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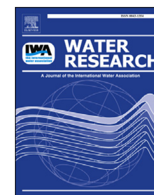

Trace levels of sewage effluent are sufficient to increase class 1 integron prevalence in freshwater biofilms without changing the core community

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ABSTRACT

Most river systems are impacted by sewage effluent. It remains unclear if there is a lower threshold to the concentration of sewage effluent that can significantly change the structure of the microbial community and its mobile genetic elements in a natural river biofilm. We used novel *in situ* mesocosms to conduct replicated experiments to study how the addition of low-level concentrations of sewage effluent (nominally 2.5 ppm) affects river biofilms in two contrasting Chalk river systems, the Rivers Kennet and Lambourn (high/low sewage impact, respectively). 16S sequencing and qPCR showed that community composition was not significantly changed by the sewage effluent addition, but class 1 integron prevalence (Lambourn control 0.07% (SE ± 0.01), Lambourn sewage effluent 0.11% (SE ± 0.006), Kennet control 0.56% (SE ± 0.01), Kennet sewage effluent 1.28% (SE ± 0.16)) was significantly greater in the communities exposed to sewage effluent than in the control flumes (ANOVA, $F = 5.11$, $p = 0.045$) in both rivers. Furthermore, the difference in integron prevalence between the Kennet control (no sewage effluent addition) and Kennet sewage-treated samples was proportionally greater than the difference in prevalence between the Lambourn control and sewage-treated samples (ANOVA (interaction between treatment and river), $F = 6.42$, $p = 0.028$). Mechanisms that lead to such differences could include macronutrient/biofilm or phage/bacteria interactions. Our findings highlight the role that low-level exposure to complex polluting mixtures such as sewage effluent can play in the spread of antibiotic resistance genes. The results also highlight that certain conditions, such as macronutrient load, might accelerate spread of antibiotic resistance genes.

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1. Introduction

The majority of EU rivers are impacted by Sewage Treatment Works (STW) effluent and it is not uncommon for effluent to provide between 20% and 70% of total river flow (Graham et al., 2010a,b; EC, 2012; Halliday et al., 2015). Growing water abstraction needs, caused by human pressure, in combination with declining river flows, owing to climate change effects, are projected to further increase STW-effluent dominance in many rivers (Johnson et al., 2009).

STW-effluent poses risks to the environment, to essential ecosystem services performed by rivers and eventually to humans (Brooks et al., 2006; Waiser et al., 2011). It is difficult to quantify the amount of sewage effluent that is harmful to a river, because a) the discharge amount and content of sewage effluent is highly variable not only per STW but also from day to day, or even hour to hour, and b) actions of individual compounds either singly or in synergy with each other, remain poorly understood (Schwarzenbach et al., 2006; Ricciardi et al., 2009).

STW-effluent and substances therein have a range of different effects on riverine biofilms, which are at the heart of biogeochemical cycles and food webs. Observed effects include increased

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growth rates due to enhanced nutrient supply (Hill and Fanta, 2008), leading to harmful blooms, lower photosynthetic and metabolic efficiency (Wakelin et al., 2008; Bonnineau et al., 2010), potentially reducing biogeochemical cycling rates, decreased biofilm adhesion (Schreiber and Szewzyk, 2008), decreased bacterial diversity (Ricciardi et al., 2009), changes to community composition (Lawrence et al., 2005; Wakelin et al., 2008), decreased colonization rates (Verma et al., 2007) and spread of antibiotic resistance genes (Amos et al., 2014). This range of responses reflects the diversity of chemicals and microorganisms emitted by STWs. To confound the issue, contamination of river microbiota by STW-effluent components such as metals or antibiotic resistance genes (ARG), gets passed through the food web to higher organisms (Brooks et al., 2006). Fish and water fowl, for example, have been found to harbour ARGs after exposure to microbial reservoirs in the environment (Ghosh and Mandal, 2010; Bonnedahl and Järhult, 2014) which poses a potential threat to humans.

There are indications that exposure to STW-effluent contributes to multi-drug resistance in environmental bacteria (Akiyama and Savin, 2010; Amos et al., 2014). While there has always been a background level of genes in the environment that can confer resistance to antibiotics (Bhullar et al., 2012; Martínez, 2012), it is now well known that resistance to antibiotics in the environment is increasing (Alanis, 2005; Da Silva et al., 2006; Knapp et al., 2009).

ARGs are more prevalent where bacteria are exposed to antibiotics, metals or detergents (Pei et al., 2006; Graham et al., 2010a,b). In that light, there have been calls to develop sewage systems that produce effluent cleaned of bacteria, pharmaceuticals, macronutrients, nanoparticles and other contaminants, but cleaner systems are costly (Amos et al., 2014; Huang et al., 2015) and do not always achieve what is required. Huang et al. (2015) have demonstrated that tetracycline resistance genes were still prevalent in final cleaned effluent treated with ozone, even after tetracycline and tetracycline-resistant bacteria had been removed, marking out STW-effluent as a potent source of ARG regardless of cleaning efforts such as UV disinfection (Baquero et al., 2008; Huang et al., 2015). A growing number of studies show that proximity to STW-effluent increases the occurrence of antibiotic resistance also in riverine microbiota (Edge and Hill, 2005; Servais and Passerat, 2009; Graham et al., 2010a,b; Amos et al., 2015).

The effects of STW-effluent are hard to unravel, because of the complexity of its compounds and the variability of its composition. Many studies have therefore focused on the effects of particular polluting substances, such as antibiotics, antivirals or nanoparticles (Graham et al., 2010a,b; Kaegi et al., 2011; Slater et al., 2011), not on the mixture itself. To overcome the lack of information on the effects of STW-effluent as a whole, we conducted this study, using an experimental approach to compare biofilms grown in novel in-situ mesocosms at two sites within the Thames catchment that are differently impacted by sewage effluent. The mesocosm system allows for in-situ experiments with replication under controlled conditions in the river, thereby combining the need for control and replication with the need to study natural systems in their complexity. Based on findings of a laboratory-based pilot study (unpublished), we added nominal concentrations of ~2.5 ppm of STW-effluent to the experimental flumes. This low concentration was designed to avoid changing the microbial communities through addition of nutrients, with an aim to focus on the effects from the complex mixture of micropollutants within sewage effluent.

We used a molecular sequencing approach to estimate the impact of STW-effluent on the biofilm communities, focusing on the 16S rRNA locus to characterise the community composition. We also assessed the prevalence of class 1 integrons to investigate if low level STW-effluent exposure can induce genetic changes to a microbial community, such as an increase of antibiotic resistance genes stored on mobile genetic elements. Class 1 integrons, which, like other mobile genetic elements, carry such genes, can be passed between bacteria by horizontal gene transfer and confer antibiotic resistance. Class 1 integrons encode the class 1 integron-integrase gene (*IntI1)*, which is able to insert up to 6 gene cassettes at an integron-associated recombination site (Gillings et al., 2009). Class 1 integrons were first known to primarily confer resistance to antibiotic drugs (Recchia and Hall, 1995; Partridge et al., 2001), but they also confer resistance to a variety of other antibiotic compounds and biocides (Gillings et al., 2009; Gaze et al., 2011). Class 1 integrons are elevated in sewage sludge and elevated levels have also been found in sewage effluent (Gaze et al., 2011; Amos et al., 2015; Paiva et al., 2015). Gillings et al. (2015) have recently proposed to use the class 1 integron-integrase gene as a genetic marker for anthropogenic pollution.

We hypothesized that adding sewage effluent to a river, even in as low a concentration as 2.5 ppm, would significantly change the diversity and structure of riverine biofilms or lead to increased transfer of mobile genetic elements. We tested our hypothesis with a factorial design, using two different Chalk streams as experimental platforms and two treatments (sewage effluent and control) per experiment.

2. Methods

2.1. Sites

The Lambourn (experimental site: 51.446022, −1.382894 Lat/Long, Decimal Geographic Coordinates) and the Kennet (experimental site: 51.422744, −1.698095 Lat/Long, Decimal Geographic Coordinates) are aquifer-fed Chalk streams with a high base flow index. They are calcium and bicarbonate rich and have relatively constant chemical compositions (Neal et al., 2000, 2004).

The Kennet is impacted by point source and diffuse pollution. Marlborough STW is located 1.7 km upstream from the experimental site (supplement/Fig. 6), serving a population of ca. 8000 (Census, 2001). Iron dosing (tertiary treatment) reduces phosphorus loads between 80 and 90% in the final effluent (Neal et al., 2010). The site is frequently affected by excessive benthic algae growth (Bowes et al., 2010). Macrophyte growth is sparse and consists mainly of *Callitriche platycarpa*, a plant common in eutrophic waters (Thiébaut and Muller, 1999).

The River Lambourn is a tributary of the River Kennet. Five kilometres upstream from the experimental site, East Shefford STW discharges into the Lambourn. The STW has tertiary treatment facilities that remove between 80 and 90% of phosphorus from the sewage effluent and serves a population of ca. 1000 people (Census, 2001; Jarvie et al., 2006). Additional waste water input might be received from septic tanks which release into groundwater close to the river (Neal et al., 2004). The experimental site has high macrophyte biomass, including abundant *Ranunculus fluitans*, a keystone species in undisturbed Chalk rivers (Hatton-Ellis and Grieve, 2003).

Nutrient and boron measurements taken in the first half of 2009 (supplement/Fig. 8) in the build-up to our study showed that the main differences in the river chemistry were soluble reactive phosphorus (SRP) and boron levels. Boron is used as a tracer for anthropogenic contamination, as it is a constituent of detergents (Barth, 1998). Both SRP and boron are higher in the Kennet (supplement, Fig. 8), suggesting that the defining difference between the two sites is the measure of human impact on each site.

2.2. Experiment methodology

We conducted mesocosm experiments in the Rivers Lambourn and Kennet using the mescososms described in Bowes et al. (2010). Briefly, they are constructed as blocks of 3 flumes that float at a constant height directly in the river, allowing river water to flow through at a constant water depth of ~6 cm (supplement/Fig. 7). Each flume measured 5 m × 0.3 m, was gated to standardise flow rates at the upstream end and had a sump to collect river debris ¾ of the length from the inlet (Bowes et al., 2010). Treatments were delivered through a tube to the upstream ends of randomly chosen flumes, using a peristaltic pump (FH100M, Fisher Scientific, Loughborough, UK) at a drip rate needed to maintain a concentration of ≥2.5 ppm at a flow rate of 0.10 m s^{-1}, as calculated by standard volumetric calculations. Treatment concentration within the flumes was controlled by regulation of the pump drip rate, making it possible to replicate conditions from one flume to the other. We therefore considered each flume to be one biological replicate. During the experiments, we set the flow rate in each flume to 0.10 m s^{-1} (measured daily by a Valeport 801 flow meter) by manipulating the gate opening. Sewage was collected from the Marlborough STW (serves ca 8000 people, phosphorus removal, no disinfection unit) for the Kennet site, and Boxford STW (serves ca 700 people, secondary treatment and settlement tank, no phosphorus unit, no disinfection unit) for the Lambourn site. The rationale behind that was to a) add sewage effluent to the river, that the river would receive anyway, b) reduce transport times for the sewage effluent. The effluent was kept in plastic containers wrapped in aluminium foil in dark metal cupboards, to minimize changes to the effluent caused by light and temperature effects.

A gap between the riverbed and mesocosms limited the number of invertebrate colonizers entering the flumes. The Kennet's smaller channel width allowed for 6 flumes, whereas the Lambourn could fit 8 flumes. We chose 8 flumes for the second experiment to gain statistical power. Microbial biofilms were grown on 2 cm × 2 cm limestone tiles placed behind the sump area of the flumes (supplement/Fig. 7). For both experiments, we placed six sterile tiles in each flume at the beginning of the experiment. The tiles were harvested after 9 days, to avoid losing the biofilms. Biofilms grown in similar studies (Bowes et al., 2010, 2012) and pilot studies, had by that timepoint matured enough to be in danger of lifting off the tiles and float downstream.

2.3. Sample collection and DNA sequencing

The experiments started on 17/06/09 in the Kennet and 02/09/09 in the Lambourn. On day 9 of each experiment, we collected the biofilm tiles and some river water in sterile sampling bags and transported them in a cool box to the laboratory (<5 h), were the whole tiles were frozen at −80 °C. We performed a CTAB/chloroform: isoamyl extraction modified from Doyle (1991) on the pooled samples, followed by PEG-precipitation after Paithankar and Prasad (1991). The complete extraction protocol can be found in the supplementary methods. We used the 454 GS-FLX TITANIUM platform (Roche 454 Life Sciences, Branford, CT, USA) to produce tag-encoded 16S amplicons of ~400 bp length. We targeted a fragment of the 16S ribosomal RNA gene (rRNA), comprising the V6 and V7 regions using the universal primers 967F, 5′-CNACGCGAAGAACCTTANC-3′, and 1391R, 5′- GACGGGCGGTGTGTRCA-3′ which capture both chloroplast and bacterial 16S rRNA (Huber et al., 2009; Huse et al., 2008). We used that regions because it gave us the best resolution, given the small fragment size that could be sequenced (Huse et al., 2008). The sequencing libraries were generated through a one-step PCR with a total of 30 cycles, a mixture of Hot Start and Hot Start high fidelity taq polymerases and amplicons extending from the forward primers. DNA amplification and pyrosequencing were carried out at Research and Testing Laboratory (Lubbock, TX, USA).

2.4. Analysis of integron prevalence

For the analysis of *intI1* prevalence in the Kennet, we pooled controls from two successive experiments in the Kennet (a pilot study done 02/06/2009 and the described study from 17/06/2009) to match the number of Lambourn replicates. Amos et al. (2015) have shown that no significant changes to integron prevalence levels in the Thames catchment between summer months could be observed, and we tested our data with ANOVA for time effects and found none. The integron prevalence analysis was done as per Gaze et al., (2011). In short, subsamples of the DNA extractions were amplified by real time PCR with primers targeting the class 1 integron-integrase gene (*intI1)*. Standard curves for absolute quantification of *intI1* were produced from serial dilutions of *E. coli* SK4903 and enumerated by viable plate counts. Molecular prevalence was calculated by dividing the number of target genes by the number of 16S rRNA copies, with corrections made for 16S rRNA copy number. One sewage effluent replicate from the Kennet did not amplify.

2.5. Bioinformatics

We used CloVR 1.0 RC4 (Angiuoli et al., 2011) on the Data Intensive Academic Grid (DIAG, University of Maryland, USA) to run the QIIME workflow 'pick_otus_through_otu_tables.py' (Caporaso et al., 2010). For a detailed description of the settings please see section 2.5 of the supplementary methods. After quality control, the data set consisted of 66,073 raw reads for the 6 flumes of the Kennet sewage study, and 250,350 raw reads for the 8 flumes of the Lambourn sewage study. Two of the Lambourn samples (S1 and S2) together contained more than 50% of the raw sequences from the Lambourn study. The Sequencing Facility started the sequencing process with equal amounts of DNA, which suggests that the differential amplification might be due to the barcodes used. The OTU table was rarefied (see below) to reduce the effects that this relatively deeper sequencing of two samples might have had on statistical evaluation of species richness and diversity.

Following from earlier studies (Pillet et al., 2011; Lindemann et al., 2013) we used the chloroplast 16S rRNA to focus on the algal communities. Therefore, of those OTU's that were identified to genus level, we divided the community into algal-derived chloroplast reads and bacterial (including cyanobacterial) reads. We equilibrated the number of sequences per sample by rarefaction (randomly sampling without replacement (Hamady et al., 2009; Koren et al., 2013), resulting in 1589 algal sequences per sample, and 1390 bacterial sequences per sample. The rarefied OTU table lists 2765 bacterial OTUs and 350 eukaryotic OTUs. Any OTUs that are discussed on the species level were blasted individually against the RDP database (Cole et al., 2009). Only fragments that could be matched at 97% or above were classified to species level. The rarefied OTU tables were imported into the R environment (www.r-project.org) for statistical analysis and into Galaxy (Afgan et al., 2016) where we used PICRUSt (Langille et al., 2013) and MaAsLin analysis (Tickle et al., In progress) to predict functional traits in each community and to link those to treatments and sites. For a detailed explanation of the analysis, see the supplementary methods, section 2.5.

2.6. Analysis of nutrients

To measure water chemistry during the experiments, we

manually collected one sample of river water from each flume channel. All samples were analysed within 24 h in the Centre for Ecology and Hydrology, to minimize errors associated with sample instability. For a detailed description of the analysis, see the supplementary methods, section 2.6.

3. Results

3.1. Nutrient levels

The nutrient levels measured in the experimental flumes in both rivers at the beginning and end of the experiment (supplement/Fig. 9) showed no difference in nutrient levels between the treatment groups (control and sewage effluent). In general, the Lambourn flumes had 56% less soluble reactive phosphorus (SRP) than the Kennet flumes, but very similar total dissolved nitrogen (TDN - 100%) and silicon concentrations (102% - silicon is required primarily by diatoms). The nutrient levels where also similar to background levels measured routinely near to the sites (supplement, Fig. 8).

3.2. Biofilm community differences

We separated the algal and bacterial biofilm components for analysis, because sewage effluent, as a mix of different pollutants, can affect algae and bacteria in different ways (Crane et al., 2006). The most dominant algal organisms in both rivers were the Bacillariophyta and the most common bacterial phylum was that of the Proteobacteria (Fig. 1). A large number of OTUs remained unclassified. Overall, the Lambourn and Kennet samples had distinctly different periphyton communities (PERMANOVA Bacteria: Pseudo-F = 18.5, $p = 0.001$, Algae: Pseudo-F = 112.2, $p = 0.001$). This is also apparent in their rank abundance curves (Fig. 2) in which different OTUs dominate each community both in the algal and bacterial component of the biofilms. The biofilm communities in each river were also differently diverse (Fig. 3, 2-way ANOVA, F = 7.6, $p = 0.02$ for bacteria, F = 25.4, $p = 0.0004$ for algae), but there were no differences between sewage effluent and control flumes. Visualizing the data with an NMDS (Fig. 4), the data points cluster very clearly by river, not by treatment. PICRUSt/MaAsLin analysis of the 16S data to predict functional traits (Langille et al., 2013) notably showed negative correlations between the Lambourn communities and functions relating to xenobiotic processes which point towards sewage effluent contamination, such as naphthalene, benzylethene or benzoate degradation, thereby confirming that the impact of sewage effluent on the Lambourn is small.

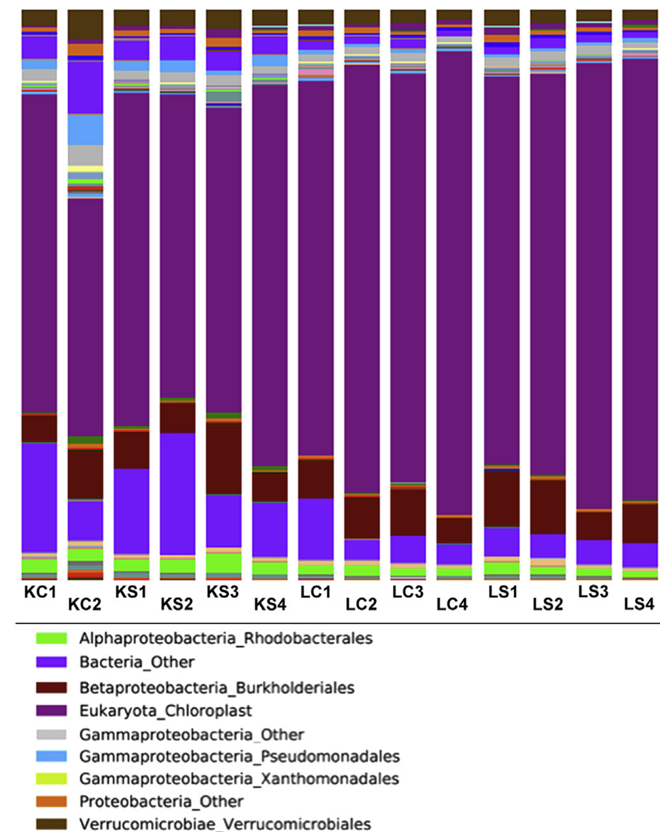

Fig. 1. Taxonomic barchart of all replicates at order level where identification down to that level was possible, based on relative abundance. KC=Kennet Control, KS=Kennet Sewage effluent, LC = Lambourn Control, LS = Lambourn Sewage effluent.

3.3. Class 1 integron prevalence

Focusing on class 1 integron prevalence (Fig. 5) rather than diversity and metabolic differences between the community, a different picture emerges. Here, the treatment (ANOVA, F = 5.11, $p = 0.045$), the river (ANOVA, F = 18.83, $p = 0.001$) and interactions between treatment and river (ANOVA, F = 6.42, $p = 0.028$) are all significant. Both in the Kennet and in the Lambourn replicates, class 1 integron prevalence is greater in the communities exposed to sewage effluent than in the control flumes (Fig. 5), even though there is much more overlap in the Lambourn. The Lambourn control samples show the lowest abundance of class 1 integrons, with a proportion of 0.07%, SE $\pm$ 0.01. The Lambourn sewage samples had a greater proportion of integrons at 0.11%, SE $\pm$ 0.006. In the Kennet control samples, integron prevalences are seven times (0.56%, SE $\pm$ 0.01) that of the Lambourn control and in the Kennet samples treated with sewage effluent, class 1 integrons are 16 times as abundant (1.28%, SE $\pm$ 0.16) as in the Lambourn control. The difference in integron prevalence between the Kennet control and Kennet sewage-treated samples is proportionally greater than the difference in prevalence between the Lambourn control and sewage-treated samples.

4. Discussion

The two experimental river sites harboured very different microbial communities (Fig. 2), probably owing to different nutrient levels (supplement/Figs. 8 and 9) and exposure to differing levels of sewage effluent. Further low-level addition of sewage effluent during the experiment did not cause the treated communities to diverge significantly from the controls, even though it is possible that greater replication or deeper sequencing would have shown up different patterns. The Kennet location is permanently impacted by STW effluent. The Kennet sites' significantly lower diversity profiles, as compared to the Lambourn, are likely the effect of nutrient enrichment rather than pollution by other chemicals (Hill and Fanta, 2008; Ricciardi et al., 2009). It is possible that the Kennet community differs from the Lambourn because it has already been permanently altered by effluent, a phenomenon previously suggested in Fechner et al. (2012) studying changes *in situ* and Serra et al. (2009) in artificial indoor flume systems.

The differences in integron prevalence observed by us, however, clearly show that modification on the species level is not the only change to look out for, to assess a complex microbial community subjected to pollutants. The rise in intergon prevalence in the Kennet and Lambourn samples exposed to STW-effluent clearly show a significant impact at the level of functional genes, even in the absence of significant taxonomic changes. This appears to

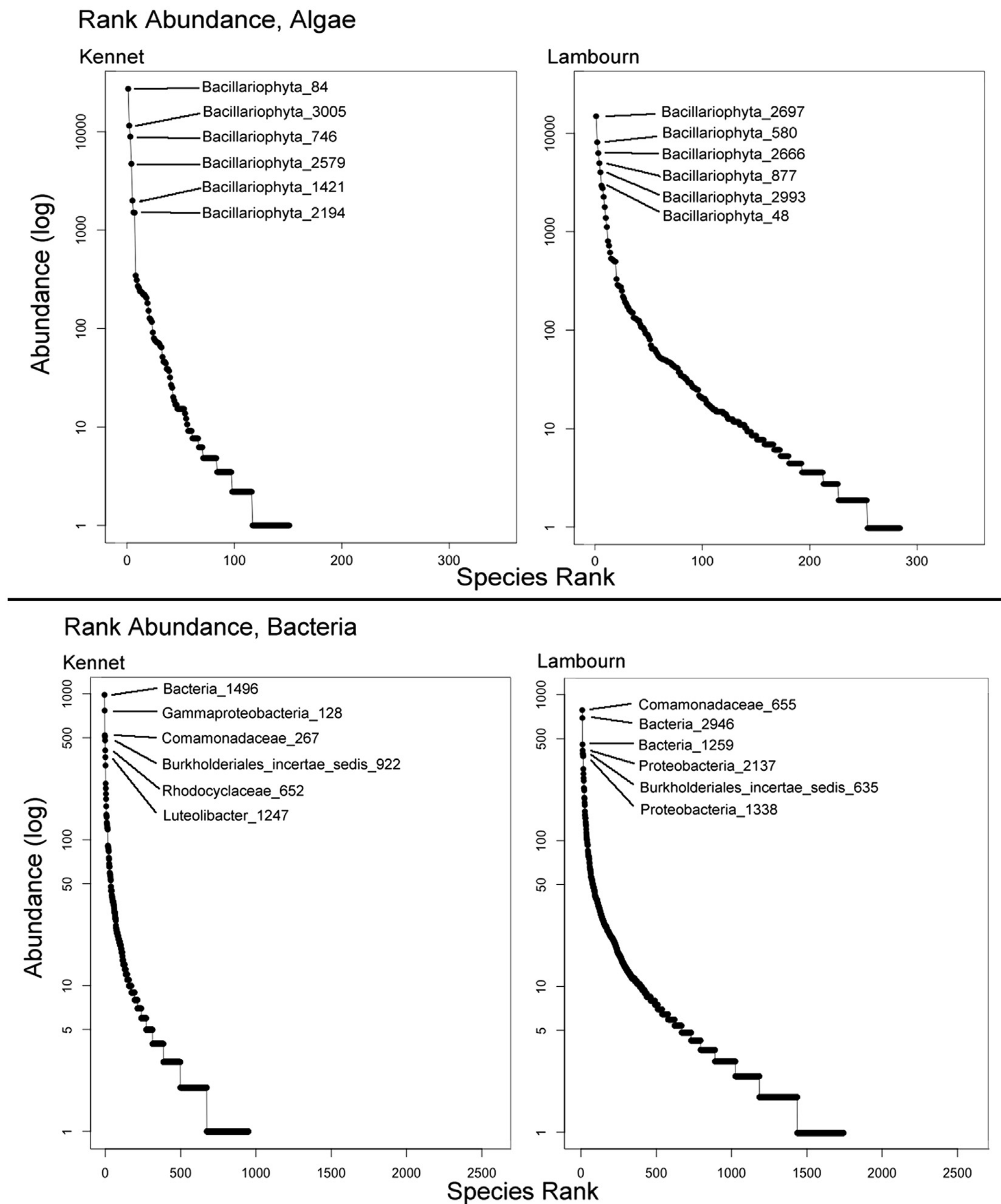

Fig. 2. Rank Abundance curves for both algal and bacterial communities in Kennet and Lambourn. Numbers behind the taxonomic level denote different OTUs.

confirm previous observations that effluent exposure induces transcriptional changes and promotes antibiotic resistance (e.g. Yergeau et al., 2010; Gaze et al., 2011; Amos et al., 2015). The higher class 1 integron-integrase gene prevalence in the Kennet control samples, as compared to the Lambourn, confirm a higher sewage-effluent impact (Graham et al., 2010a,b; Gaze et al., 2011), but the proportionally higher increase of *IntI1* in the Kennet sewage effluent samples as compared to the Lambourn, is surprising.

The sewage effluent that was added to the flumes was collected from the nearest STW outlets on the Kennet and Lambourn respectively at three times during the experiments. The rationale was, to add the same sewage effluent that each river receives on a regular basis. Importantly, though, sewage effluent batches, even if from the same STW, are highly variable, depending on the mix of substances that are being processed at the STW at a given time. This makes it unlikely, if theoretically possible, that the Kennet experiment was conducted with three batches of sewage effluent that were higher in *IntI1* prevalence than both the average STW effluent it generally received and the Lambourn STW effluent, which led to the observed differences in proportion of *IntI1* between the Kennet sewage effluent and control treatments in comparison to the Lambourn treatments. A possible reason for a higher *IntI1* prevalence in effluent could be selection pressure through elevated levels of antibiotics (Singer et al., 2014; Bengtsson-Palme et al., 2016), even though Bengtsson-Palme et al. (2016) did not observe consistent enrichment of resistance genes under elevated levels of antibiotics.

Alternatively, the time difference between the experiments (mid-June vs early September) might have influenced the observed result, such as through preferential uptake of *IntI1* by a specific bacterial species that is seasonal. The high within-sample variability of the 16S data (16S should per se not to be used for absolute

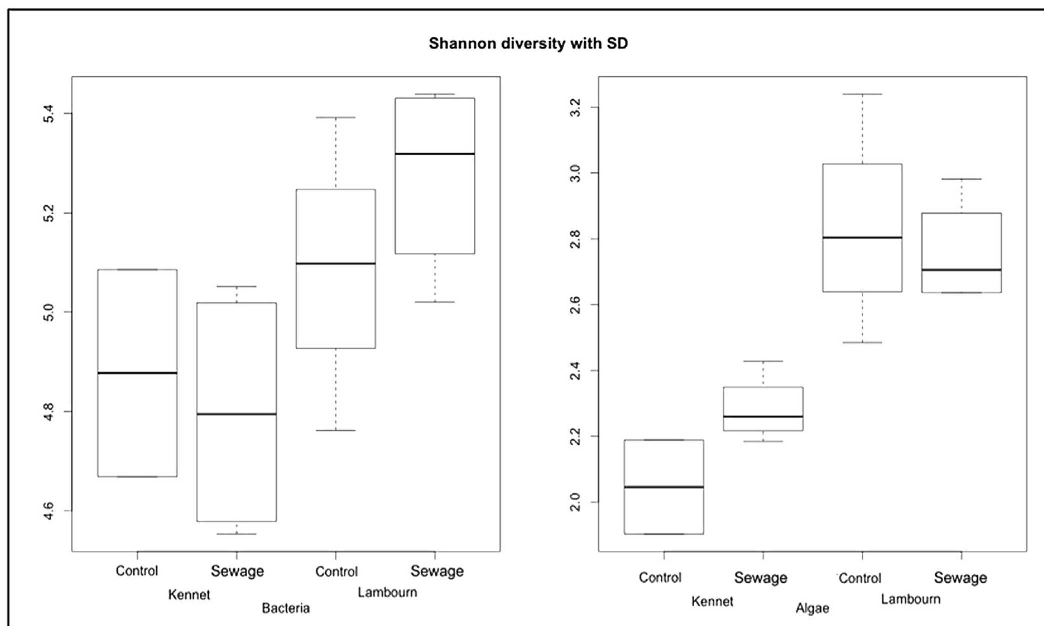

Fig. 3. Standard boxplots of shannon diversity indices for both bacterial and algal components of the Kennet and Lambourn biofilms.

abundances anyway) did not allow conclusions here.

The result could, on the other hand, point towards mechanisms in the Kennet, which particularly promote the exchange of integrons that are added through effluent as DNA or in intact cells. The Kennet, as a sewage-effluent impacted environment, might contain a greater concentration of pollutants than the Lambourn and therefore select for greater *IntI1* uptake. One such option would be co-selection in response to metals (Pal et al., 2015). Other mechanisms could be related to poorly-understood interactions such as those between bacteria and phages. Phages can contain mobile genetic elements (Parsley et al., 2010), specifically also class 1 integrons (Schmieger and Schicklmaier, 1999). Muniesa et al. (2013) suggest that the number of antibiotic resistance genes in phages is 10 times lower than those in bacterial populations from the same environment. As bacteriophages are thought to outnumber bacteria by a factor ranging from 1 to 10, this suggests that they carry a significant number of intergons, and therefore the possibility of phage-mediated uptake of *IntI1* probably warrants investigation.

The one obvious difference between the otherwise similar rivers, however, is the Kennet's higher nutrient load. As shown by Amos et al. (2015), phosphorus levels in particular correlate with class 1 integron prevalence and it appears that a heightened nutrient load might significantly enhance the exchange of class 1 integrons and presumably also other genetic material. Indeed, Van Elsas et al. (2003) have shown that horizontal transfer of genes in soil biofilms was increased in the nutrient-rich rhizosphere of plants as a result of enhanced metabolic rates and increased cell

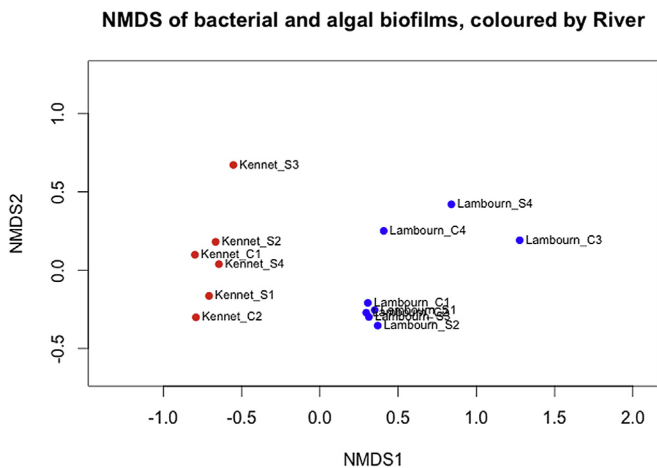

Fig. 4. NMDS of all biofilm replicates with algal and bacterial components, stress:0.04. Kennet samples are marked red, Lambourn samples blue. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

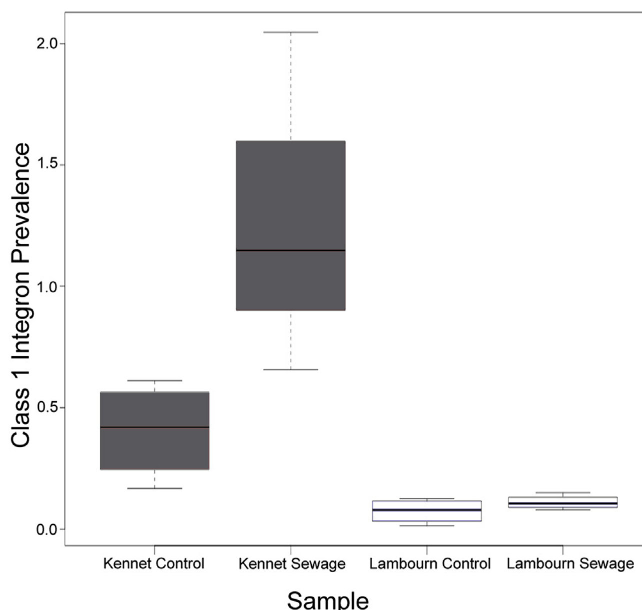

Fig. 5. Standard boxplots of class I integron prevalence (%) in the Kennet and Lambourn.

motility. It is possible that a similar process takes place in nutrient-rich stream biofilms such as those growing in the Kennet.

5. Conclusions

Our findings highlight the role that low-level exposure to complex polluting mixtures such as STW-effluent can play in the spread of ARGs. If even small amounts of routinely administered sewage effluent increase the potential to spread class 1 integrons, and thereby antibiotic resistance, this needs to be acknowledged more widely. Moreover, a higher nutrient load in the river might lead to increased ARGs exchange, which means that continued attention needs to be directed towards the still elevated nutrient loading of many streams and rivers. If such low levels of STW effluent as those used in our experiment trigger enhanced gene cassette exchange through synergies with nutrient or antibiotic medication loads, it will be difficult to stop ARG transfer by simply lowering concentrations of nutrients or medication. It is unlikely that the exposure of river- and stream-biofilms to STW effluent can be stopped and it seems necessary at this point that we not only turn our attention to the traits which enhance the distribution of genetic material in biofilms, but study more closely what can restrict the transfer of ARGs or what reduces the need for bacteria to retain them.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2016.09.035.

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