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„Assessing the anthropogenic impacts of a large cultural event on a riverine environment – a case study of the Traisen river "

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Abstract

Anthropogene Effekte der letzten Jahrzehnte haben mehr Ökosysteme erfasst als je zuvor. Angesichts der Tatsache, dass sich die meisten Studien des Anthropozäns auf industrielle Umgebungen konzentrieren, werden kulturelle Ereignisse - obwohl sie auch als Umweltstressfaktoren wirken können - nicht ausreichend untersucht. Im Mittelpunkt stand das Musikfestival „FM4 Frequency“ in der Landeshauptstadt St. Pölten (Niederösterreich). Diese Veranstaltung am Ufer der Traisen bietet mit mehr als 150.000 Besuchern eine großartige Gelegenheit für eingehende Forschungen. Die Menschen betreten die Flussumgebung, um sich zu erholen, sich zu waschen und zu kühlen, wodurch Abfall und andere Fremdstoffe unsachgemäß in den Fluss gelangen. Die Studie untersuchte Indikatorbakterien, gelösten organischen Kohlenstoff (DOC) und seine Derivate sowie die Beobachtung der Biofilmvielfalt an den Zementbänken im Fluss. Die Beobachtung der Ergebnisse vor, während und nach dem Festival und der Vergleich zwischen dem stromaufwärts gelegenen (nicht betroffenen) und dem stromabwärts gelegenen (betroffenen) Gebiet bildeten eine solide Grundlage für die Beobachtung der Daten ohne laterale Stressfaktoren. Die Ergebnisse zeigen, dass es einen biogeochemischen Effekt in Bezug auf erhöhte DOC-Konzentrationen sowie einen latenten Effekt auf die Biofilmgemeinschaft gibt, der höchstwahrscheinlich auf einen erhöhten Nährstoffeintrag zurückzuführen ist. Die Enterococcus-indikatoren nahmen während des Festivals signifikant zu, aber die E. coli zeigten ein völlig anderes Muster, da sie überhaupt nicht vom Festival betroffen waren. Diese Ergebnisse weisen darauf hin, dass bei der Verwendung von Fäkalindikatorbakterien Vorsichtsmaßnahmen getroffen werden müssen, da die Ergebnisse zwischen den verschiedenen Indikatoren erheblich varieren. Aufgrund der molekularen Daten deuten die Ergebnisse auf einen latenten Effekt der Diversitätsdifferenz zwischen dem stromaufwärtigen und dem stromabwärtigen Teil des Standorts hin, was bedeutet, dass das Festival das Ökosystem so stark gestört hat, dass es zu einer Verschiebung der mikrobiellen Diversität im Fluss kommt. Im Allgemeinen deuten die Ergebnisse auf eine kurzfristige Verschiebung der Umweltparameter des Flusses hin und liefern somit den Beweis, dass bei der Organisation einer kulturellen Veranstaltung in der Nähe eines empfindlichen Ökosystems erhöhte Aufmerksamkeit erforderlich ist.

Abstract

Anthropogenic effects arising in the recent decades have taken a grasp of more ecosystems than ever before. Given most of the Anthropocene studies focus on industrial settings, cultural events - although they may also act as environmental stressors - are understudied. We focused on the "FM4 Frequency" music festival held in the regional capital St. Pölten (Lower Austria). Located along the shores of Traisen river and with more than 150,000 visitors, this event provides a great opportunity for in-depth research. People are entering the riverine environment for recreation, having a wash and for cooling thus improperly disposing waste, and other foreign matter into the river itself. The study undertook fecal indicator bacteria, dissolved organic carbon and its derivatives, as well as the observation of biofilm diversity on the artificial cement banks in the river. Observing the results before, during and after the festival, and comparing them upstream (the unaffected) versus downstream (the affected) area, gave the research a solid basis for observing the data without lateral stressors. The results indicate that there is a biogeochemical effect in terms of increased DOC concentrations, as well as a latent effect on the biofilm community, most likely due to increased nutrient inputs. The *Enterococcus* fecal indicators increased significantly during the festival but the *E.coli* showed a completely different pattern by not being affected by the festival at all. These findings indicate that precaution needs to be taken when using fecal indicator bacteria, as the results differ significantly between different indicators. From the molecular data, the findings indicate a latent effect of diversity discrepancy between the upstream and downstream portions of the site, meaning that the festival has disturbed the ecosystem enough to cause a shift in the riverine microbial diversity. In general, the results indicate short-time shifts in the environmental parameters of the river, and hence provide evidence that increased attention needs to be given when organizing a cultural event nearby sensitive ecosystem.

1. Introduction

1.1 The Anthropocene

The current period of Earth is considered by some scientists to be the Anthropocene (Ellis and Trachtenberg 2014) – a period in Earth's history where humans are directly and indirectly shaping the behavior of the environment. Whether that being improper waste management, industrialization, or other public-induced actions, there is increasing evidence that humans as a species are major stressors of its surrounding ecosystems (Vitousek 1997). Since the second part of the 20th century, Austrian water quality parameters have been regularly tested, resulting in the improved conditions of national waters across the country (BMNT 2018). Nevertheless, certain unique anthropogenic actions may occur in the present day, and albeit the good regulation, need to separately be observed and accounted for.

This anthropogenic phenomenon is also recorded in aquatic habitats. For example, land use, such as urbanization and agriculture can directly affect the hydrology and ecosystem behavior of streams, by changing the flow patterns and habitats of flora and fauna (Whiles et al. 2006; Poff et al. 2006). Another important impact is distribution of feces and fecal bacteria, which can be traced and correlated to the major urban/human activity, with a stronger signal closer to the point source (Walson et al. 2001). These fecal bacteria pose a risk for both the environment, and health of the public. Additionally, from a social-ecological standpoint, human recreation has been shown to exhibit negative effects on the riverine ecosystem, through soil erosion, increased turbidity, and improper waste management, among other factors (Buckley 1991). As such, assessment of impacts on riverine ecosystems is essential for better understanding of its functions under anthropogenic pressure, as well as the necessary precautions to be undertaken in the future.

1.2 Fecal bacteria as indicators of public health

State-of-the-art water testing protocols rely on fecal bacteria as indicators of both fecal contamination of the water body, and the possible presence of water-borne, harmful pathogens (Tallon et al. 2005). In some cases, it has been found that pathogens and indicators together are the leading cause of impairment to the environmental health of rivers and streams (Meals et al 2013). Hence, due to the nature of the festival, this project was deemed

as a good opportunity to test for this type of an environmental indicator, by focusing on two standard measurements – *Escherichia coli* and *Enterococcus*.

E.coli is indigenous to the intestinal flora of warm-blooded organisms. It has served as a primary indicator of intestinal bacterial dynamics (Edberg et al. 2000). It has been shown that other indicators were present in higher concentrations even when no fecal load had actually occurred, due to their thermotolerance rather than actual fecal indication (Gauthier and Archibald 2001). Hence, there are findings that support the notion that, since *E.coli* is the only fecal coliform exclusively stemming from a fecal environment (Bej et al. 1990; Baudisova 1997) it should be considered as a primary object of research in this type of assessment.

Enterococcal bacteria (EC) are another type of frequently used fecal indicators. They are commonly found in the intestines and feces of warm-blooded animals (Tallon et al. 2005). An additional characteristic of this group is that it is less susceptible to certain environmental factors, such as osmotic stress (Bordalo, Onrassami, and Dechsakulwatana 2002) or discharge (Laureano-Rosario et al. 2017). As such, for a further fortification of potential findings, as well as for an addendum to the continuing debate of which indicators to use, we also included *Enterococcus* in the study.

1.3 Benthic biofilm – a disturbance indicator

Ecosystem community with all of its components such as species number, similarity, and diversity, exhibits responses to rapid environmental changes that come about (Proia et al. 2013; Yule et al. 2010). Analyzing the community provides essential information as to which group is affected, by what means, and by whom. In a riverine ecosystem, different communities can be analyzed with respect to their habitats, as in the case of plankton versus benthos. This type of impact assessment here focuses specifically on the benthic biofilm community, as to see how it would react to a potential short-term alteration such as this festival. Biofilms, an assemblage of microbial organisms attached to substrate surfaces and immersed in an extracellular matrix, are regulated by a wide variety of different environmental and genetic factors (Maric and Vranes 2007) making them ideal high-quality indicators of ecosystem health (Bailey 2011; Burns and Ryder 2001). Additionally, they are an important component in freshwater carbon fluxes (Ask et al. 2009). In terms of ecological dynamics, biofilm was previously used in assessing community composition, performing

different non-metric and diversity and abundance indices (McClellan et al. 2008; Tlili et al. 2011).

1.4 The biogeochemistry of the river – a standard for limnology

The components that are a standard for measuring in almost all riverine environments are concerned with the biogeochemistry of the ecosystem, such as dissolved organic carbon (DOC), nitrogen compounds, oxygen and other parameters indicative of its functioning. More specifically, these patterns can further explain the stream carbon balance (Kolka, Weishampel, and Fröberg 2008), the type of organic compounds found or emerging in the system (Weishaar et al. 2003), the changes that are occurring in the system (Gergel, Turner, and Kratz 1999), or in general, the non-altered behavior of DOC behavior of a system (McDowell and Likens 1988). Furthermore, these values have been shown to be of importance, when associated with other environmental variables, such as a positive relation to fecal bacteria number (Guber et al. 2006), and the bacterial community in general (Wetzel 1992), making them an ideal medium to observe ecosystem changes. The derivatives of DOC, specifically Specific Ultraviolet Absorbance (SUVA) (Weishaar et al. 2003), as well as different absorbance spectra such as the Ultraviolet absorbance at 254nm (SAC_{254}) have been shown to serve as a predictor of fecal pollution (Stadler et al. 2010), as well as an assessment tool for organic pollutants (Rößler and Metzger 2016). Thus, these measurements can certainly unearth answers to questions of the chemical behavior of the river.

1.5 Approach and questions examined

We focused on examining if there was a direct effect of the festival on the aforementioned variables. We expected a significant difference between the upstream and downstream sites during the festival, as compared to before or after due to the sudden influx of people, organic matter and other potential environmental stressors. We assumed the concentration of DOC and its derivatives (values that are mathematically derived from the raw measurements) to be significantly higher downstream versus the upstream sections during the festival, as compared to before or after the festival, due to the input of organic matter such as waste or excrement. Additionally, the fecal indicators *Escherichia coli* and *Enterococcus* were hypothesized to be significantly higher in the downstream portions of the river during the festival, as compared to before or after the festival, due to disturbance caused by entrance

into the stream during the festival. As for benthic biofilm, the hypothesis was that there would be a significant difference in community composition as a result of the festival.

2. Materials and Methods

2.1 Site description and sampling procedure

The Traisen river, one of the Danube tributaries, is located in the province of Lower Austria. The catchment consists of agricultural, riparian and urban areas. The river itself has been subject to intense measures of river regulation, mostly due to urbanization initiatives, such as the construction of the nearby Altenwörth power station among other factors. That, combined with the impermeable surfaces set up along the inhabited part of the basin, serves as an example of a riverine ecosystem altered by the Anthropocene.

We studied a stretch in the close vicinity the city of St. Pölten, the capital of Lower Austria. We focused on the stretch of the river mostly affected by the festival and sampled the river just after the festival area (downstream site 48°09'39.9"N, 15°37'45.7"E). As an unaffected reference site, we sampled also upstream the festival area (48°11'33.2"N, 15°38'00.0"E). Excluding the festival event, the river serves as a recreational site – with bike pavements consistent along the bank, and pedestrian cement crossings set up at equidistant portions across the river.

In the year 2018, the festival took place from 16th to the 19th of August. During this time, the following irregularities, as compared to the remainder of the Traisen survey were observed: a sudden, large, densely distributed group of people, concentrated specifically between the project's upstream and the downstream sites, and high input of waste (compostable, metallic, plastic and cardboard). We found constant motion of people along the banks of the river, and repetitive entering and staying in the river for prolonged periods (refreshing, bathing, having a wash). Although portable toilets were installed in the festival area, misuse of the river for this purpose could not have been ruled out. There were many visitors already present one day before the official start of the festival (15th), possibly setting up camping areas. Some people stayed a few days longer than the festival took place (20th and 21st of August), but these occurrences were much less in numbers, as compared to the festival period itself.

2.2 Fecal bacteria in the water column

Sampling for fecal bacteria was done before any other sampling. 250 mL autoclaved bottles were taken, and four sample replicates were collected at both the upstream and downstream sites. Then, after entering the river, the bottles were submerged to 30cm below the surface, where they were uncapped and filled. In order to avoid any reactions, the bottles were kept in a cooling bag and afterwards were refrigerated at 4°C and processed within a 24 h according to the manufacturers advice and other recommendations (Myers and Sylvester 2003).

Concentration of fecal bacteria in the water column was tested by microplate arrays. For the processing of the water samples, industrially tested ISO-certified MUG/MUD plates (Biorad GmbH) were used. These 96-well platelets had an implemented reagent within each well which would illuminate under ultraviolet radiation if the well would contain the targeted bacterium. As a rule, the number of dilutions to inoculate varies according to the presumed level of contamination to be tested: first, the bottle of the sampled river water was taken out of refrigeration. 9mL of river water was transferred into a into a Greiner tube which contained 9mL of microplate diluent (S.M.D. code 53784). After vortexing the mixture, the now diluted sample was transferred into another 9mL of the MiliQ water – SMD mix, thus obtaining a 1/2 dilution and a 1/20 dilution, respectively. Then, the two different dilutions were transferred into two separate autoclave-sterilized petri plates, from where, with an 8-fold multipipette, the petri dish content was transferred into the wells of the microplate. Out of the 96 wells, 64 wells (8 columns) were filled with the 1/2 dilution, whereas the remaining 32 wells (4 columns) were filled with the 1/20 dilution. The now filled chambers were left to incubate at 44±0.5°C for a minimum of 36 hours, after which the samples were read using a handheld UV light lamp.

As the festival approached, a concern emerged that the two-dilution readings will not give a high enough resolution. To avoid values that are beyond the readings of the plate, we went for four dilutions during the festival period. Sampling was identical, as well as the transfer and preservation of the samples, as compared to the two-dilution technique, but there were four different fractions now involved : 1/2, 1/20, 1/200, 1/2000 respectively, all transferred into individual autoclave-sterilized petri-dishes, and pipetted into the wells, which were now separated into equal proportions of 24 wells per dilution. This procedure was extended up until the 23rd of August, 3 days after the closing of the festival, in order to observe any

potential latency effects, after which the two-dilution method was conducted until the end of the sampling season.

After incubation, plates were analyzed in a dark room using a handheld UV light. The illuminated cells were manually counted and according to the manufacturers table, the most probable number (MPN) was determined. This MPN is based on Poisson's law according to which the statistical table has been made. The illuminated wells for each dilution, when combined, gave the MPN. For instance, an example of MPN calculations for 2-dilutions and 4-dilutions are represented in the Appendix.

2.3 Analysis of chemical components

Basic parameters such as water temperature and pH were also measured on-site by handheld apparatus. As for the biogeochemical components, two methods of measurement were used: (1) continuous in-situ monitoring via a spectrolyzer (S::CAN company), and (2) ex-situ, laboratory analyses.

The S::CAN spectro::lyzer is an industrial-scale apparatus used for in-situ measurement of water quality by measuring the entire absorption spectrum, providing the examiner with a variety of different characteristics of the water (SCAN 2019). It has been used elsewhere (Stadler et al 2017; Miles et al. 2011), with comparable results and as such, served as a good medium for in-situ observation of the desired variables. Probes, once installed, took readings of UV absorption spectra, specifically focusing on specific absorption coefficient at 254 nanometers (SAC_{254}). The measurements were taken in 15 min increments, with certain pauses, either for maintenance or repairing of technical malfunctions. Locations of the probes were both upstream and downstream of the festival, however, with a larger discrepancy in the time of the set-up between the two sites. On the downstream site, the probe was installed in early June, and on the upstream site in mid-July. This, and the fact that the downstream probe had less tampering and stoppage of measurement, ultimately gave it a higher importance when temporal values were observed and correlated with other datasets.

Besides the real-time spectrolyzer monitoring, vials were also acquired in order to sample the water for DOC analysis. Pre-treatment of vials was done according to a guideline established by the Lunz Research center (Appendix). The samples were taken via a pre-treated syringe, which was used to take the river water. Afterwards, after implementing a sterilized glass microfiber GF/F filter on the syringe tip, the sample was injected back into the vial, in order

to purify the sample from particles. The vials were then placed in a cooling box and transferred to be measured within a week of sampling. DOC analysis was performed in collaboration with Astrid Harjung (University of Vienna) on a GE-Sievers 900 TOC analyzer (SUEZ Water Technologies & Solutions, Trevose, PA USA) which was equipped with a persulfate oxidation and an inorganic carbon removal unit.

Additionally, an autosampler was used between the two last days of the festival, with the goal of observing the biogeochemical parameters in a higher, hourly resolution for 24 h. The samples were then transferred to the laboratory in less than 5 h, where they were further individually analyzed for DOC in the laboratory in the same manner as the regular in-situ samples.

2.4 Benthic biofilm analysis

We sampled the biofilm from the in-river cement crossings in both the upstream and downstream sites. Using an Eppendorf tube and leaning the edge of it against the diagonal side of the pathway, 15 to 20 cm of biofilm streak was dragged out of the river, with the effort to suffer minimal loss of biofilm in the process (Rao 1993). Then, after waiting briefly for the particulate matter to rest on the bottom of the tube, excess water was poured back into the river. Enough water was kept in order to successfully freeze the biofilm samples without damaging them. Four replicates were taken on each day of the sampling and all of them were frozen at -80°C in less than 10 h after sampling, while in the meantime being kept cool.

Sample processing for molecular biology was performed approximately one month after sampling. In order to avoid any damage to the biofilm, vials were first transferred from the freezer into a refrigerator in order to thaw enough but remain as intact as possible. The vials were then placed horizontally in a vortex until the biofilm was fully dispersed. Then, 100µL of the homogenized sample were extracted and placed into a set of bead solution tubes, the first step of the PowerSoil© DNA extraction kit (QIAGEN, 2014), the rest of it being explained in the appendix (Appendix). The DNA extract was then placed in a freezer at -20 °C and kept as such until the confirmation of the sequencing date. Once the date has been confirmed, the extracted DNA material was pipetted into microplates, each with its specific code relating to the date and locale of the festival and were sent immersed in dry ice to a third-party laboratory for 16S sequencing. An affiliated company performed the necessary PCR and primer selection. The results of sequencing conducted by this third-party were then returned in the form of fastq files. Pre-processing was conducted by Katharina Besemer

(University of Vienna). This preprocessing through a QIIME2 bioinformatics pipeline (Caporaso et al. 2010) provided results on specific characteristics of each Operational Taxonomic Unit (OTU) involved in a sample. After the preprocessing, singleton and doubleton OTUs were removed (Chiu and Chao 2016), as well as the OTUs where no blast hits were observed. This shortened the dataset from around 14000 OTUs to around 11000 and thus gave a higher resolution of the overall status of the microbiome diversity in the samples.

2.5 Statistical analysis

The statistical significance was assessed spatially and temporally. The spatial set-up followed the upstream-downstream model, meaning that the upstream part of the river was presumed unaffected and as the river progresses through the festival´s stressors, it increases in concentrations of the observed matter. The temporal pattern was tested stage-wise: the pre-festival stage (mid-July to 15th of August), the festival stage (16th to 19th of August) and the post-festival stage (until late September). After MPNs were obtained, a Two-Way ANOVA was applied. However, due to the lack of data normality, a post-hoc test was used, namely the Tukey-Kramer test with two variables: “Sites” – upstream versus downstream, and “Stage” – before, during and after the festival. Using the statistical software SigmaPlot ©, the results were both statistically analyzed as mentioned above, and later plotted via the same software.

The real-time, spectrolyzer data was viewed as a time series and in general, was conducted mostly for an overall pattern to be observed. In the cases of statistical observation, the real-time data readings were extracted to be identical in the date and time to other, ex-situ analyzed samples treated as single, point data, in order to increase the precision of correlation values. More specifically, DOC and $SAC_{254,}$ as well as the discharge data from the city authorities, were taken into account and represented.

The sampled DOC values were taken at a minimum of three field replicates per site and date, and statistically analyzed in an identical process as the fecal indicators – using the SigmaPlot© statistical software, a post-hoc test, more specifically the Two-Way Tukey-Kramer test was conducted, with locale of the festival and the stages of the sampling as the two components. The same concept was applied for the 24-hour autosampler dataset, where the two factors were locale of the festival and the phase of the day (i.e. daylight versus night), respectively. The aforementioned software was then used to plot the graphics for best possible representation of the results.

Statistical analysis of molecular data was done with the "Vegan" package, focusing on ecological dynamics (Oksanen et al. 2007) as well as the taxonomy-specific package "Phyloseq" (McMurdie and Holmes 2013), both packages part of the R statistical software. Non-metric multidimensional scaling (nMDS) using the Bray-Curtis dissimilarity (Equation 1) as the distance parameter (Bray and Curtis 1957) was performed for the observation of the community distances. The nMDS simulation was ran in a 2-dimensional setting with 20,000 iterations, until the simulation stress was low enough. This was accompanied by a statistical analysis in the form of Analysis of similarities (ANOSIM) implemented in three different scenarios – the "before", "during", and "after" scenarios observing the upstream versus downstream portions the results of which are represented in the results section. The actual scripting of the code is represented in the Appendix.

$$BCd = \frac{\sum|xi - xj|}{\sum(xi + xj)}$$

Equation 1 : The Bray-Curtis dissimilarity index, whose resulting distance is further used to be implemented in the nMDS calculation

3. Results

3.1 Fecal indicators

Escherichia coli did not show a pattern connected to the festival (Figure 1). In the pre-festival period and the period of the festival, differences were not significant (Two-Way Tukey Test $p > 0.05$) between the upstream and downstream components of the festival. Additionally, there appears to be no significant difference between the values during the festival, either. One thing to take note of, is the fact that both the upstream and downstream values were very high at the last measuring point in autumn, albeit both nearly equally high.

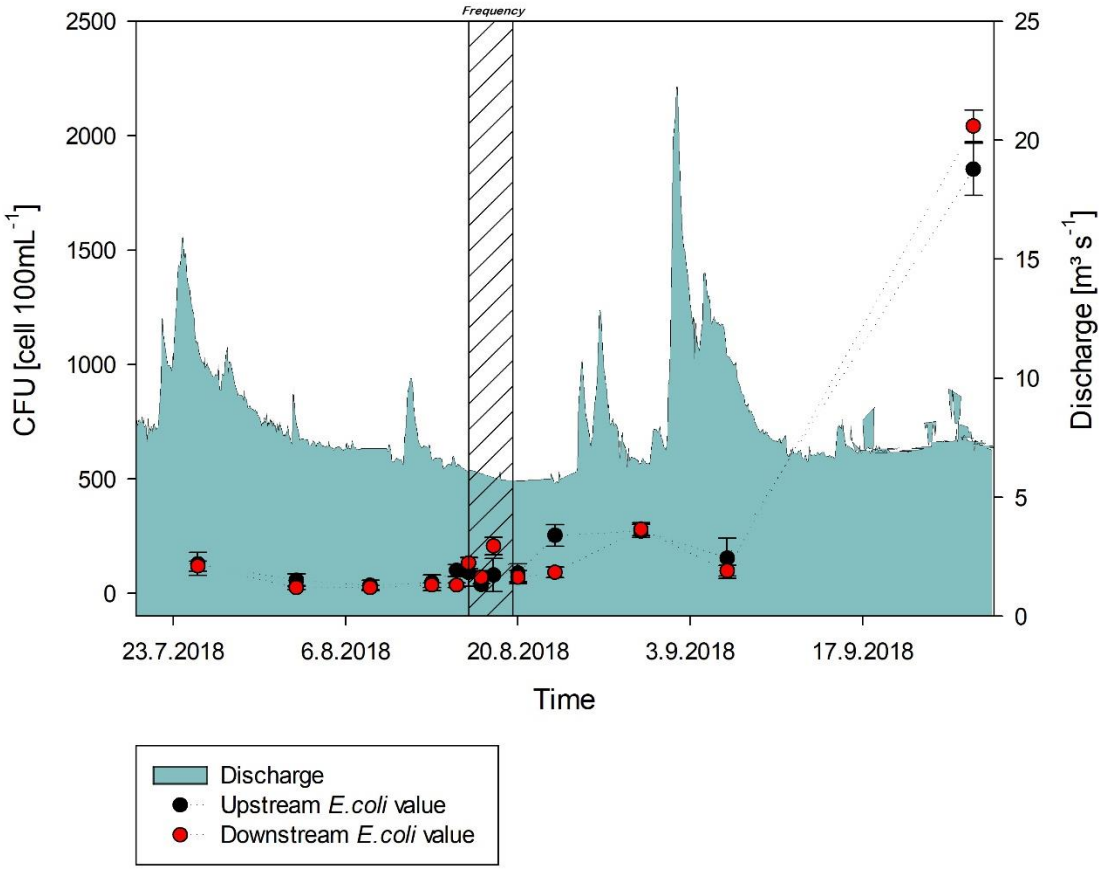

Figure 1 : *E.coli* cells per 100mL, both upstream (black) and downstream (red), over the course of the sampling. The festival period is marked in the rectangle portion.

In the case of *Enterococcus* (Figure 2), although both upstream and downstream values reached very high concentrations, significant differences were observed between the upstream and downstream portions of the river during the festival ($p < 0.05$), whereas there

was no significant difference neither before nor after the festival. However, the *Enterococcus* cells were observed to be very latent in their concentration, and gradually lowered some time after the festival, instead of exhibiting an immediate lowering effect after the people attending the festival have cleared out from the premises.

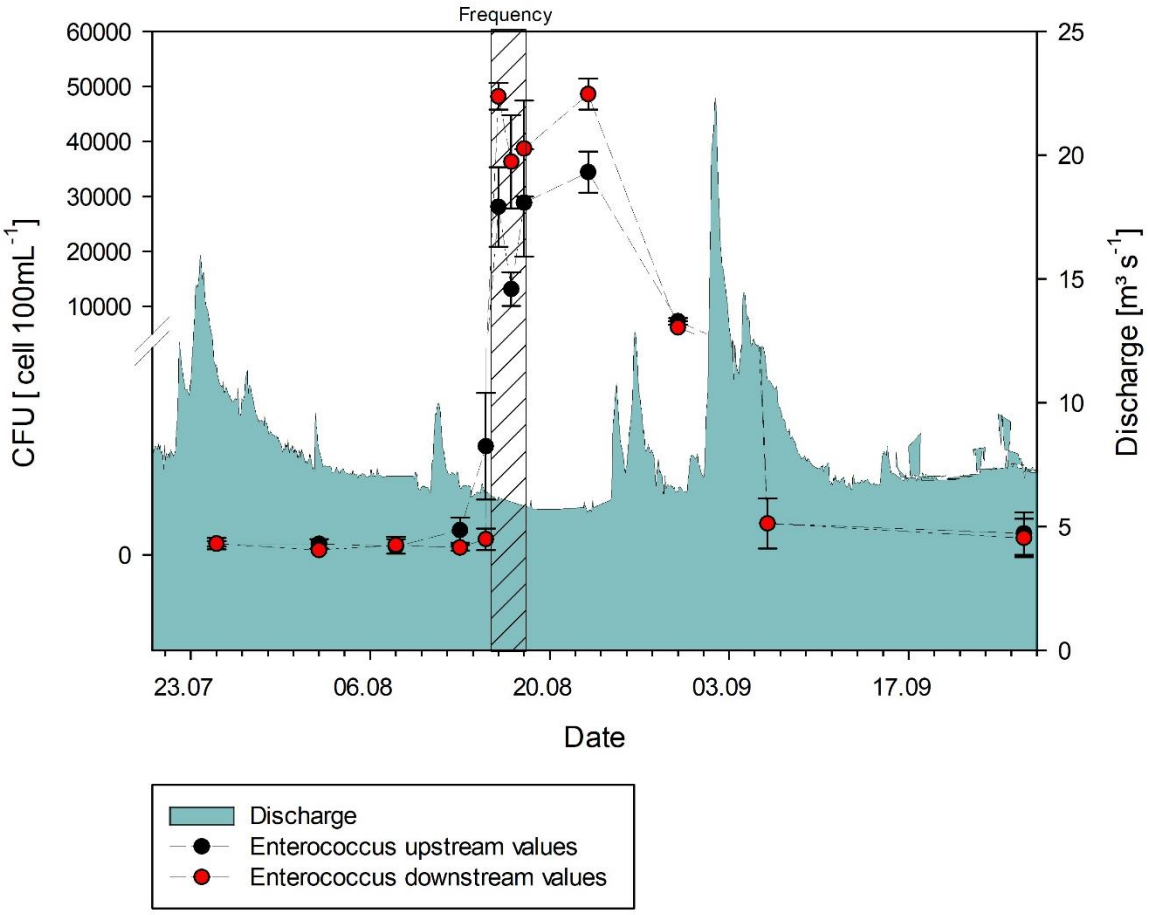

Figure 2 : The concentration of *Enterococcus* fecal indicator per 100mL both upstream (black) and downstream (red) during the sampling period. Notice the retention of high concentration even after the festival.

3.2 Chemical analysis and environmental parameters

During the sampling season, the discharge values, according the St. Pölten water authorities, ranged from 6 to 22 m^3s^{-1}. During the actual festival, coincidentally, the values were some of the lowest recorded, and there were two peak events, namely end of July and beginning of September, which reached above the 15 m^3s^{-1} measured value, whereas on average, the value was around 9 m^3s^{-1}. The pH values did not significantly change throughout the sampling season and remained in the close vicinity of 8.4 pH, varying by 0.2. The temperature

measurements were mostly around 22 degrees Celsius, except during peak rainfall events, when the value would decrease down to 12 degrees.

The laboratory-analyzed DOC samples exhibited values that were significantly higher during the festival ($p < 0.001$) as compared to both before or after the festival, with the discrepancy between the two sites lowering immediately after the festival's occurrence (Figure 3). The downstream spectrolyzer probe exhibited an anticlockwise hysteresis (Figure 4), whereas the upstream probe was disregarded due to having much of the data missing.

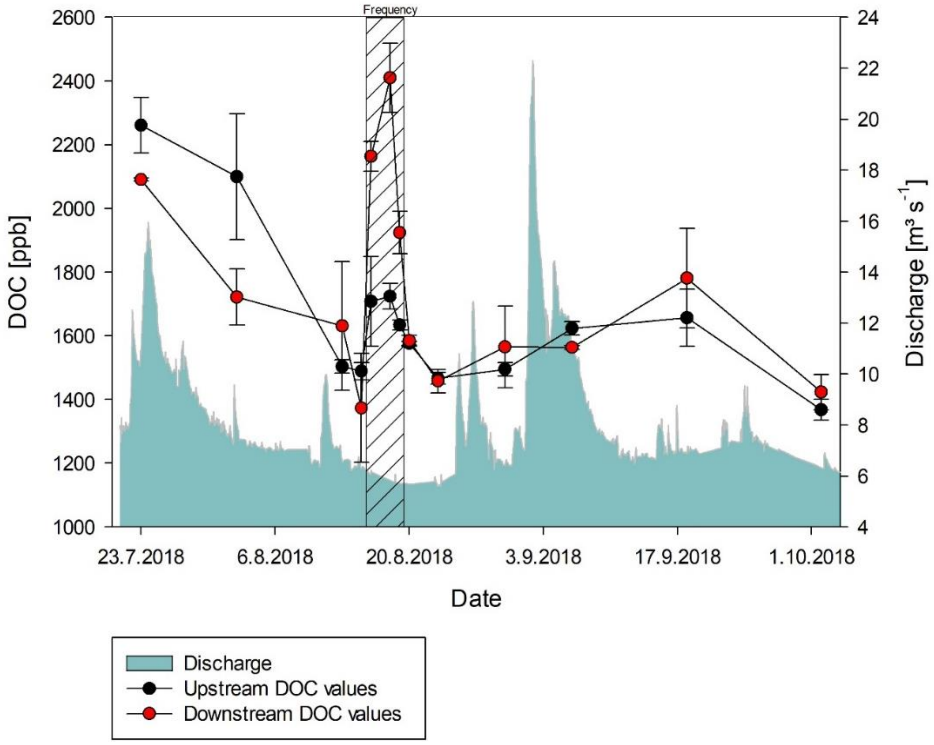

Figure 3 : Dissolved organic carbon (DOC) concentration, analyzed in a laboratory setting.

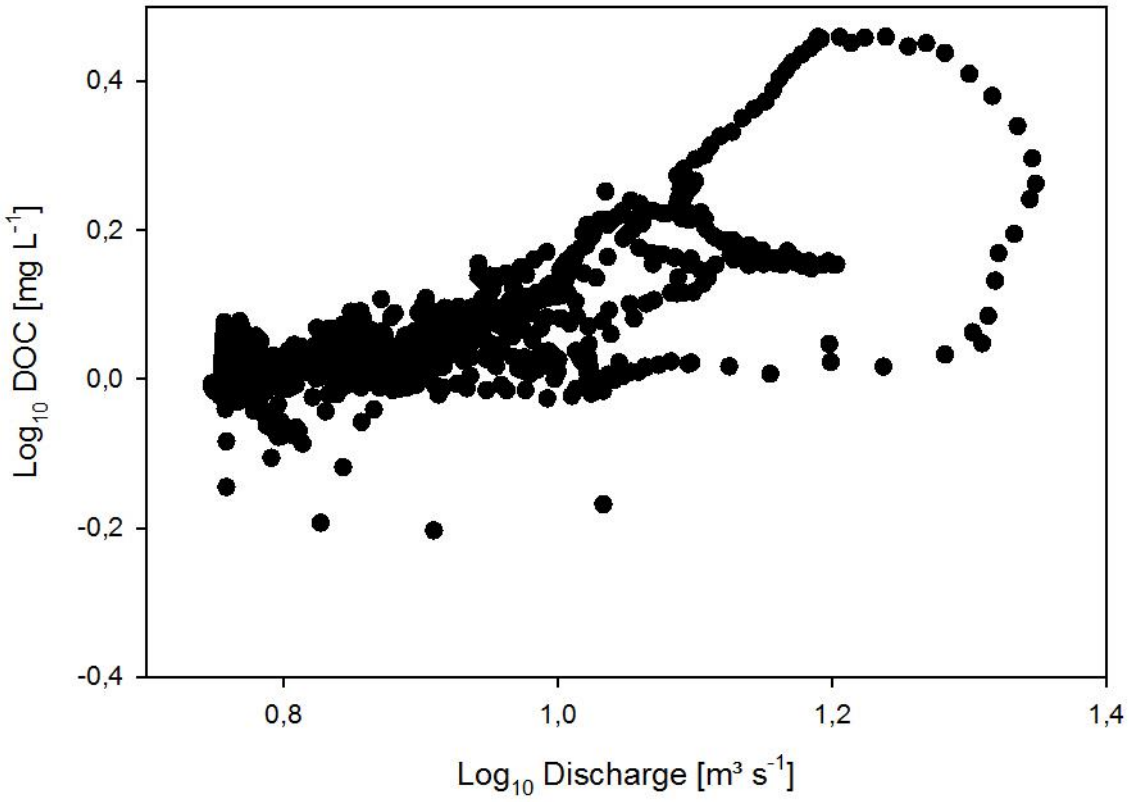

Figure 4 - an anti-clockwise hysteresis pattern observed when spectrolyzer DOC was correlated with the discharge of the Traisen river. Notice the clear hysteresis loop, where the shift is observed.

The analysis of the autosampler-based DOC measurements resulted in a significant difference between the upstream and downstream sites both during daytime (until 21:00 hours) and at nighttime (from 21:00 until 06:00 hours), with the closest value, almost an intersection between the two points, arising at 03:00 AM, and then again diverging in separate directions as the new day arose (Tukey Kramer post-hoc test $p < 0.05$) (Figure 5).

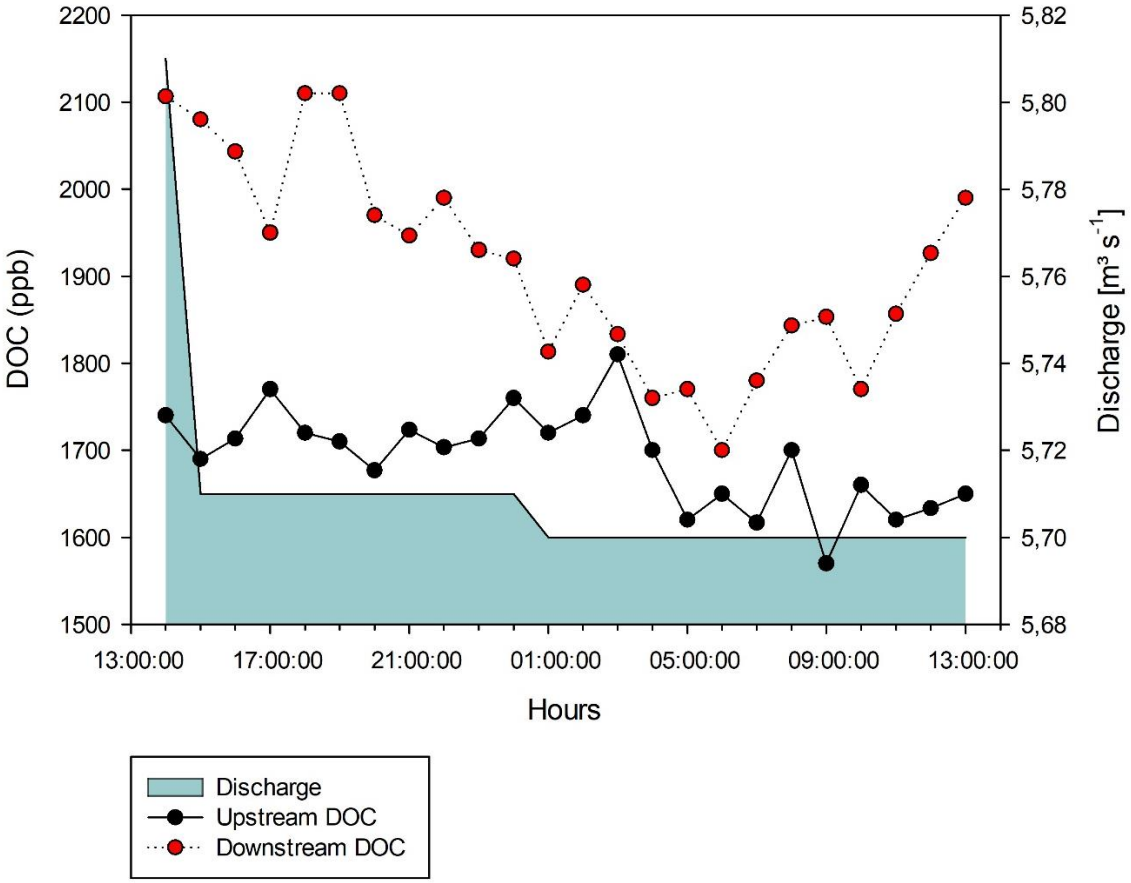

Figure 5: The hour-based incremental values of DOC in between two festival dates, with respect to the upstream and downstream portion of the sampling site.

We found a positive correlation between *E.coli* and SAC_{254} (R^2 = 0,6258), and a negative correlation (R^2 = 0,4914) of *Enterococcus* cells versus the riverine discharge, respectively. Additionally, SAC_{254} to *Enterococcus* observation resulted in a positive correlation (R^2 = 0,5328), as well as DOC versus *Enterococcus* (R^2 = 0,5583), while almost no correlation was observed in either *E.coli* with respect to discharge (R^2 = 0,008) or with *E.coli* with respect to DOC (R^2= 0,0822 ; Figure 6).

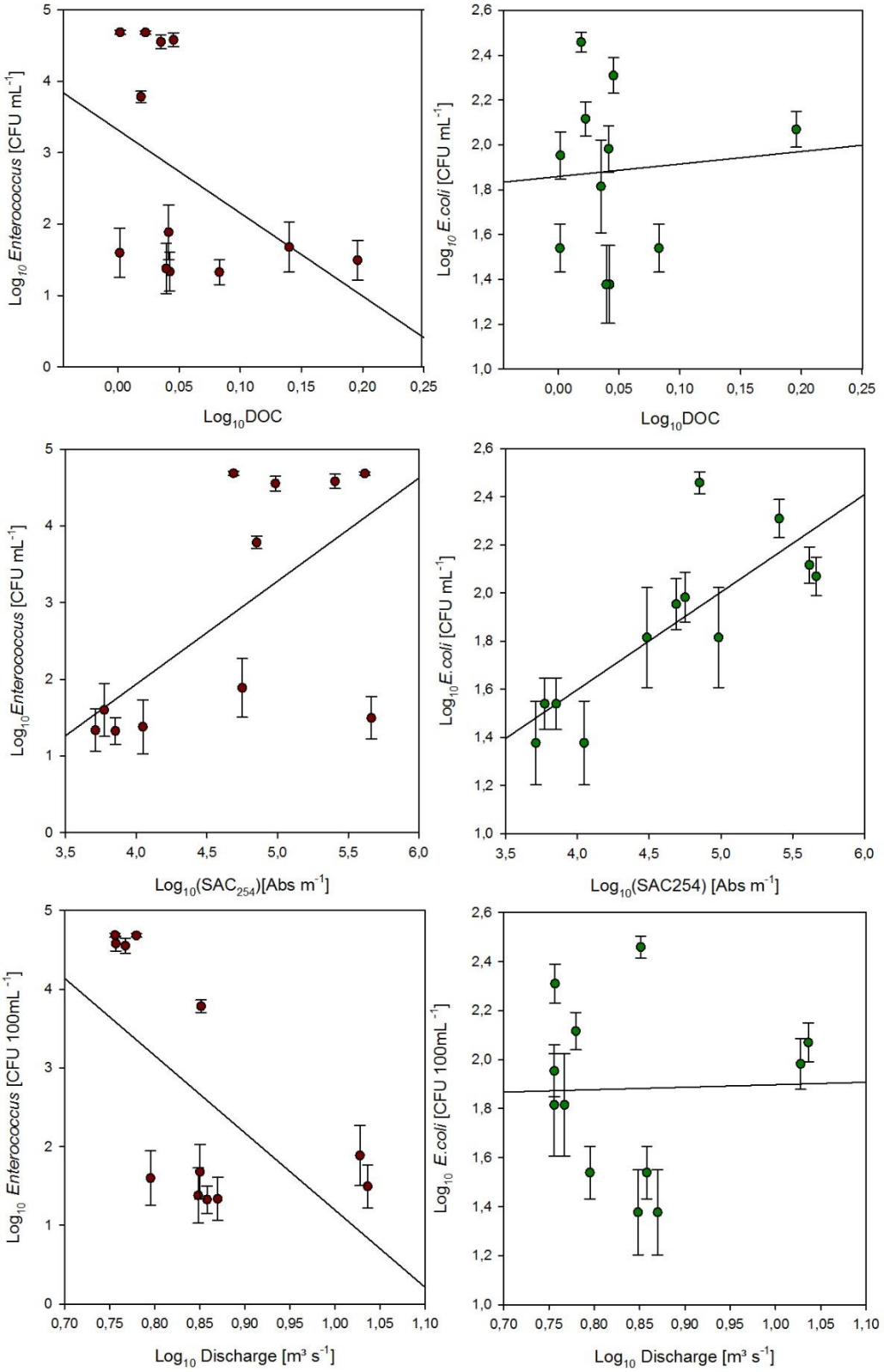

Figure 6 : Correlations of different environmental parameters (DOC, Discharge and SAC254) with *E.coli* and *Enterococcus cells*, respectively, each done in a set of 12 replicates.

3.3 Biofilm community analysis

The relative abundance of the bacterial biofilm species was modelled (Figure 7), as to give a visual representation of the 10 most abundant phyla (more than 95% of the whole abundance) and their diversity dynamics throughout the duration of the experiment. No patterns of individual bacterial species, in terms of species richness, are pointing to a sharp effect of the festival (samples 6 through 8).

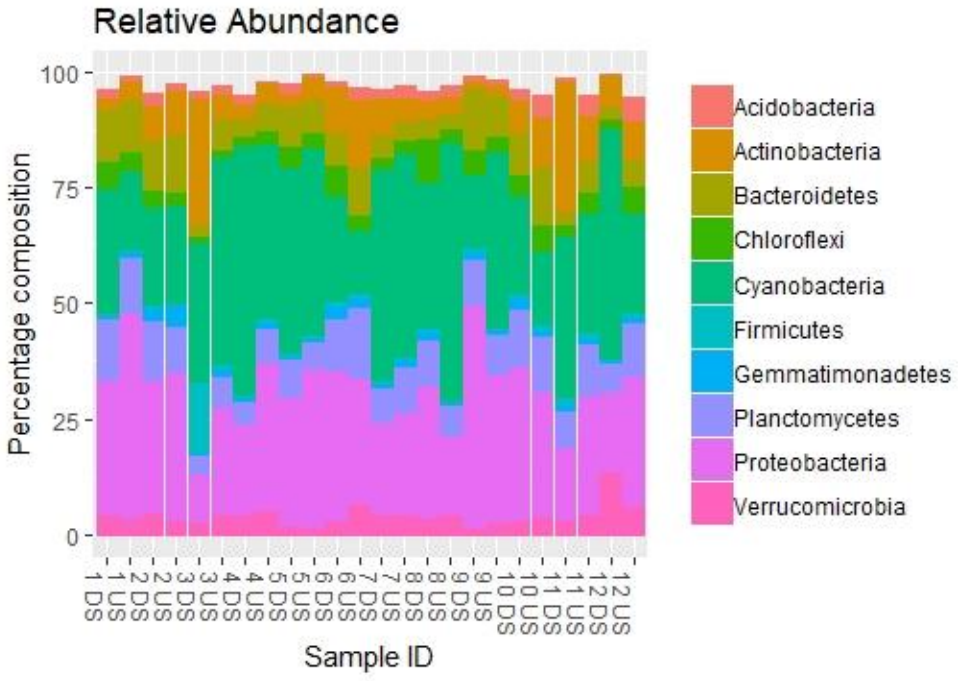

Figure 7 : Representation of relative abundance with respect to the top 10 most occurring Phyla. Samples 1 through 5 are before the festival, 6 through 8 are during, and 9 through 12 are after the festival. The US specifies the festivals in the Upstream portion of the river, while the DS specific the samples in the downstream portion of the river.

The nMDS analysis of 16S DNA community composition resulted in a clockwise pattern of community divergence after the festival (Figure 8). The distances between upstream and downstream communities did not differ so much before and even during the festival. However, the more time passed after the festival, the closer the community came back to its similarity.

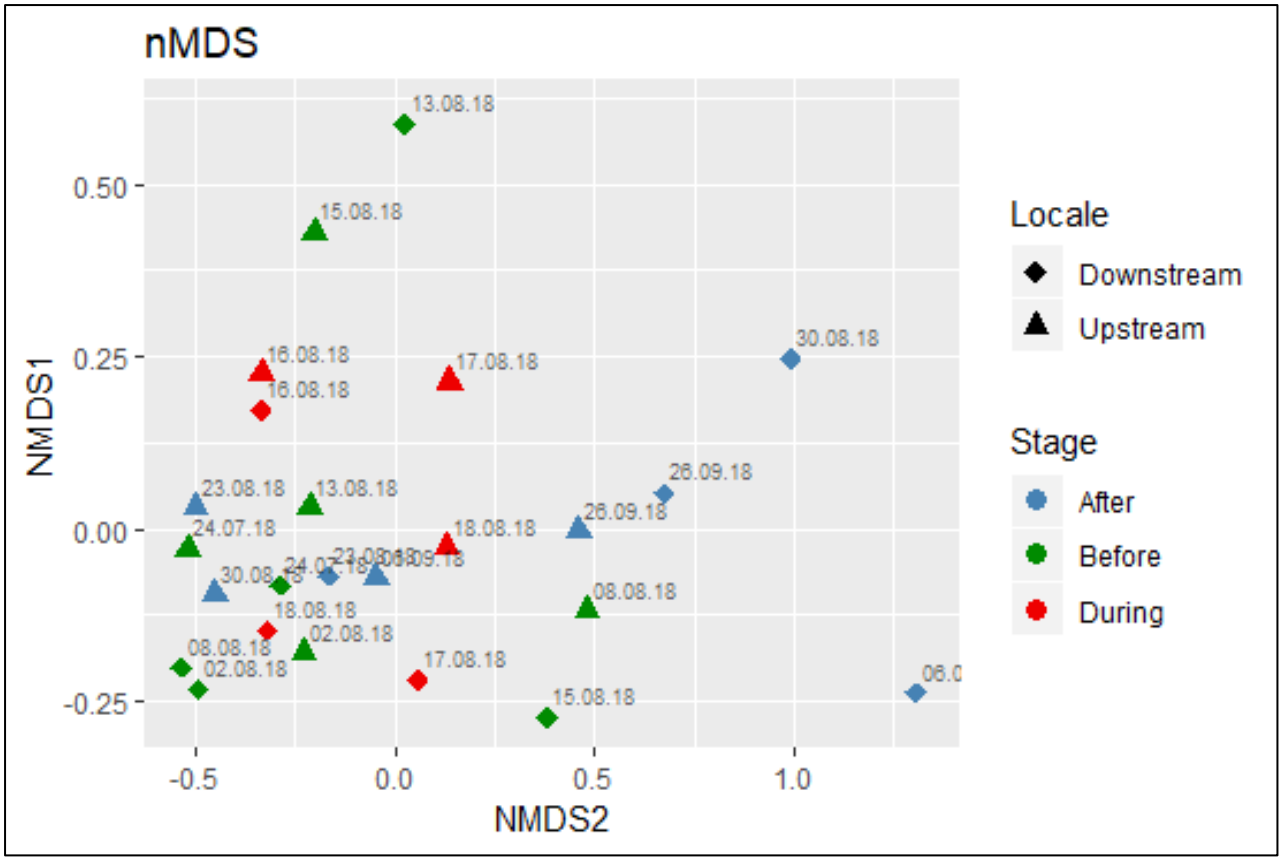

Figure 8 : The Non-metric multidimensional scaling (NMDS) simulation as per the R "vegan" package statistical software. Using the Bray-Curtis dissimilarity index, the communities described in the figure show a clear pattern of a large community distance divergence after the festival, as opposed to before or during the festival.

ANOSIM testing showed a significant difference ($p < 0.05$) between the upstream and downstream points after the festival, as opposed to before or during (Table 2).

Table 2 : The statistical results of an ANOSIM test of the upstream versus downstream values on different stages of the festival.

Stage of sampling	Number of samples per stage	P-value	Status
Before the festival	5	0.067	Insignificant
During the festival	3	0.4	Insignificant
After the festival	4	0.04	Significant

4. Discussion

4.1 Fecal indicator bacteria

In general, the whole concept of the use of fecal cell readings to establish the current state of environmental pathogens in question remains a debated topic. Different research scenarios provide different outcomes in terms of bacterial success, leading to, for instance, a heavy criticism of the concept in sediment microbiology (Davies et al. 1995). Conversely, the concept has been praised in alpine mountainous waters (Farnleitner et al. 2010). These opposing findings further continue this debate.

Both *E.coli* and *Enterococcus* cell counts are used as a standard in public health studies as an indicator of the presence of human feces in the sampled environment (Griffin et al. 2001; Myers and Sylvester 2003). Surprisingly, we found a discrepancy between the two indicators. The question that remains is, which bacterial cells indicate what. Kirschner et al. (2009) provided a table for interpretation of *E.coli* and *Enterococcus* numbers at different levels of pollution (Table 3). We took this table as a reference for the severity of the enteric pollution of the river.

		I	II	III	IV	V
Parameter	Concentration	Little	Moderate	Critical	Strong	Excessive
E.coli	100mL	<100	>100-1000	>1000-10,000	>10000-100,000	>100,000
Enterococci	100mL	<40	>40-400	>400-4000	>4000-40,000	>40,000

Table 3 : representation of the excerpt from Kirschner et al. 2009, describing the quantity of bacteria with respect to public health concern.

The concentration of *E.coli* cells was – except the final sampling – low and did not significantly differ at any of the three stage's average values. In late September, a much higher, as per the aforementioned table "critical" value was recorded. Obviously, *E.coli* growth was promoted by other factors than the festival activities 6 weeks before, because other samplings after the event showed not increase. Previous studies revealed an optimal temperature range (Carrillo, Estrada, and Hazen 1985), nutrient addition (Topp et al. 2003), and appropriate growth substrate (Whitman and Nevers 2003) as possible candidates. Most likely, the latter two have contributed to the peak of the *E.coli*. The surrounding economy

being mostly agricultural suggests that this peak actually arose from the bovine manure, applied only after mid-September in temperate climates (Madison et al. 1995). As both upstream and downstream values were high, the source of pollution was located upstream our sampling sites. Additionally, the strong correlation between SAC_{254} and the concentration of *E.coli* indicates that the activity of this species was more dictated by water chemistry, rather than by human interference. This pattern was also found by (Kellner et al. 2014). In another study, SAC_{254} turned out as an early indicator of *E.coli* (Stadler et al. 2010).

Enterococcus readings on the other hand showed a different pattern, primarily by exhibiting a significant difference between upstream and downstream portions during the festival. The values of cell concentrations were on the high level, and according literature parameters even excessive (Kirschner et al. 2009) at the time of the festival. Up to almost a week afterwards, the concentration persisted at a high level because of only low channel flow (Laureano-Rosario et al. 2017).

Although we found a statistically significant difference between up- and downstream *Enterococcus* numbers, which was caused by visitors, upstream values also largely increased during the festival, albeit to a lesser extent than the downstream values. This phenomenon may be caused by non-point sources, for which *Enterococcus* is known for. Bird droppings (Kirschner et al. 2004), and other warm-blooded animals (Tallon et al. 2005) could have been the major sources. Malfunction or waste mishandling of upstream wastewater treatment which was found elsewhere (Garcia-Armisen and Servais 2007) can be ruled out, because other parameters did not show such a pattern.

4.2 Chemical analysis

With respect to DOC, we found significant differences between the upstream and the downstream sites during the festival, as opposed to either before or after the festival. This pattern disappeared immediately after the festival was concluded. The difference in DOC proceeded to be equidistant for the remainder of the sampling season. These findings strongly indicate that the festival activities have been the main cause of additional DOC into the river, possibly due to the input of various organic matter into the stream, such as beer, whose composition on average contains roughly 800 organic compounds (Buiatti 2009), food waste, which was constantly observed around the site and in the river during sampling, as well as fecal matter, and other recreational and industrial organic pollutants (Muralikrishna and Manickam 2017).

One might argue that this is possibly the natural pattern of the DOC in the river system, given that there were high values observed prior to the festival at the first sampling point. However, even when the values were high outside of the festival period, both upstream and downstream values were high, with no significant difference in between, hence the concentration obviously being related to a flood event that occurred at that time, from the upper sediment layers transportation (Morel et al. 2009). This is further confirmed from the spectrolyzer probes, where a formation of an anti-clockwise hysteresis of the flow-DOC dynamics indicate that soil water runoff was a major contributor to the observed DOC concentrations with respect to hydrological events (Figure 4.), as suggested in prior publications (Evans and Davies 1998).

Even in the higher resolution observation, where the data were autosampled hourly for 24 h, has the concentration been significantly different between upstream and downstream. The closest that the upstream and downstream values came together was around 4 AM, when the last stage had finished its performance, and the festival was finished for the day. One thing to mention is that, during night time, although significantly different, both values were much lower than during day time, and again increased in concentration as the new day emerged. The most probable explanation would be the lack of visitor activity at that time, but it is appropriate to also mention the current trend of findings relating to the importance of photodegradation on the amount of dissolved organic matter. Once affected during daylight, it results in a variety of photoproducts, including carbon-based, to emerge (Moran and Zepp 1997).

4.3 Biofilm community analysis

The nMDS performed with the 16S sequenced data did not show visibly segregated communities with respect to the pre-, during and post-festival stages. However, as the festival ended, the upstream communities diverged from their downstream counterparts. This phenomenon extended to two weeks after the festival has ended, at which point the communities started to shift back to their original state and their respective site-specific differences. This statistically fortified shift in community composition after the festival (ANOSIM, $p < 0.05$) indicates a delayed effect of the biofilm response to the festival. Until the end of the festival, community composition of the sampling sites was comparable. In this period, the discharge was consistently low ($Q = 6 m^3 s^{-1}$), but afterwards was followed by two high-water events. As per the intermediate disturbance hypothesis (Sommer 1995), one

would expect the divergence to occur simply due to a high enough disturbance, caused by discharge. However, the upstream communities should have then shifted, if they were similar to begin with. Therefore it could be possible that the higher intensity of discharge could have benefited the community divergence in terms of nutrient output. The variability of nutrient loading and the dynamics of discharge are directly affecting the concentrations of organic matter (Bianchi et al. 2004), as well as the overall contribution of the sediment DOM to the body of water (Reader et al. 2019). Some of this DOM might have been deposited in the sediment during the festival, either via improper waste management, defecation or some other factor. This information can further serve as to the question of how much, and for how long, does an anthropogenic effect really take place, and to assess the reversibility of the impact as well.

5. Conclusion

We proved impacts of the festival on the riverine environment, addressed on multiple spatiotemporal resonances. This study thus serves as an addendum to the ever-growing list of potential anthropogenic effects that are arising in the modern era. Further it pushes the debate of which analytical tools to use, in order to assess an impact fecal contamination. We found visible and also measurable impact of the festival attendees on the biogeochemical and microbiological parameters of the river. With respect to fecal indicator bacteria, the results are inconclusive with respect to studied bacterial cultures. Although a significant signal was present in the *Enterococcus* cells between the upstream and downstream portions during the festival, we found also an unexpected upstream increase, which cannot be explained by festival attendees. The findings call for a longer study period of the river´s microbial dynamics.

Regarding the DOC findings, the hypothesis was fully confirmed, as its concentration was significantly higher downstream than upstream during the festival, a phenomenon that occurred at no other time during the sampling season. This effect on the river can give the proper authorities and research bodies further insight into research and prevention of increased DOC in a future scenario.

The genetic diversity of the biofilm microbiota provided new insight into the diversity dynamics of the microbial biofilm composition. The findings imply that after the festival has occurred, the microbial communities would experience the effects in terms of nutrient influx. Therefore, the hypothesis that the biofilm community would experience a change because of the festival is likely true, however, only once the festival has ended.

Overall, this thesis served as a representation of how a cultural event, if not properly regulated, can be detrimental to the neighboring ecosystem, and that, without proper action by the attendees, organizers and local authorities, there will be a variety of impacts on the environmental surroundings as a result of this improper management.

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7. Appendix

7.1 DOC – Preparation protocol

Removal of inorganic and organic carbon residues from glass surfaces by application of acid and heat treatment.

Material and Equipment	Tweezers
HCl (0,1M)	Aluminium foil
Potassium Peroxodisulfate (10% w/v) or	Plastic bucket
NaOH (0,1M)	500 ml glass flask

Please note:

Note that only pre-cleaned glassware must be put into the acid-treatment containers. Failure to do so will have an adverse effect on the quality of analytical data.

Septa must not be washed in the dishwasher with labelled glass vials at the same time. They will absorb the colour of the labelling.

Do not combust used and acid-washed glassware in the same muffle furnace at the same time.

Store brown vials separately from clear vials and large ones from small ones. They are used for different applications (DOC, DIC, EEM).

Wrap up 6 vials at a time in a row in tin foil. These small, flat packs are easy to pack closely, do not fall apart and fit into the small muffle furnace. Take care to cover the vials completely to avoid contamination.

Protocol – Pretreatment of glassware

Combust used glassware (450°C, 1.5 hours - only if labelled with adhesive tape). Wash all glassware in the dishwasher (Program: G) and remove persistent residues manually before submerging it overnight in 0,1M HCl. Rinse 3 times with distilled water, let dry (or dry at 70°C in the drying oven reserved for clean material – wet lab 2), wrap up in aluminium foil and combust at 450°C for 4 hours.

7.2 Description of biofilm DNA extraction

The DNA extraction was conducted under user manual of the QIAGEN DNA extraction kit (QIAGEN 2014) as described below:

Material and Equipment	
UltraClean™ Soil DNA Isolation Kit (MO BIO Laboratories, catalog # 12800-100) Ethanol 70 % (v/v) Micro centrifuge (10,000 x g) Pipette and sterile tips (volumes required 50 µl - 1000 µl) Vortex Bunsen burner Eppendorf tubes (sterile) Spatula Forceps Petri dishes (∅ 10 cm, sterile) Adhesive tape	Kit Contents: 2 ml Bead Solution tubes (containing 550µl Guanidine thiocyanate solution) Solution S1 (containing SDS) IRS solution Solution S2 (containing Ammonium acetate) Solution S3 (containing Guanidine-HCl, Isopropanol and proprietary salts) Solution S4 (cont. Ethanol – flammable) Solution S5 (10 mM Tris, pH 8) Spin filter units in 2 ml tubes Collection tubes (2 ml) (Store at room temperature)

Protocol

1. Biofilm samples containing less than 0,5 ml water: Add entire volume of bead solution (without beads) to the sample. Vortex until biofilm is fully dispersed. Remove tiles with sterile forceps. Transfer the entire volume to the bead solution tubes.
   Biofilm samples containing more than 0,5 ml water: Transfer tiles and water to a sterile Petri dish. Scrape biofilm off the tiles using a sterile spatula and forceps. Transfer liquid and biofilm to a sterile eppendorf tube. Vortex for 10 min. Transfer 100 µl of the homogenised sample to bead solution tubes for DNA isolation. The rest of the sample can be stored at -20 °C.
2. Gently vortex to mix.
3. **Check Solution S1.** If Solution S1 is precipitated, heat solution to 60°C until dissolved before use.
4. Add 60µl of Solution S1 and invert several times or vortex briefly.
5. Add 200µl of Solution IRS (Inhibitor Removal Solution). Only required if DNA is to be used for PCR.

6. Secure bead tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 minutes.
7. Make sure the 2ml tubes rotate freely in your centrifuge without rubbing. Centrifuge tubes at 4,000 x *g* for 3 minutes. **CAUTION:** Be sure not to exceed 4,000 x g or tubes may break.
8. Transfer 550 µl of supernatant to a clean microcentrifuge tube (provided). Supernatant may still contain some biofilm particles.
9. Add 250µl of Solution S2 and vortex for 5 sec. Incubate 4°C for 5 min.
10. Centrifuge the tubes for 1 minute at 10,000 x *g.*
11. Avoiding the pellet, transfer 700 µl of supernatant to a clean microcentrifuge tube (provided). Add 1.3ml of Solution S3 to the supernatant (careful, volume touches rim of tube) and vortex for 5 seconds.
12. Load approximately 660 µl onto a spin filter and centrifuge at 10,000 x *g* for 1 minute. Discard the flow through, add the remaining supernatant to the spin filter, and centrifuge at 10,000 x *g* for 1 minute. Repeat until all supernatant has passed through the spin filter. **Note**: A total of three loads for each sample processed is required.
13. Add 300µl of Solution S4 and centrifuge for 30 seconds at 10,000 x *g*.
14. Discard the flow through.
15. Centrifuge again for 1 minute.
16. Carefully place spin filter in a new clean tube (provided). Avoid splashing any Solution S4 onto the spin filter.
17. Add 50µl of Solution S5 to the center of the white filter membrane.
18. Centrifuge for 30 seconds.
19. Discard the spin filter. DNA in the tube is now application ready.
20. Store DNA frozen (-20 °C). Solution S5 contains no EDTA.

7.3 Computational modelling scripting

Distance calculations, resulting in the generation of the nMDS graphics, as well as the related statistics.

```
install.packages('vegan')
install.packages('MASS')
install.packages('OTUtable')
install.packages('otuSummary')
install.packages('ggplot2')
install.packages('tidyverse')
install.packages('dplyr')
library(vegan)
library(MASS)
library(OTUtable)
library(otuSummary)
library(ggplot2)
library(dplyr)
metadata=Metadata
rownames(metadata)<-metadata[,1]
#Lets associate the two dataframes via cbind
cbinded_data<-cbind(taxtree[,1:5],otutab[2:25])
#remove binded data with a no - hit
combi<-cbinded_data[!(cbinded_data$Rank1=='No blast hit'),]
#This will remove the no-blasts,
#now turn the cbind back to a normal OTU table,by removing
#the taxtree columns
#fix_otu<-cbinded_data[,-c(1:5)]
#Modify the OTU table-change the first column to a row names
row.names(combi)=combi$OTU
combi$OTU=NULL
#Do the same for the taxonomic trees
row.names(taxtree)=otutab$OTU
taxtree$OTU=NULL
taxtree<-taxtree[!(taxtree$Rank1=="No blast hit"),]
#Remove OTU singletons
otu_no_sing = combi[rowSums(combi[,5:28])!=1,]
#Remove doubletons
otu_no_doble = otu_no_sing[rowSums(otu_no_sing[,5:28])!=2,]
#Now name the no_sing, no_double into more
#reasonable name
otu_final<-otu_no_doble
otu_just<-otu_final[ -c(1:4) ]
tranotu=t(otu_just)
#Transpose the final otu table
###nMDS
nmds2<-metaMDS(tranotu,distance = 'bray',k=2,try=20000)
stressplot(nmds2)
NMDS1 = nmds2$points[,1]
NMDS2 = nmds2$points[,2]
NMDS = data.frame(MDS1 = MDS1, MDS2 = MDS2,
                  Stage = metadata$Stage,
                  Locale= metadata$Locale,
```

```
                  Descriptor = metadata$Description)
# Initiate Ggplot
ggplot(NMDS, aes(x=MDS1, y=MDS2))
# area and poptotal are columns in 'midwest'
#simple scatterplot
P<-ggplot(NMDS, aes(x=MDS1, y=MDS2)) + geom_point()
# Add Title and Labels
P + labs(title="nMDS",y="NMDS1", x="NMDS2")+
geom_point(aes(col=Stage,shape=Locale), size=3)+
scale_shape_manual(values = c(18,17))+
scale_color_manual(values=c(During="red2",
                            After="steelblue",
                            Before="green4"))+
geom_text(label=metadata$Date,
                          check_overlap = F,nudge_y = 0.02,
                          nudge_x=0.015,
                          size=2.25,alpha=0.6,
                          vjust=0,hjust=0)+

theme_gray()
#ADONIS
a_dist=vegdist(tranotu,distance="bray")
adonis=adonis(formula = a_dist ~ Locale * Stage,
              data = metadata, permutations = 1000)
adonis
#Altogether insignificant, but taken apart, phase wise,
#we can perform an ANOVA between different stages
###ANOSIM
#before stage
trapre<-t(pre)
pre.dist=vegdist(trapre,distance="bray")
metapre <- metadata[ which(metadata$Stage=='Before'),]
anosimpre<-anosim(pre.dist, metapre$Locale, permutations = 500)
anosimpre
#during stage
tradur<-t(during)
dur.dist=vegdist(tradur,distance="bray")
metadur<-metadata[which(metadata$Stage=="During"),]
anosimdur<-anosim(dur.dist,metadur$Locale,permutations=500)
anosimdur
#after stage
traft<-t(post)
post.dist=vegdist(traft,distance="bray")
metaft<-metadata[which(metadata$Stage=="After"),]
anosimaft<-anosim(post.dist,metaft$Locale,permutations=500)
anosimaft
```

Dilution number	Dilution	All number of wells	Number of positive wells	MPN
2	1/2	64	42/64	**1313** cells/100mL
	1/20	32	12/32	
4	1/2	24	24/24	6.01 * 10 * 100 = **6010**
	1/20	24	9/24	cells/100mL
	1/200	24	1/24	
	1/2000	24	3/24	

Representation of the selection of positive cells for the process of fecal indicator analysis, according to a Poisson distribution. Arbitrary numbers are used as a representation of how the methodology functions.