Fine-Scale Temporal Dynamics of a Fragmented Lotic Microbial Ecosystem

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Microbial ecosystems are often assumed to be relatively stable over short periods of time, but this assumption is seldom tested. An urban stream influenced by both flow and varying levels of anthropogenic influences is expected to have high temporal variability in microbial composition, and short-term ecological instability. Thus, we analyzed the bacterioplankton composition of a weir-fragmented urban stream using Automated rRNA Intergenic Spacer Analysis (ARISA). A total of 46 sequential samples were collected in July 2009 for 7 days, every 7 hours, from both the up-stream side of the weir (stream water) and the downstream side of the weir (estuarine) water. Bray-Curtis similarity based analysis showed a clear division between upstream and downstream communities. A sudden pH drop induced change in both communities, but composition stability partially recovered within less than a day. Thus, our results show that microbial ecosystems can change rapidly, but re-establish a new equilibrium relatively quickly.

Stability of river ecosystems and the roles of diversity¹, complexity², river continuity³ and disturbances are often debated. Previous studies of lotic microbial communities have shown that the riverine ecosystem is an ever changing environment^{4–6}. However, these studies mostly emphasize spatial trends, sometimes disregarding the temporal changes characteristic of microbial communities that are quick to respond to disturbances. Moreover, short-term temporal dynamics are an especially important for highly dynamic ecosystems such as rivers or streams⁷, where the short-term microbial ecosystem can be less stable⁸.

Most current studies of lotic bacterial communities are based on weekly or monthly samplings^{9–11}. Studies looking at a finer temporal scale, such as multiple samples per day, have been limited to estuary variation between low and high tides, and focused on cultivable indicator bacteria^{12–14}. Since changes in sunlight and tides (for estuaries) can have large effects on bacteria, the conclusions of many microbial studies of lotic ecosystems, which are based on a low frequency sampling, hang on the assumption of high community stability in the short-term. However, this assumption remains mostly untested.

The Yarqon stream, which runs through the center of Israel's coastal plain¹⁵, into the Mediterranean Sea, consists mostly (about 70%) of treated wastewater of variable quality^{16,17} and a fixed 400 cubic meters per hour influx of fresh water, pumped from the "Yarqon-Taninim" aquifer. The Seven Mills ("Sheva Tahanot") weir (Fig 1.) introduces a 2 meter water barrier that separates the bulk of the stream from its estuarine section, and forces a continuous, unidirectional downstream flow. The section located up-stream to the weir contains mostly treated wastewater and is therefore affected by changes in the treated wastewater it receives. The downstream section of the river is brackish, due to a tidal water inflow¹⁸ which occurs in a 12–13 hour cycle, and therefore effectively contains a mix of water originating from the upstream flow combined with incoming sea water¹⁹. The upstream and the downstream sections are thus substantially different in terms of their physical - chemical characteristics such as salinity, water temperature and pH^{17,19,20}. Such weir construction is a common practice in urban rivers which have coastal tidal effect and may be considered as a barrier between ecotones²¹.

We wished to examine the fine-scale temporal stability of the bacterial communities on both sides of the weir, using frequent sampling, and thus test whether current weekly-to-monthly sampling practices are sufficient to describe microbial river ecosystems. The study site chosen enabled us to look both at a niche diurnally interrupted by sea water flow, hence subjected to constant predictable variation (downstream) and at a niche which is more static, but can be influenced by rare anthropogenic effects (upstream). For this purpose we used a molecular fingerprinting method ARISA – (Automated Ribosomal Intergenic Spacer Analysis^{22,23}), known for its high taxonomic resolution, which can distinguish between bacterial species, and even strains^{24,25} and which is frequently used in studies of aquatic environments^{22,26}.

Figure 1 | Yarqon stream scheme, indicating the relative location of the “Seven Mills” sampling point.

Results

The upstream and downstream bacterial communities are distinct. We explored the two sampling sites using Canonical Correspondence Analysis (CCA). CCA is an ordination method where the axes are linear combinations of environmental variables, and these variables are plotted as correlations with site scores²⁷. The CCA graph obtained for the 23 sampling points showed a somewhat expected separation between the upstream and downstream microbial communities (Fig. 2A) and a similar separation was observed in the Bray-Curtis cluster analysis (Fig. 2B). The CCA representation of the environmental parameters showed that total dissolved solids (TDS) and conductivity clearly separated the up and down-stream sides of the weir, and pH and temperature showed a similar trend. The overall correspondence between TDS and conductivity was very high and thus only the representation of the conductivity measurements is represented (TDS can be found in Supplementary Figure S1). When examining the microbial communities, the ARISA-derived patterns showed downstream samples to be clustered more closely both by the cluster analysis and by CCA. Only two OUT's, one from each segment, were unique when the upstream was compared to the downstream. Notably, only 3.5% of the ARISA OTUs observed (5 of 143 binned OTUs: lengths, 239, 409, 461, 481, and 702 bp) were present in all samples, both upstream and downstream, and none of these pervasive OTUs was dominant in any sample (i.e. had a relative fraction that exceeded 1%). SIMPER analysis showed that the two OTUs corresponded to ARISA fragment lengths of 339 bp and 341 bp, were predominantly found in the upstream samples, and contributed 5.15% and 2.23%, respectively, to the overall dissimilarity between upstream and downstream samples.

Changes in bacterial communities in response to fluctuations in water parameters. We plotted the physical-chemical parameters of the water, bacterial diversity and the similarity between each pair of consecutive ARISA profiles (Fig. 3D). Water temperatures shifted

according to the time of day, both upstream and downstream, indicating that the influx of seawater does not markedly affect this parameter downstream. The downstream section was found to be brackish with tide-dependent salinity cycles, while the upstream section's salinity remained continuously low. An unexplained increase in downstream salinity appeared at the 20_5(105)dw sample. In addition, an unknown event which occurred between 42–49 hours [sample 5_3(42)] caused a drop of about 1 pH unit which persisted for the remainder of the experiment, affecting both downstream and upstream segments, indicating that this event had occurred someplace upstream of the weir.

We examined the short-term dynamics of the bacterial community by measuring the ARISA-based Bray-Curtis similarity values between each pair of consecutive samples. In general, before the pH drop both communities tended to have high short-term stability (mean similarity of 0.65 and median 0.54 for consecutive upstream and downstream samples, respectively). Following the pH drop, the dynamics of the downstream community had a similar trend to that of the upstream bacteria, but lagged one sampling point (7 hours) behind, as can be expected from the relatively slow water flow downward [~ 1400 cubic meters per hour¹⁷]. The pH drop caused a sharp dip in similarity (Fig. 3D, 56 h/63 h), indicating a sudden change in the microbial community. Stability was only partially regained (Fig. 3D, 98 h/105 h), and samples maintained overall similarity (mean similarity of 0.573 and 0.482 for consecutive upstream and downstream samples, respectively). This was also reflected by the hierarchical clustering and the CCA (Fig. 2B). The decreased stability was also evident from the Bray-Curtis similarities between adjacent time points (Fig. 4) (interquartile range upstream before pH drop = 0.1, downstream before pH drop = 0.11; upstream, after = 0.32 and downstream after = 0.28). Notably, diversity values remained alike, even when communities underwent changes, showing microbial diversity to be a poor indicator of community fluctuations (See Supplementary Figure S2). Nevertheless, an analysis of similarity

Figure 2 | (A) CCA analysis of ARISA and environmental data. ARISA-based CCA was calculated with the following environmental parameters; pH, temperature, TDS, conductivity and U.V radiation. See methods section for the nomenclature of samples. N= 46. (B) A Bray Curtis cluster analysis with a 1000 bootstrap trials, represented as % bootstrap support. Red, upstream samples; black, downstream samples. N= 46.

Figure 3 | (A–D) Temporal changes in environmental parameters and microbial communities on the two sides of the weir. (A) pH, (B) Temperature, (C) conductivity, (D) Bray-Curtis similarity between ARISA profiles of each consecutive sample pairs. The missing sample in the upstream graph (133/140, 140/147) is due to an unsuccessful PCR amplification of the 140 h time point. The continuous line and empty squares represent upstream samples, while the dashed lines with full triangles represent downstream samples.

(ANOSIM), based on the Bray-Curtis similarity measure did not show significant differences between pre- and post-pH drop in communities within each site ($p=0.587$ and $p=0.545$, respectively, for upstream and downstream respectively).

Discussion

Microbial communities of rapidly changing ecosystem, such as streams and rivers, tend to be under-studied, since their characterization is complicated by their ever-changing nature^{7,28,29}. Such ecosystems are predicted to have low community stability^{30,31}. This study analyzed the short-term microbial community dynamics of an anthropogenically-impacted stream site, known as “Seven Mills”³², which is fragmented by a weir²⁸, thereby enabling a comparison of the stability of two microbial ecosystems with differing disturbance patterns. The upstream section of the weir experiences these anthropogenic disturbances more strongly, due to the rate of water flow, while downstream is diluted by seawater and under constant diurnal influences of low and high tides.

As seen from the CCA analysis (Fig. 2A), cluster analysis (Fig. 2B) and SIMPER results, the up- and downstream were distinct, and the differences between them could not be ascribed to a single OTU.

Therefore, this separation may be linked to the transportation of marine bacteria from the estuary downstream of the weir during high tide¹³. The influence of the tides was apparently greater than that of the continuous flow of the upstream water to the bacterial communities on the downstream side of the weir.

During our sampling schedule an unexpected drop of pH was observed, which was fortuitous, from our perspective, allowing us a glimpse of the robustness of the weir communities. When looking at the overall consequences of the pH drop, we observed that the average similarity values and diversity indices (See supplementary Fig. 2) remained relatively stable. However, when additionally partitioning the by the pre- and post-pH drop data by site (See supplementary Fig. 3), it is apparent that the upstream was more sensitive to this event, showing a decrease of diversity and richness. This supports the view³³ that a routinely disturbed ecosystem is more robust than an ecosystem that is generally undisturbed. In addition, the pH drop was rapid and had a downstream effect which was surprising in light of the small water volume which flows down from the upstream, compared to the large dilution capacity of the downstream water due to incoming tidal flow. Hence, it appears that changes in pH can have substantial and rapid consequences for water-borne bacterial ecosystems.

Examining the sequential Bray-Curtis mean similarity values (Fig. 3D), we observed a relative stability in both upstream and downstream communities before the pH drop, but an obvious oscillation after it occurred ($t=49$). The oscillation can be additionally observed by the interquartile range (IQR) values of the Bray-Curtis similarity box plots (Fig. 4) before and after the pH drop. Yet, this effect did not manifest in either the CCA or cluster analysis. These results suggest that the microbial communities at both sides of the weir were affected, yet showed some degree of robustness, later regaining ecological stability.

Our in-situ experiment was performed in mid-summer when the water flow in the stream is at its yearly low. Hence, the connectivity between both sides of the weir may be lower than the yearly average. A sudden influx of rainwater, which is common in the Mediterranean winter, will strengthen the connectivity between both sides of the weir, because water may overflow, bringing more bacteria from the upstream to the downstream. Thus, the strength of the connectivity between river sections can be highly variable, depending on season and intrinsic water volume¹⁶.

A microbial community can recover more rapidly from disturbance than do flora or fauna, due to the unicellular physiology and rapid growth rates of many bacteria, which can have generation times shorter than one hour under optimal conditions. It is therefore imperative to further study short term community stability, if we are to understand the true dynamics of many natural microbial assemblages and to reinforce the validity of previously-conducted larger- interval temporal experiments.

Scaling is an important concept in landscape ecology, where it is usually used in a geographical, rather than temporal perspective³⁴. A lesson from experiment described in this study is that a bacterial community can change drastically and rapidly and yet have an equally rapid recovery. Our data emphasize the need for a re-evaluation of the time scale of microbial sampling experiments beyond diurnal cycles, especially in dynamic environments such as fresh water streams, lakes and irrigated soil.

Methods

Sampling site and sample collection. Sequential samples were collected from the “Seven mills” weir of the Yarqon stream (32°09'N; 34°81'W; 5 m altitude) during a 7-day period, every 7 hours, encompassing day and night as well as the low and high tides changes, from July 6th to July 12th 2009, resulting in a total of 23 samplings. At each time point, two samples were taken, one from the upstream section of the weir,

and the other from the downstream section below the weir, at the origin of the estuary. The delay in taking these paired samples, taken 50 meters apart, was less than 10 minutes. Samples were taken from 0.5 m deep surface water, using 0.25 L plastic collection bottles. The water samples were placed in a cooler to maintain a steady temperature of ~4°C until processed in the laboratory no more than 40 minutes later.

The sample nomenclature used is based on all or part of four parameters; time of day (hours), sampling day 1–7, cumulative time of sampling since the first sampling time (“time zero”) and the stream location (up or down). For example, 20_5(105)dw represents a sample taken at 20:00, on the 5th day of the experiment and 105 hours since the first sample was taken, which was obtained from the downstream part of the stream. Figure legends may show only partial nomenclature.

Environmental variables. The following physical - chemical parameters were measured: water temperature, pH, Total Dissolved Solids (TDS), and conductivity (μS), selected as prime environmental factors, since they previously showed the highest correlations with microbial communities in the Yarqon³⁵. The parameters were measured using the portable sampling device CRISON MM 40 (Crison Instruments, Barcelona, Spain). In addition to the data obtained in-situ, UV radiation intensity was estimated based on the Israeli Meteorological Service data long term averages from its web site for each respective time period (http://www.ims.gov.il/ims/all_tahazit/) in MJ per square meter in order to evaluate differences between day and night UV levels. Sampling was done during the month of July, which was regarded as the most suitable time for sampling due to the lack of rain and the minimal cloud cover.

Sample filtering. Samples were pre-filtered through 47-mm-diameter Whatman glass microfiber GF/C filters (nominal pore size, 1.2 μm) to remove large particles, plant cells and protists. Subsequently, the filtrate was passed through a 0.2- μm -pore-size ME-24 membrane filter (Schleicher & Schuell) so that the majority of bacterial cells are retained on the filter. The filters were then placed into sterile sealed Petri dishes and stored at -20°C for one hour until DNA extraction.

DNA extraction and purification. Filters were shredded under sterile conditions and DNA was extracted from the samples using the PowerSoil DNA extraction kit (MO-BIO, 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 of 20 ng per reaction. PCR was performed with 1.25 U of *Taq* DNA polymerase (BIOTAQTM, BIOLINE), 3 mM of $MgCl_2$, 2.5 μl 10xPCR buffer, 0.1 mM of each dNTP, ultra pure water (Biological Industries, Israel) and 10 pmol of the primers 1392F (5'-GYACACACCGCCGT-3') and 125R (Tet- 5'-GGGTBCCCCATTTCG-3')²³. Reactions were prepared in duplicate in a dedicated PCR cabinet with filtered laminar airflow. Negative controls, containing no template, were also prepared, to verify lack of contamination. The reaction was performed as follows: 3 min at 94°C; 30 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 (Göttingen, Germany). All PCR products were observed by gel electrophoresis (1% TBE agarose gel) to verify successful amplification and to rule out contamination. PCR products were analyzed using the ABI PRISM 3100 Genetic

Figure 4 | A box plot diagram of the ARISA-based Bray-Curtis similarity values between consecutive samples. N = 46.

Analyzer. The labeled fragments were separated on the capillary sequencer, while an internal size standard, a custom made marker - CST ROX 250-1150 (Bioventures, Murfreesboro, TN, USA), was used in each capillary.

Fingerprinting - Data Analysis. Raw data generated by the ABI PRISM 3100 Genetic Analyzer was initially analyzed using GeneMarker™ (SoftGenetics, State College, PA, USA). All operational taxonomical units (OTUs) with arbitrary relative fluorescence intensity of 40 or lower were excluded. Subsequently, all OTUs were binned as described previously^{24,36}, and intensities were summed up for each bin. Next, relative intensity for 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. In order to validate reproducibility, all pairs of duplicates were compared, and only OTUs that appeared in both technical duplicates from the same sample were used and their new relative intensities were calculated. Finally, the averaged values for each sample were normalized to reflect relative intensity values. All data were then exported to PAST²⁷, a statistical data analysis package. Using PAST, similarity calculations were performed by the Bray Curtis similarity index³⁷, which takes into account taxon richness and abundance in the samples.

Normalization and similarity matrices generation. All environmental data used for statistical analysis were square root transformed and standardized by the Z score method. Briefly, the mean of the population was subtracted from each observation, and the difference was divided by the standard deviation of the population. The ARISA similarities were calculated by the Bray Curtis measure, while the environmental data similarities were calculated by the Euclidean distance measure.

Statistical analysis. CCA analyses were performed by PAST by using default settings. Cluster analyses (group average, significance level 5%, 1000 permutations) were then performed on the same dataset with Bray-Curtis measure with a 1000 permutations for calculation of bootstrap.

To test differences between clusters, the analysis of similarities [ANOSIM, see³⁸] based on Bray-Curtis similarity was performed using the Primer-E software (PRIMER-E Ltd, Lutton, UK). ANOSIM is a permutation-based statistical test, an analog of the univariate ANOVA, which tests for differences between groups of (multivariate) samples from different locations and experimental treatments. ANOSIM was performed separately on each environmental, richness or diversity parameter. ANOSIM was performed separately on environmental data and ARISA dataset, as suggested by the exploratory analysis. SPSS version 15 (SPSS Inc, Chicago, USA) was used to calculate Spearman correlations. SIMPER analysis was performed using PAST.

The Bray-Curtis distance matrix was used in order to measure the temporal dynamics of ARISA profiles. Each time point was compared to its consecutive time point (e.g. ARISA profiles from 0 time point were compared to 7 h time point, ARISA profiles from 7 h time point were compared to ARISA 14 h, etc...). Each similarity score was labeled after the two points that were compared (e.g. the similarity between time point 0 and 7 was referred to as 0/7). The median and mean were calculated by PAST, also partitioning the data before/after the pH drop (from 0 h up to 42 h and from 49 h to 155 h), for both the upstream and downstream sections. The Box plot was produced by SPSS Ver. 15 (SPSS Inc, Chicago, USA).

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Author contributions

AO - Study design, Sample collection, sample processing, analysis of results, preparation of the manuscript; UG - Study design, analysis of results, preparation of the manuscript; LS - Sample collection, sample processing, analysis of results.

Additional information

Supplementary information accompanies this paper at <http://www.nature.com/scientificreports>

Competing financial interests: All the authors declare that there are no competing financial interests.

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