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# Ecotoxicity and antibiotic resistance of wastewater during transport in an urban sewage network

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## Abstract

Urban wastewater (UWW) management usually entails biological and physicochemical monitoring due to its potential impact on the quality of the receiving environment. A major component of a sewage system is the pipe network leading the water to the treatment plant. Up to now, few studies have been conducted on the diverse phenomena that may affect the characteristics of the water during its transportation. In this study, ecotoxicity and potential antibiotic resistance were used in a global method to assess the change of UWW quality in a sewage system and determine if sewer pipes can act as a bioreactor spread. Three bioassays were conducted to assess the ecotoxicity of the samples and the concentration and relative abundance of two classes of integrons (as a proxy for antibiotic resistance) were measured. The results of the bioassay battery do not show a pattern, despite the fact that differences were noticeable between upstream and downstream samples. Antibiotic resistance appeared to decrease during transport in the pipe as the concentration and relative abundance of integrons decreased during several campaigns. This result should be confirmed in other sewer networks but already provides useful information for the management of urban sewage system.

**Keywords** Ecotoxicity · Antibiotic resistance · Integrons · Urban wastewater · Sewer network

## Introduction

UWW is a mix from several sources: domestic and industrial emissions (Deblonde et al. 2011; Giorgetti et al. 2011) and effluents from care structures like hospitals, retirement homes, and veterinary practices (Gwenzi et al. 2018a; Petrie et al.

2015). A wide range of pollutants can be found in this type of wastewater: detergents, organic matter, metals, and pharmaceuticals (Auriol et al. 2006; Miege et al. 2009; Orias and Perrodin 2013; Teijon et al. 2010). The occurrence of these compounds in effluents and their transmission to the environment, especially aquatic, is becoming a global issue (Buerge et al. 2006; Da Silva Oliveira et al. 2007; Hreiz et al. 2015; Luo et al. 2014). The treatments implemented to eliminate them are subject to extensive study due to the potential impacts of this micropollution on the environment.

The constitution and composition of the wastewater matrix at the end of the pipe is relatively well-known but there is less data on its change in the sewer during transport (Auguet et al. 2017). In every sewage system, UWW is collected at its source and is conducted to a treatment station and then to the receiving environment. Sewers are considered as a transport system for UWW and are usually not considered as part of the treatment. UWW treatment is studied at the inlet and outlet of wastewater treatment plants (WWTP) but during its transport in the pipes, reactions may occur due to residence time and modifications of physicochemical conditions (oxygenation, volatilization, etc.). Many parameters can be affected, such as pollutant concentrations and therefore the impact of the global mix (ecotoxicity), and the sanitary risk (transfer of resistance genes).

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The control of antibiotic resistance spread is a major concern when dealing with UWW because it has now been established that effluents can be a source of resistance genes and a threat to human and environmental health if released into the receiving environment (Bengtsson-Palme and Larsson 2016; Gwenzi et al. 2018b; Michael et al. 2013; World Health Organization 2014). Integrons are a mobile genetic element which allows bacteria to exchange genes (Gillings 2017). Some of the genes integrated in these integrons can be resistance genes, which is why integron quantification is used as a proxy for the propagation of potential resistance genes (Amos et al. 2015; Rizzo et al. 2013; Stalder et al. 2014; Subirats et al. 2018).

Ecotoxicity studies are used to detect differences in biological and physicochemical characteristics that are not revealed by targeted micropollutant analysis methods (Gosset et al. 2016). Indeed, ecotoxicity may be present due to a non-detected compound or because different compounds may have synergistic or antagonistic effects when mixed (Boillot and Perrodin 2008; Vasquez et al. 2014).

The aim of this study was to test the hypothesis that a sewage system acts as a bioreactor and is part of the treatment of UWW. In order to do that, we used integron quantification and ecotoxicity tests as global methods for assessing the change of UWW quality during the transport of UWW in sewers (Perrodin et al. 2011), independently of pollutant concentrations. Analyses were performed on samples collected upstream and downstream of a long sewer pipe to assess potential change.

## Materials and methods

### Study site

The site chosen was a wastewater transfer tunnel crossing the border between France (Saint-Genis Pouilly) and Switzerland (Satigny). It is a 2.7-km-long sewer without any additional wastewater or rainwater input. It collects UWW from a mostly residential area. The water velocity is around 1 m/s and it remains under aerobic conditions during the transport (Fig. 1).

### Sampling procedure

As the aim of the study was to determine the change of UWW during transport, we had to collect the same portion of water upstream and downstream. To do so, the travel time of the water was recalculated for every sample, according to the flow at the exact time of sampling. Due to the study site layout, the travel time was consistent from one campaign to another and was approximately 45 min. The sampling time was chosen to target the peak morning flow due to water consuming activities (toilets, showers, laundry, etc.). The campaigns were conducted on dry days (no rain in the area at least three days

before the campaign), in order to have the lowest flow and speed and also the least diluted samples. All the samples of approximately 20 L were collected using the same type of pump (HACH automatic sampler AS950), homogenized, sub-sampled, and stored in glass bottles in cooled conditions ( $-20\text{ }^{\circ}\text{C}$ ) before analyses. Extractions that were needed for chemical analyses and integrons quantification were performed on cool samples as soon as they got to the laboratory (less than 24 h after sampling). Samples needed for ecotoxicology analyses were frozen as soon as they got to the laboratory (less than 24 h after sampling) and stored until analyses (maximum 6 weeks after sampling).

Two types of sampling protocol were performed: short time sampling and continuous sampling. All the campaign characteristics are reported in Table 1. For the first 9 campaigns, which were called “short,” an upstream sample of 20 L was collected using a pump immersed in the sewer. The downstream sample was pumped after the travel time of the water in the pipe. This type of sample was called “short” because it was pumped during a short period of time (a few minutes).

For the last four campaigns, another protocol was used and called “continuous.” The samples upstream and downstream were collected for 3 consecutive hours. A small volume between 1 and 3 L (in proportion to the flow at this time) was collected every 15 min and pooled in a final 20-L sample. Once again, the downstream sample was collected after the travel time of the water in the pipe. This type of sample was meant to be more representative of the wastewater running in the pipe.

### Physicochemical analyses

The physicochemical analyses were performed on all the water samples. Classical parameters, pharmaceuticals, and some of their metabolites, metals, and surfactants were analyzed. More details about the methods were explained in Bergé et al. (2018) and Wiest et al. (2018). A detailed list of all the parameters with a result above the limit of quantification can be found in supplementary data (Table S1).

### Bioassays

The three bioassays used are standardized tests and are presented in Table 2. They were chosen in order to get acute and chronic tests with organisms from different levels of the food chain. Furthermore, these tests have been well-known and successfully used on fresh water samples for a long time in ecotoxicology studies.

The tests were conducted on UWW samples kept at  $-20\text{ }^{\circ}\text{C}$  before bioassays.

The results of the bioassays are expressed in EC20 or EC50 depending on the test. A lower EC means a higher effect. When no effect is measured at the highest concentration tested, the result is considered as “non-toxic.”

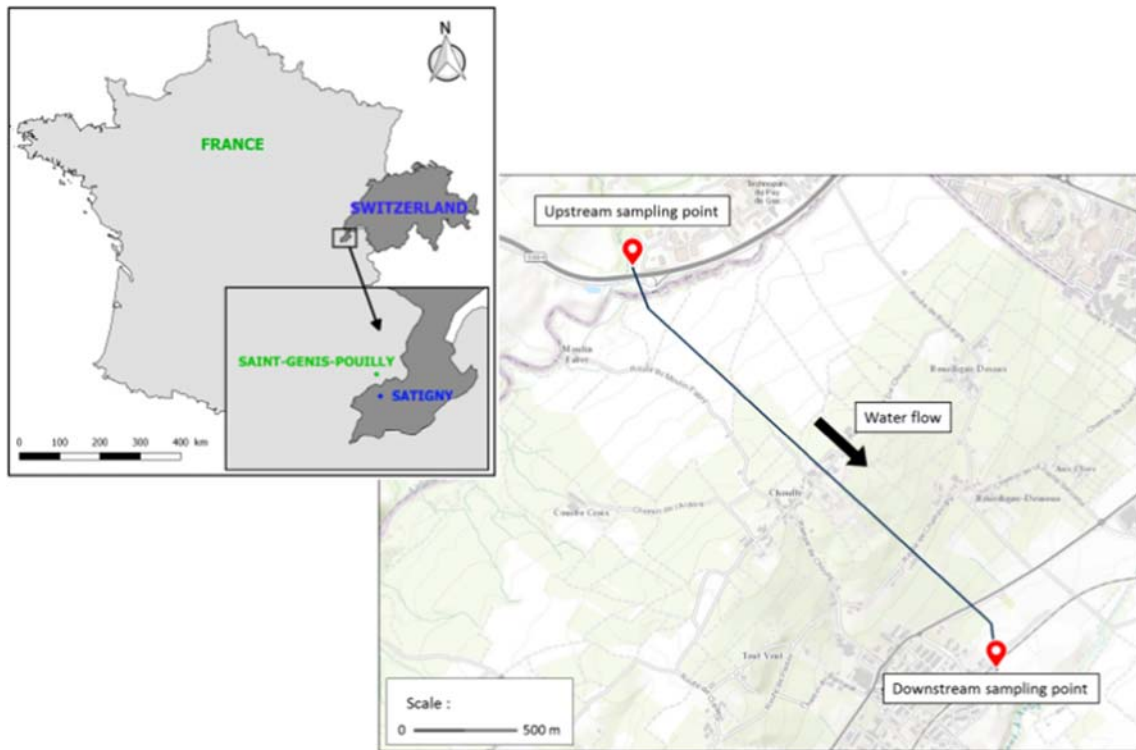


Fig. 1 Sampling site location. Upstream and downstream sampling points are represented by red dots

**Integron quantification**

Class 1, 2, and 3 integrons (Int1, Int2, Int3) and 16S ribosomal RNA (rRNA) were quantified using qPCR as described in Laquaz et al. (2017) and used as a proxy for potential antibiotic resistance gene spread in the bacterial community. 16S rRNA-encoding gene quantities is divided by 4.1 which is the average copy number of 16S rRNA in bacteria (Klappenbach et al. 2001) used to estimate bacterial cell numbers; the relative

abundance of integrons is calculated by dividing the number of copies of integrons by the estimated bacterial concentration.

**Results and discussion**

**Physicochemical parameters**

The results of the effluent physicochemical analyses are summarized in Table S2 (supplementary data). The results were classified according to the change of the values during the transport in the sewer: (1) a net decrease (change > 80%), (2) a net increase (change > 80%), and (3) the absence of, or slight, change. This classification highlights a strong dominance of parameters synonymous with an absence of change during transport in the sewer, as well as parameters that vary erratically.

This table also makes it possible to identify the parameters that decrease or remain stable for all the campaigns (paracetamol, 4-hydroxy diclofenac, organic ammonia (TKN), chemical oxygen demand, Comperlan100, conductivity, dissolved organic carbon, total organic carbon), as well as parameters that always increase or remain stable (sulfamethoxazole, ibuprofen, LAS C13, NH<sub>4</sub><sup>+</sup>, benzyldimethyldodecylammonium chloride, total organic suspended solids, total suspended solids).

These results could be linked to the following phenomena: (1) the decrease in the values of global organic parameters

Table 1 Sampling campaign characteristics

Campaign number	Sampling date	Sampling time	Type of sampling protocol
1	06/07/2016	9:22	Short
2	06/07/2016	10:23	Short
3	06/07/2016	10:45	Short
4	15/11/2016	8:35	Short
5	15/11/2016	9:32	Short
6	15/11/2016	10:30	Short
7	08/12/2016	8:40	Short
8	08/12/2016	9:30	Short
9	08/12/2016	10:30	Short
10	15/03/2017	8:30	Continuous
11	15/03/2017	11:40	Continuous
12	05/04/2017	8:30	Continuous
13	05/04/2017	11:31	Continuous

**Table 2** Bioassays performed

Organisms	<i>Daphnia magna</i>	<i>Pseudokirchneriella subcapitata</i>	<i>Brachionus calyciflorus</i>
Biological parameter	Mobility	Growth	Reproduction
Endpoint	EC50 (48 h)	EC20 (72 h)	EC20 (48 h)
Reference	AFNOR 2012a	AFNOR 2012b	AFNOR 2009
Short description	Organisms are exposed to a range of dilution of the sample tested in test tubes, 4 replicas per dilution are made (5 organisms per replica). The replicas are stored in the dark. Their mobility is checked after 24 and 48 h.	Organisms are exposed to a range of dilution of the sample tested in microplate wells, 3 replicas per dilution are made. The replicas are stored with a day/night schedule with controlled luminosity and temperature. The algae concentration is measured with a Thoma cell counting chamber after 72 h.	Organisms are exposed to a range of dilution of the sample tested in microplate wells, 3 replicas per dilution are made. The replicas are stored in the dark. The number of organisms is determined after 48 h by the laboratory staff under a binocular magnifier.

(dissolved organic carbon and total organic carbon) indicates the global level of degradation of the organic matter in the studied wastewater network, (2) the important decrease in the concentration of specific pollutants (paracetamol, Comperlan100) indicates their strong biodegradability (Wu et al. 2012; Žur et al. 2018), (3) the decrease of the TKN could be due to the volatilization of nitrogen due to bacterial activity during its transport (Valero and Mara 2007; Yetilmezsoy and Sapci-Zengin 2009), (4) finally, the increase in the values of suspended solids (total organic suspended solids and total suspended solids) could be attributed to the suspension of compounds previously decantable.

### Ecotoxicity

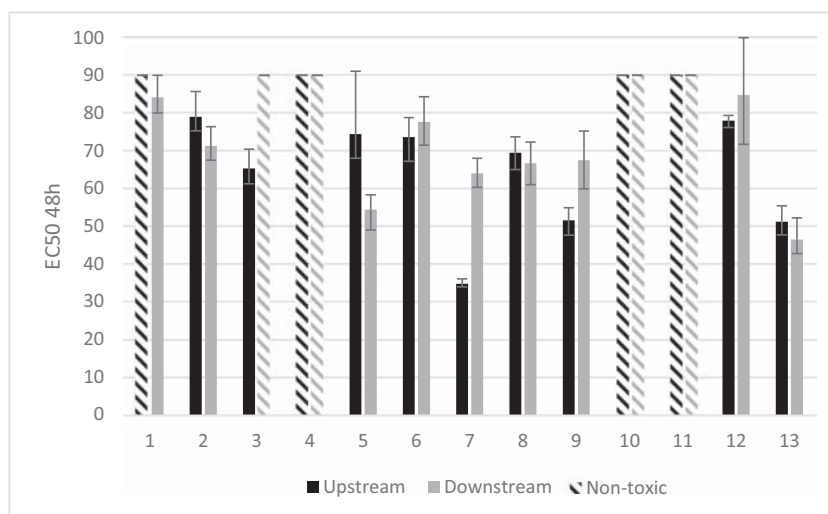
Bioassays results are displayed in Figs. 2, 3, and 4 for the mobility test on *Daphnia magna*, the growth test on *Pseudokirchneriella subcapitata*, and the reproduction test on *Brachionus calyciflorus*, respectively.

The *D. magna* mobility tests on UWW samples showed an EC50 between 35 and 90%. We observed differences between upstream and downstream samples: in campaign 5, the sample was more toxic downstream, contrary to campaign 7 where UWW was less toxic after 45 min in the sewer. But these differences depended on the campaign, with no clear pattern. UWW ecotoxicity for *D. magna* changed during transport in the sewer network but not in the same way every time.

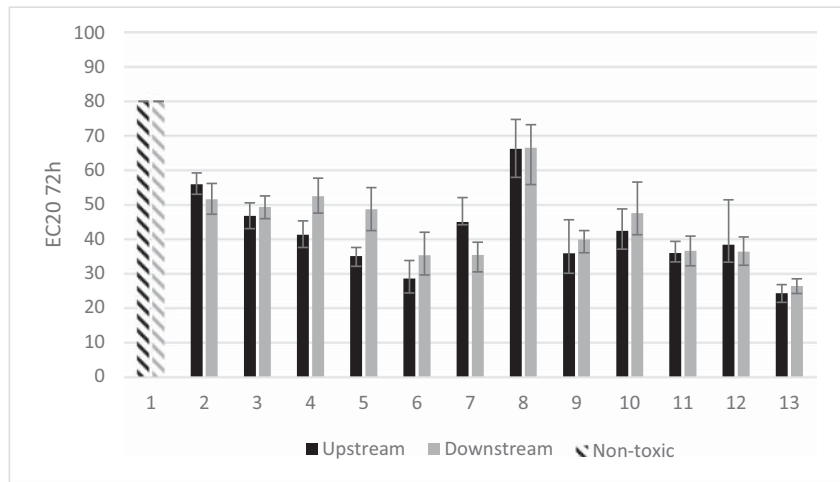
The *P. subcapitata* growth tests on UWW samples showed an EC20 between 25 and 80%. We observed small differences between upstream and downstream samples but they depended on the campaign, with no clear pattern. Surprisingly, UWW ecotoxicity for *P. subcapitata* remained stable during transport in the sewer network.

The *B. calyciflorus* growth tests on UWW samples showed an EC20 between 29 and 100%. We observed differences between upstream and downstream samples but they depended on the campaign. These results show that UWW ecotoxicity for *B. calyciflorus* changes during transport in the sewer network, but with no clear pattern.

**Fig. 2** Mobility test on *Daphnia magna* 48 h



**Fig. 3** Growth test on *Pseudokirchmeriella subcapitata* 72 h



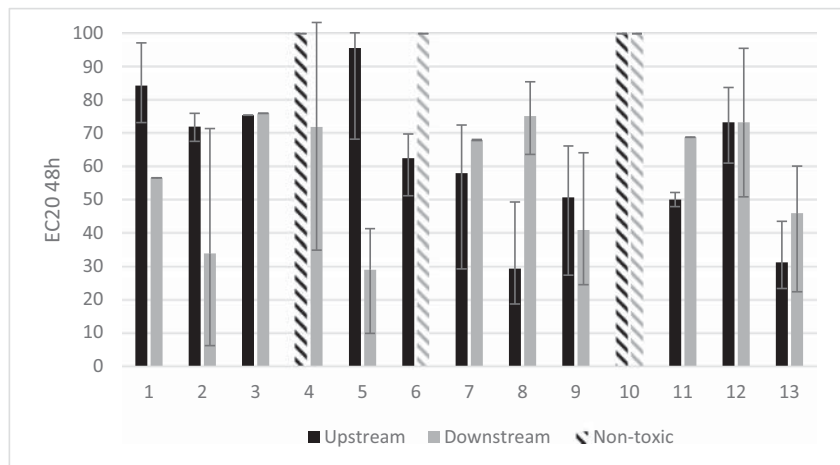
The results of bioassays are consistent with those measured in urban raw UWW at the entrance of a treatment plant (Cao et al. 2009; Laquaz et al. 2017). Bioassays on algae appear to be the most sensitive of the three bioassays tested, maybe because of surfactants that are quite toxic for algae (Liwarska-Bizukojc et al. 2005; Pavlič et al. 2005). The results of the bioassays on upstream and downstream samples do not show an overall pattern: the ecotoxicity increases or decreases depending on the samples. However, despite a relatively short travel time between the sampling points (approximately 45 min), differences are noticeable. This result confirms the relevance of using ecotoxicological tests to assess a difference on this type of sample. To conclude, the pipe works as a bioreactor but does not drastically increase or decrease the ecotoxicity of the UWW, so it cannot be considered as a pre-treatment or as an additional risk factor.

The short and continuous sampling protocols obtained ecotoxicity results of the same order for the three bioassays. They did not seem to affect the sensitivity of our bioassays by selecting a water section with high concentrations (short

campaigns) or, on the contrary, by diluting the potential peaks in concentration in a global sample (continuous campaigns). For further studies, we suggest using a continuous sampling protocol in order to ensure greater representativeness of the water flowing through the pipe.

Furthermore, despite the considerable analytical means deployed, it was difficult to identify the pollutants responsible for the ecotoxicity measured in the effluents. Indeed, the ecotoxicity of the pollutant can, on the one hand, be linked to physicochemical parameters not monitored in this study and, on the other hand, depend on interaction phenomena between the pollutants present in the effluent (phenomena of synergy and/or antagonism). In this study, the upstream samples were collected as close as possible to the primary sources (dwellings, etc.) but as it is a diffuse source of pollution, it was not possible to be in the direct vicinity. A study considering the phenomena that occur in the vicinity of a primary source would be interesting to identify brief phenomena like the volatilization of ecotoxic substances (Diaz et al. 2018; Perales et al. 2017).

**Fig. 4** Reproduction test *Brachionus calyciflorus* reproduction inhibition



### Antibiotic resistance

The results of integron quantification are displayed in Figs. 5 and 6: class 1 integron copy number and relative abundance, and class 3 integron copy number and relative abundance. Class 2 integrons were not in sufficient concentration to be quantified. Figure 5 presents the results of class 1 integron concentration (a) and relative abundance (b) as a function of sampling campaign.

The copy number of the class 1 integron results obtained from UWW upstream and downstream of the sewer was variable from one sample to another, between log 7.1 and 10.2 depending on the day of collection, and presented high variability for some of the samples. No significant differences can be seen for the first three campaigns. Samples collected during campaigns 4, 5, and 6 (collected on the same day at different times) show the same pattern: integrons are less abundant downstream. Campaigns 8, 9 (collected on the same day at different times), and 10 showed the opposite pattern: there are more integrons in the water downstream.

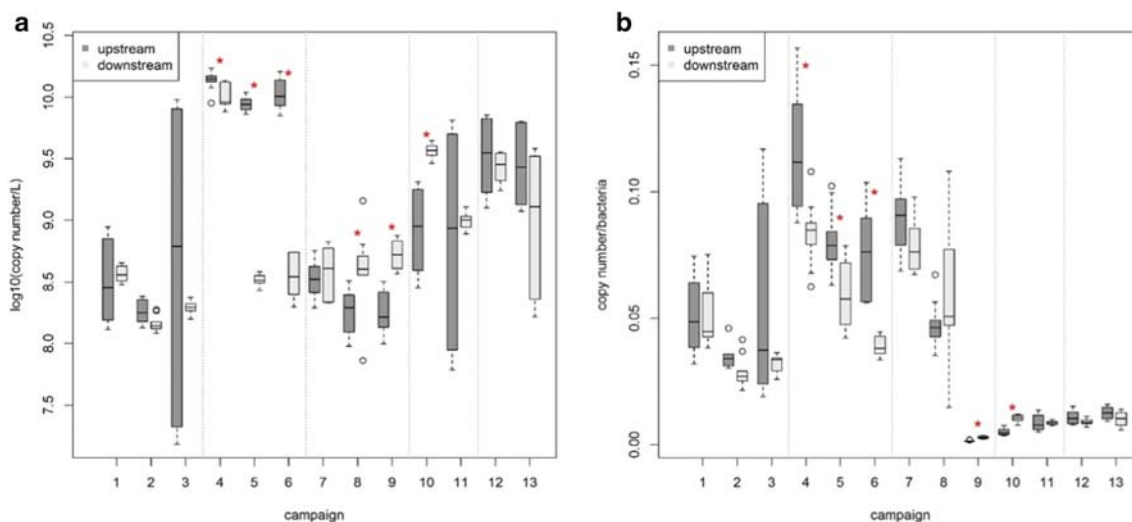
The relative abundance of class 1 integrons varies between 0.001 and 0.16 integron copy per bacteria. Campaigns 4, 5, and 6 (collected on the same day at different times) showed the same pattern: there were fewer integrons per bacteria downstream. For these samples, not only the total concentration of integrons decreased, possibly due to a global decrease of bacteria, but the potential antibiotic resistance of the bacteria in water was also lower. Campaigns 9 and 10 showed the opposite pattern: the relative abundance of class 1 integron increased during transport in the sewer but at a lower value (around 4 times less than in the previous samples). For the 4 last campaigns, the number of integrons in the water was in the same range as the previous one but the relative abundance was lower because of a higher concentration of bacteria (based on the quantification of 16S RNA).

The copy number of the class 3 integron results obtained from UWW from upstream and downstream of the sewer was variable from one sample to another, between log 6.5 and 9.6 depending on the day of collect and presented high variability for some of the samples. Campaigns 4, 5 and 6 (collected on the same day at different times), and 2 showed the same pattern: there were fewer integrons downstream. Campaigns 7, 8, 9 (collected on the same day at different times), and 10 showed the opposite pattern: there were more integrons in the water downstream.

The relative abundance of class 3 integrons varied between 0.001 and 0.04 integron copy per bacteria (based on 16S RNA abundance). Campaigns 2 and 3 (collected on the same day at different times) and 5 and 6 (collected on the same day at different times) showed the same pattern: there were fewer integrons per bacteria downstream. For these samples, not only the total concentration of integrons decreased, possibly due to a global decrease of bacteria, but also the potential antibiotic resistance of the bacteria in water was lower. Campaigns 7, 9, 10, 12, and 13 showed the opposite pattern: the relative abundance of class 1 integrons increased during transport in the sewer. For the four last campaigns, the values of abundance were around 2 times lower than in the previous samples although the number of integrons in the water was in the same range of values.

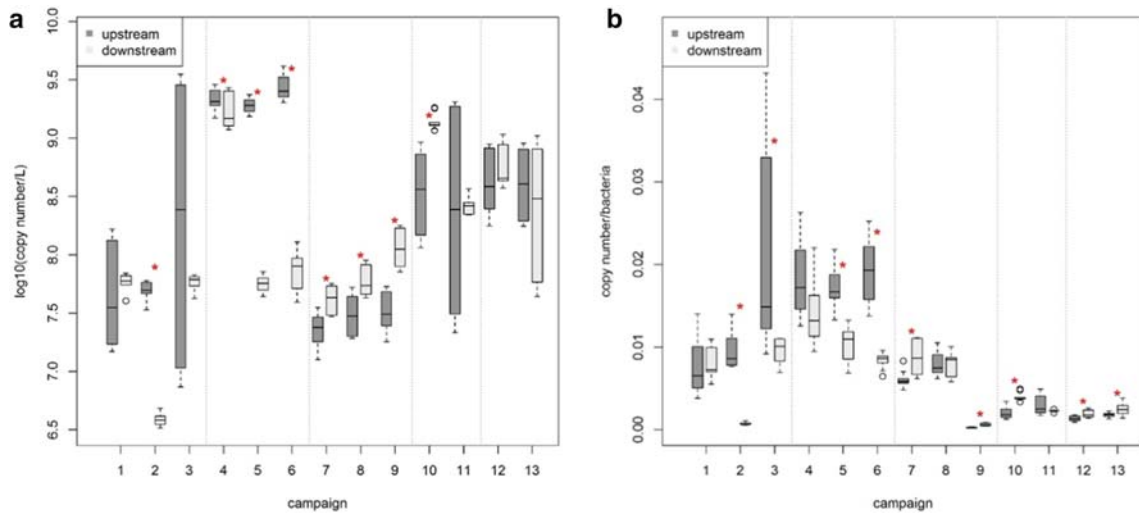
Two types of pattern appear among the 13 campaigns, a significant increase or a significant decrease. The latter shows much higher differences between upstream and downstream in terms of concentration and relative abundance. The sewer pipe acts like a bioreactor but as the results are not consistent from one campaign to another, it cannot be considered as a pre-treatment or as an additional risk factor for the spread of antibiotic resistance.

The noticeable differences between upstream and downstream values mean that certain phenomena occurred during 45 min of travel in the sewer. This hypothesis is consistent



**Fig. 5** Class 1 integron concentration (a) and relative abundance (b) in water. Integron concentration is presented by copy number per liter and relative abundance by copy number per bacteria (quantified using 16S

ARN copy number). The significant differences between upstream and downstream are symbolized by asterisks (*t* test, *p* value < 0.05)



**Fig. 6** Class 3 integron concentration (a) and relative abundance (b) in water. Integron concentration is presented by copy number per liter and relative abundance by copy number per bacteria (quantified using 16S

ARN copy number). The significant differences between upstream and downstream are symbolized by asterisks (*t* test, *p* value < 0.05)

considering that the generation time and horizontal spread among bacteria can be very short (Saha et al. 2016; Vieira-Silva and Rocha 2010). No direct links can be made with the change of physicochemical parameters.

### Conclusion

First of all, this study showed that the bioassay battery selected met the objective established at the beginning of the study, that of being sensitive enough to detect significant differences in characteristics of the effluent between “upstream” and “downstream” segments of the sewer. It also confirmed the synergy between the bioassays used, a synergy that had already been highlighted in previous studies (Laquaz et al. 2017). The bioassays carried out on the site studied showed that the modification of ecotoxicity, from upstream to downstream of the sewer studied, was noticeable despite the short period of transport. The search for possible links with physicochemical parameters was not very conclusive, probably due to the implication of other parameters not monitored in this study and/or to the interactions between the different pollutants present in the wastewater (“cocktail” effect) (Vasquez et al. 2014). Regarding the change of antibiotic resistance, no general rule could be deduced from the work carried out, but significant differences were sometimes observed between upstream and downstream. Furthermore, no link could be established between the antibiotic resistance monitoring parameters and the results of physicochemical analyses.

This work provides useful information for making recommendations for the management of sewer networks. It shows that the contribution of the sewer networks as a bioreactor to reduce the ecotoxicity of effluents is generally slight, especially for networks of short length. There is therefore no reason to change their current design in order to reduce the ecotoxicity of the effluents.

Conversely, this work highlights a significant reduction of antibiotic resistance during the transport of the effluents in sewer networks for certain campaigns. If this reduction were confirmed by further studies, it would be relevant, when designing new networks, to match the operating conditions of the pilot site of this study more closely, especially if the sewer network is not equipped with an efficient wastewater treatment plant. A longer residence time in the sewer could intensify the phenomena that occur and provide more significant differences. Hence, we are considering launching a study on a longer sewer to consolidate the current results.

It would be also interesting to investigate the occurrence of resistance genes to see if there is an change of the resistome during transport in sewage. For example, Auguet et al. (2017) studied several antibiotic resistance genes in biofilm and water along a sewer. Finally, the ecotoxicity linked to certain substances could prevent the development of germs sensitive to the same substances, and thus promote the development of germs that are resistant to these substances. The test of this hypothesis could be the subject of a specific study.

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