious spatial separation could be seen when examining the environmental parameters (Fig. 2 and 3). BOD and NO_2 concentration levels were low at both 1F and 2F, close to the source of the stream, and increased at 3F, the point where water from the three WWTPs (9W, 10W and 11W) is introduced into the stream. Finally, a clear drop of BOD and NO_2 levels could be observed at 8B, which is brackish due to dilution with seawater. A similar pattern was observed for additional parameters: pH (Fig. S1), total N, NH_3 (Fig. S2), total P, and dissolved oxygen (Fig. S3). No clear trend was visible for turbidity and total suspended solids (TSS) (Fig. S4), which can be explained by local water mixing and

Table 2. One-way ANOVA for season-based separation between groups based on ARISA

	Period	Location
BOD (mg L^{-1})	0.556	**0.007**
COD (mg L^{-1})	**0.005**	**0.026**
TS-105 (mg L^{-1})	0.655	0.197
TSS-550 (mg L^{-1})	0.994	**0.023**
Total-N (mg L^{-1})	0.181	**0.002**
NH_3-N (mg L^{-1})	**0.045**	**0.013**
NO_3^- (mg L^{-1})	**0.005**	0.47
NO_2 (mg L^{-1})	**0.026**	0.201
Total—P (mg L^{-1})	0.101	**0.001**
Cl^- (mg L^{-1})	0.798	**<0.0001**
Temperature (°C)	**<0.0001**	0.714
pH	**<0.0001**	0.412
Conductivity (μS cm^{-1})	0.944	**<0.0001**
Oxygen percent (mg L^{-1})	0.965	**0.001**
Turbidity (NTU)	**0.015**	**0.039**
Dissolved oxygen (mg L^{-1})	0.868	**0.002**
ARISA Taxa (S)	**0.001**	0.92
ARISA Shannon (H)	**<0.0001**	0.838
ARISA Simpson (1-D)	**0.002**	0.737

Statistically significant results (P<0.05) are presented as a percentage are marked by a bold font.

Table 3. Spearman correlations between diversity and richness estimates based on ARISA and geochemical parameters

	ARISA Richness	ARISA Shannon
NO_3^- (mg L^{-1})	–0.362 (0.008)	–0.33 (0.019)
Cl^{--} (mg L^{-1})	0.34 (0.016)	0.315 (0.025)
Temperature (°C)	0.251 (0.078)	0.349 (0.025)
pH	0.521 (0.0004)	0.569 (0.00006)
Conductivity (µS cm^{-1})	0.267 (0.06)	0.29 (0.041)
Turbidity NTU	0.258 (0.07)	0.288 (0.0427)
Dissolved oxygen (mg L^{-1})	0.319 (0.0239)	0.284 (0.045)

The meaning of the numbers outside of the parenthesis, are the *R* values and in the parenthesis are the significance of each result.

turbulence caused by waves and occasional boating activities. Sets of commonly used diversity indices, such as the Shannon index or OTU richness, were calculated and compared to the patterns of the geochemical parameters measured. Notably, ARISA-based estimates of the Shannon index of diversity and the OTU richness showed a similar pattern (Fig. 3), in which richness and diversity increased with the rise in temperature (from winter to late summer). In addition, ARISA showed an unexplained decline in diversity in the mid-summer period, which is possibly explained by algal bloom which accrued during that period and a meaningful drop in the winter period. One-way ANOVA was performed, analyzing data either by period or by location (Table 2), and showed that in most cases different parameters are significantly associated with either season or physical location, but not both.

ARISA-based diversity correlates with geochemical parameters

In order to better understand the potential connection between physico-chemical and microbiological data, we analyzed the entire sample set and calculated Spearman correlation values between diversity indices and all the physico-chemical parameters detailed above. The highest positive correlations observed were between pH and the Shannon index of diversity (R=0.569), and temperature was also a significant factor (R=0.349, Table 3). The only significant negative correlation observed was between the Shannon diversity and NO_3 (R=–0.33), which may imply that there is a reduction in species-level diversity when NO_3 concentrations increase.

Canonical correspondence analysis shows that conductivity, temperature and total phosphorus may influence the microbial composition

Any molecular tool that aims to capture a reliable snapshot of microbial communities should also reflect changes driven by environmental conditions, including anthropogenic pollution or other spatial and temporal effects. CCA (Canonical Correspondence Analysis) of ARISA-based data along with the tested physico-chemical parameters was therefore performed. CCA uses unimodal probability distribution to assess

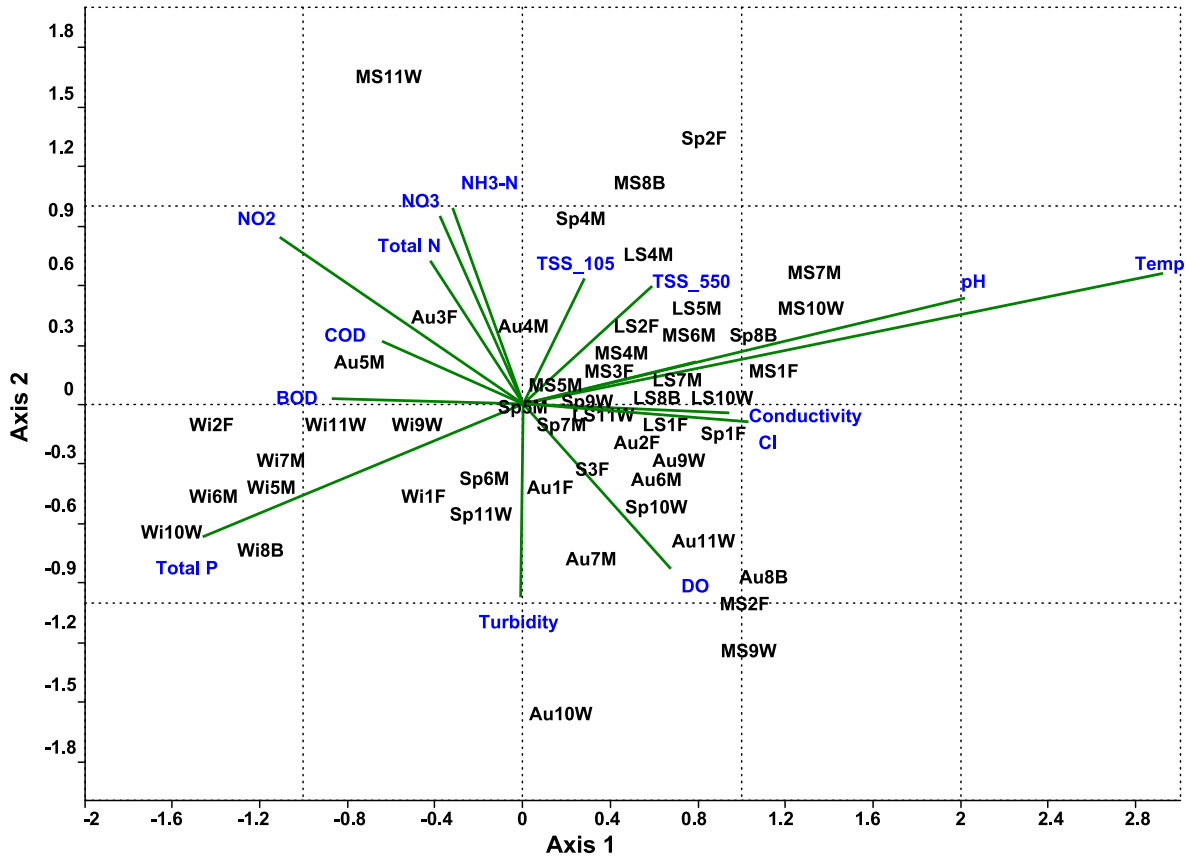

Fig. 4. CCA showing clustering and parameter vectors of ARISA. Parameters: pH, temperature, turbidity, conductivity, COD, BOD, soluble solids (TSS-550), dissolved oxygen (DO), total N, NO_3, NH_3, NO_2 and total P, which are vectored by green lines. Sampling locations are marked using the same nomenclature in Table 1 in the Materials and Methods section, with scaling focus on sample distance (Ramette, [16]).

Table 4. ANOSIM test for season-based separation between groups based on ARISA.

Pairwise Tests	ARISA	
	R	*P*-value
Global test	0.32	**0.1**
Winter/Mid-Summer	0.624	**0.9**
Winter/Autumn	0.623	**0.1**
Winter/Spring	0.461	**0.1**
Winter/Late-Summer	0.53	**0.2**
Mid-Summer/Autumn	0.273	**0.1**
Mid-Summer/Spring	0.157	**0.2**
Mid-Summer/Late-Summer	–0.047	66
Autumn/Spring	0.116	**1.5**
Autumn/Late-Summer	0.06	21.2
Spring/Late-Summer	0.021	35.2

* Statistically significant results are presented as a percentage ($P<0.05$) in bold type.

species response to environmental variation as a mathematical simplification for the estimation of a large number of parameters and the identification of a small number of ordination axes (19). In this analysis (Fig. 4), the winter samples clustered between the 3rd and 4th quartiles, due to the positive influence of the total phosphorus and the negative effect of temperature. The mid-summer samples clustered in the 1st and 2nd quartiles with a positive effect of temperature and a negative effect of total phosphorus. The late-summer samples showed a similar trend to the mid-summer samples, but with a lower magnitude. Jointly, these data indicate that temporal variation in temperature and phosphorus affects bacterial composition at the species or strain level, reflected by ARISA.

Analysis of similarity supports temporal clustering of ARISA-based data

To test the hypothesis that microbial composition is largely temporal, as observed in CCA, additional ANOSIM was performed for each fingerprinting dataset. ARISA-based similarities were found to be significantly distinct among seasons; hence, the five time periods during which we sampled were found to be separated. Nevertheless, when examining pair-wise temporal ANOSIM values, some overlap could be observed (Table 4) reflected by *R* values ranging between 0.5 and 0.75 (5). Interestingly, consecutive seasons, such as mid-summer and late-summer, were not necessarily the most similar.

Discussion

Mediterranean streams experience large temporal variation due to the climatic regime and, as a result, microbial communities may undergo major shifts due to temporal changes. Thus, distinguishing between anthropogenic disturbances and natural spatial and temporal variation is challenging. The highly inconsistent bacterial communities make it difficult to discern the presence of contaminants that affect water quality. Monitoring of lotic microbial communities therefore requires techniques which are rapid, reproducible and cost effective.

The aim of the current study was to utilize ARISA along with geochemical measurements in order to examine the microbial community structure and composition changes in a riverine environment. The geochemical data alone showed very clear trends. Water originating from springs has low nutrient concentrations and characteristics that normally reflect high water quality. In contrast, after the "Qane" and "Hadar" tributaries, into which treated wastewater is discharged from WWTPs (9W, 10W and 11W), meet the stream, an apparent shift in water parameters becomes evident. Both total nitrogen and BOD levels, for example, increase about 30-fold between 2F and 5M, as noted previously (11), which led us to hypothesize that a major change in microbial diversity will be apparent by ARISA. However, our analysis did not show a decrease in diversity or richness of the bacterial communities at the sampling points affected by WWTPs. In fact, bacterial diversity levels were similar across sampling sites, and could not be used for source-tracking. It is noteworthy that although ARISA has been shown to be a reliable indicator of the diversity of common taxa (3, 18, 22) and to correlate well with 16S rRNA gene-based richness values (14), it only represents relatively common taxa (1). The lack of change in diversity in different river sections is more likely to be the result of a "replacement effect", whereby the diversity level is stable due to continuous displacement of bacterial taxa by other microbes. This finding seems even more plausible when reviewing 16S rRNA gene clone library-based diversity indices, which clearly show that despite changes in the bacterial community composition, a very small difference in diversity exists between the samples. In addition, as mentioned above, because diversity indices pool multispecies information into a single value for each observation, it is not surprising that complex diversity patterns may sometimes be missed (13, 15) Apparently, unlike macroorganisms, such as invertebrates (10), reduction in microbial diversity cannot be used as a reliable biomarker for stream pollution by man-made chemicals and sewage, since the microbial assemblage does not always respond similarly to the same factors, both qualitatively and quantitatively (21).

As previously shown for streams and other water habitats, temperature and salinity are the most influential parameters on both temporal and spatial scales in the Yarqon stream (6, 24). Thus, these parameters are dominant even in an open ecological system which is rapidly changing, such as a Mediterranean lotic niche. CCA, as well as Spearman correlations, also suggested that temperature and pH are the most influential parameters.

Classic microbiological methods for water quality assessment that rely on indicator bacteria-based viable counts (such as coliform counts) are known to be problematic due to climactic and geographic factors (20) but, due to their relatively low cost and simplicity, are still primarily used for microbial contamination assessment. In addition, the most common tests for general water quality assessment are either physical or chemical-based, and may not correlate well with microbial contamination levels. Requirements for recreational water, for sailing, kayaking and other sports activities, are usually far more lax than for drinking water. While the standards for this water reflect some microbiology-based risk assessment, they do not currently take into account differences between risks from animal or fecal pollution and

climactic variation (20). As mentioned by the EPA expert scientific group, there is a need to "Gain better understanding of temporal and spatial variability in environmental sampling using culture-based and non culture-based methods and the implications for their use in representing water quality" (20). Thus, the need for rapid molecular tools to monitor such water still exists, and is on the rise since the use of reclaimed wastewater for recreational purposes is on the increase, due to worldwide water shortages.

Currently, molecular fingerprinting methods that do not require enzymatic digestion, such as ARISA and length-heterogeneity (LH) PCR, can be incorporated into lab-on-a-chip type solutions (2), enabling a rapid yet sensitive and accurate contamination control. Here we demonstrated that in a slow-flowing stream temporal variation may disguise weaker source-specific signals, making it difficult to use a generic lab-on-a-chip approach. Thus, ARISA primers specific to particular taxonomic groups should be used (3), enabling identification of fecal contamination from human sewage and even partially treated sewage water, rather than the usual, more generic, approaches. These cultivation-independent methods should be combined with readouts from chemical tests that are rapid and simple to perform. Such combined methodology, if standardized, may form the basis of different regulatory criteria which may better reflect the true ecological status of lotic environments.

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